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Comparative Analysis of Mass Spectrometry-Based Proteomic Methods for Protein Target Discovery using a One-Pot Approach

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Abstract

Recently, several mass spectrometry- and protein denaturation-based proteomic methods have been developed to facilitate protein-target discovery efforts in drug mode-of-action studies. These methods, which include the Stability of Proteins from Rates of Oxidation (SPROX), Pulse Proteolysis (PP), Chemical Denaturation and Protein Precipitation (CPP), and Thermal Proteome Profiling (TPP) techniques, have been used in an increasing number of applications in recent years. However, while the advantages and disadvantages to using these different techniques have been reviewed, the analytical characteristics of these methods have not been directly compared. Reported here is such a direct comparison using the well-studied immuno-suppressive drug, cyclosporine A (CsA), and the proteins in a yeast cell lysate. Also described is a one-pot strategy that can be utilized with each technique to streamline data acquisition and analysis. We find that there are benefits to utilizing all four strategies for protein target discovery including increased proteomic coverage and reduced false positive rates that approach 0%. Moreover, the one-pot strategy described here makes such an experiment feasible, because of the 10-fold reduction in reagent costs and instrument time it affords.

Graphical Abstract

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SUPPLEMENTARY MATERIAL

The Supplementary Material includes: i) Supplementary Text describing the relationships between chemical denaturation curve shift, binding free energy, and K_d ; and ii) four tables (Tables S1, S2, S3, and S4) presented as excel spreadsheets summarizing the assayed peptide and proteins in the TPP, CPP, PP, and SPROX experiments, respectively.

Keywords

thermodynamics; protein folding; chemical denaturation; thermal denaturation; mode-of-action

INTRODUCTION

For many years, mass spectrometry-based affinity capture techniques have been the preferred means by which to identify the protein targets of drugs and other small molecules. However, these techniques have been limited to applications in which the target protein was relatively abundant and in which the small molecule was amenable to affinity tag labelling. Recently, a series of mass spectrometry-based proteomics methods exploiting the chemical and thermal denaturation properties of proteins have been described that overcome these limitations. These protein denaturation-based methods, which include the Stability of Proteins from Rates of Oxidation (SPROX) [1–3], Pulse Proteolysis (PP) [4–6], Thermal Proteome Profiling (TPP)[7], and Chemical Denaturation and Protein Precipitation (CPP)[8] techniques, have proven to be attractive alternatives to affinity capture techniques for protein target discovery. The capabilities of these protein denaturation- and mass spectrometry-based approaches to detect and quantify protein-ligand binding interactions in complex biological mixtures such as cell lysates and even intact cells have been demonstrated and recently reviewed [9–11]. In recent years, these protein denaturation-based approaches have been validated in proof-of-principle studies using ligands with already well-characterized protein targets [2, 7, 8, 12, 13], and they have been used in an increasing number of protein target discovery efforts to better understand the biological activities of small molecules [7, 14–19] and to characterize biological phenotypes [20–22].

Common to the SPROX, PP, TPP, and CPP techniques is that they all involve the use of a protein modification reaction to define the chemical (SPROX, PP, and CPP) or thermal (TPP) denaturation properties of proteins. They all also rely on the ligand-induced perturbation of these denaturation properties to detect (and in some cases quantify) protein-ligand binding interactions. SPROX, PP, and CPP rely on the chemical denaturant dependence of a methionine oxidation, protease digestion, or protein precipitation reaction (respectively) to evaluate the thermodynamic properties associated with protein folding and

ligand binding interactions. In TPP the temperature dependence of a protein precipitation reaction is used to evaluate the thermal stability of proteins and protein-ligand complexes. The most popular quantitative proteomics workflows utilized with these methods, to date, have involved isobaric mass tagging strategies to generate multi-point (e.g., 8 to 30) protein denaturation curves from which absolute and relative stability measurements can be extracted [1, 7, 23]. Recently, one-pot data acquisition and analysis strategies with highly multiplexed workflows were reported for the PP and TPP techniques [24, 25]. These one-pot workflows significantly streamline the protein stability determination by reducing the number of measurements from one measurement per denaturation point (usually 8 to 30 measurements), to only one measurement per denaturation curve. This simplification reduces the number of isobaric mass tags and instrument time needed for a single analysis, which can facilitate the acquisition of data from more biological replicates and ultimately increase the statistical significance of the results. Described here is a one-pot data acquisition and analysis strategy that can be used with the SPROX, PP, TPP, and CPP techniques to detect protein-ligand binding interactions on the proteomic scale. The strategy is adapted from the one-pot protocols recently described for PP and TPP [24, 25].

In theory the SPROX, PP, TPP, and CPP techniques are generally applicable to the detection of a wide variety of protein ligand binding interactions. In practice, both fundamental and operational differences between the techniques impact their relative analytical capabilities (e.g., proteomic coverage, false positive and false negative rates). For example, SPROX and PP rely on the detection of specific peptides in the bottom-up proteomics readout (i.e., methionine-containing peptides in SPROX and semi-tryptic peptides in PP); whereas, TPP and CPP involve a protein readout (i.e., all peptides mapping to a specific protein can be used to report on its ligand binding properties). These two different readouts have important implications for the proteomic coverage, false positive and false negative rates. While the relative advantages and disadvantages of these techniques have been recently reviewed [10], the analytical capabilities of SPROX, PP, TPP, and CPP have yet to be directly compared. As part of this work we provide the first direct comparison of these four methods using a well-studied ligand, cyclosporine A (CsA) [26, 27], and the proteins in a yeast cell lysate. Assessed as part of this work are the relative merits of SPROX, PP, TPP, and CPP for protein target discovery using the one-pot data acquisition and analysis protocol described here.

EXPERIMENTAL

Materials

The following materials were from Sigma-Aldrich (St. Louis, MO): guanidine hydrochloride (GdmCl), S-methylmethanethiosulfonate (MTS), dimethyl sulfoxide (DMSO), urea, centrifugal filter units (Amicon Ultra-0.5 mL, 10 K MWCO), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), thermolysin from *geobacillus stearothermophilus*, trifluoroacetic acid (TFA), triethylammonium bicarbonate buffer (TEAB, 1 M, pH 8.5), hydrogen peroxide (H₂O₂) (30% w/w), acetic acid, and 2-mercaptoethanol. The following materials were from ThermoScientific (Waltham, MA): acetonitrile (ACN, LC-MS grade), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), bestatin, E-64, leupeptin, pepstatin A, EDTA solution (Corning, 0.5M, pH 8.0),

TMT10-Plex isobaric label reagent set, NHS-activated agarose dry resin (Pierce), and porcine pancreas trypsin (proteomics grade). Methanol (gradient grade OmniSolv®) was from EMD Millipore. Both Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and Cyclosporine A (CsA) were from Santa Cruz Biotechnology (Dallas, TX). Phosphate-buffered saline (PBS, pH 7.4) was from Gibco (Gaithersburg, MD). MacroSpin columns (silica C18) and Pi³™ Methionine reagent kit were from Nest Group (Southborough, MA).

Cell Culture and Lysis

Yeast strain S288C was obtained from ATCC and cultured in YPAD medium (0.4 g adenine sulfate, 10 g yeast extract, 10 g peptone and 20 g glucose in 1 L DI water) according to standard protocols. Briefly, a yeast colony was incubated in 50 mL YPAD medium at 30 °C. Following an overnight incubation to reach an optical density at 600 nm (OD600) of ~1.6, a 20 mL portion of the culture was inoculated with the YPAD medium (1 L) to give an OD600 of ~0.3. The inoculated medium was incubated at 30 °C until the OD600 of the solution was between 1.2–2.0. Fractions of the final YPAD medium (250 mL each) were centrifuged to generate yeast cell pellets. The pellets were stored at –20 °C until further use.

Five yeast cell pellets were lysed in 20 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride and the following protease inhibitors: 1 mM AEBSF, 20 µM leupeptin, 10 µM pepstatin A, 500 µM bestatin, and 15 µM E-64. Cell lysis was accomplished by mechanical disruption using glass beads (0.5 mm) with 20–25 sec disruption and 1 min intervals on ice for a total of 15–20 cycles. The lysed cells were centrifuged at 14,000 g for 10 min at 4 °C. The total protein concentration in the supernatant from each cell lysate sample was determined by a Bradford Assay and ranged from 2–15 mg/mL. The supernatant samples for each replicate was then divided into two equal aliquots; one aliquot was spiked with CsA in DMSO to generate the (+) ligand sample, and the other was spiked with DMSO to generate the (–) ligand sample. The concentration of CsA in these (+) ligand sample stocks for the TPP, CPP, PP, and SPROX experiments were 120, 240, 370, and 300 µM, respectively. The concentrations of DMSO in these (+) ligand sample stocks for the TPP, CPP, PP, and SPROX experiments were 1, 1.6, 2, and 2%, respectively. Note that the final concentrations of CsA and DMSO in the TPP, CPP, PP, and SPROX buffers were identical at 120 µM and 1%, respectively (see below). The concentrations of DMSO in the (–) ligand sample stocks for each technique were identical to those in the (+) ligand samples. The (–) and (+) ligand sample stocks generated for each technique were equilibrated at room temperature for 1 h prior to CPP, TPP, SPROX, or PP analysis.

CPP Analysis

The (+) and (–) ligand samples were subjected to a CPP analysis similar to that previously described [8], with the exception that a one-pot analysis strategy [24, 25] was employed in the mass spectrometry-based proteomics readout. Briefly, the (+) and (–) ligand samples were distributed into a series of 12 GdmCl-containing buffers (PBS, pH 7.4) where the final GdmCl concentrations were equally spaced at 0.15 M intervals between 0.50 and 2.15 M. The final volume in each buffer was 20 µL. The final amount of protein was 150 µg, and the final concentration of CsA was 120 µM. The solutions were equilibrated at room temperature for 1 hour before 480 µL of deionized water was added into each solution to

initiate protein precipitation. After 10 min, 150 μ L aliquots of the (+) ligand samples solutions were combined and centrifuged at 14000xg for 15 min, as were 150 μ L aliquots of the (–) ligand samples solutions. The above procedure was done on 5 separate yeast lysates, which generated 5 (+) and (–) ligand sample pairs for a total of 10 samples. The supernatant (800 μ L) from each combined sample was transferred into a 10K centrifugal filter unit, where bottom-up proteomic sample preparation with isobaric mass tag labeling protocol (termed iFASP) was performed on each sample as previously described [28]. This protocol involved reduction with TCEP, reaction with MMTS, digestion with trypsin, and labelling with a TMT 10-Plex reagent kit according to the manufacturer's protocol. The 10 TMT 10-Plex-labeled samples, which included 5 (+) and (–) ligand sample pairs, were combined, and desalted using a C18 Macrospin column according to manufacturer's instructions prior to LC-MS/MS analysis.

PP Analysis

The (+) and (–) ligand samples were subjected to a PP analysis that included a semi-tryptic peptide enrichment strategy for proteolysis procedure (STEPP) similar to that previously described [29], with the exception that a one-pot analysis strategy [24, 25] was employed in the mass spectrometry-based proteomics read-out. Briefly, aliquots of the (+) and (–) ligand samples were distributed into a series of 12 urea-containing buffers (PBS, pH 7.4) where the final concentrations of urea were equally spaced at 0.4 M intervals between 1.0 and 5.4 M. The final concentration of CsA in the samples was 120 μ M and the total amount of protein in each sample was 100 μ g. The samples in the urea-containing buffers were incubated for 2 h at room temperature before 5 μ g of thermolysin was added to each of the (+) and (–) ligand samples in the denaturant-containing buffers. The thermolysin proteolysis reactions were allowed to proceed for 1 min at room temperature before they were quenched upon addition of 60 μ L of a urea/EDTA solution (~0.2 M EDTA, 8 M Urea, pH 8.0). Equal aliquots of the (+) ligand samples solutions were combined, as were equal aliquots of the (–) ligand samples. The above procedure was performed on 5 separate yeast lysates. This ultimately generated 5 (+) and (–) ligand sample pairs for a total of 10 samples.

Aliquots containing ~80 μ g of total protein from each of the 5 (+) and (–) ligand pairs were subjected to the STEPP protocol we recently reported.[29] Before the STEPP protocol, an additional 20 μ L of a urea/EDTA solution (~0.2 M EDTA, 8 M Urea, pH 8.0) was added to each sample to ensure proper unfolding for labeling with isobaric mass tags. As part of the STEPP protocol, samples were reacted with 1.5 mM TCEP for 1 hr at 30°C and then with 2.5 mM MMTS for 10 min at room temperature. The protein material in the (+) and (–) ligand samples from each of the 5 biological replicates were labeled with a TMT 10-Plex reagent kit according to the protocol outlined in reference[29]. The protein samples in the TMT 10-Plex were combined to generate a single protein sample that was lyophilized, re-dissolved in 2% v/v TFA, and desalted using C18 columns according to the manufacturer's protocol. The desalted sample was lyophilized, re-dissolved in 0.1 M TEAB solution (pH 8.5), and digested with trypsin overnight at 37 °C. The ratio of trypsin to total peptide was 1:100 (w/w). NHS-activated agarose resin and 50 μ L 0.5 M NaCl was added to the digested sample, such that the NHS-activated agarose resin to total peptide ratio was approximately

150:1 (w/w). Samples were reacted for 1.5 h at room temperature, acidified with 2% v/v TFA, and transferred to C18 Macropsin columns for desalting prior to LC-MS/MS analysis.

SPROX Analysis

The (+) and (–) ligand samples were subjected to a SPROX analysis similar to that previously described,[30] with the exception that a one-pot analysis strategy [24, 25] was employed in the mass spectrometry-based proteomics read-out. Briefly, aliquots of the (+) and (–) ligand samples were distributed into a series of 12 PBS buffers (pH 7.4) containing increasing GdmCl concentrations. The initial GdmCl concentrations in the buffers, which were determined by measuring refractive index as described elsewhere[31], were equally spaced at 0.3 M intervals between 1.1 and 4.3 M GdmCl. For each biological replicate (total of 5), there were twelve GdmCl-containing buffers each with 110 µg of total protein from the yeast lysate, CsA (120 µM), and DMSO (1%).

The five pairs of 12 (+) and (–) ligand samples in the GdmCl-containing buffers generated above, were equilibrated at room temperature for 1 h. The methionine oxidation reaction in SPROX was initiated by adding a 4 µl aliquot of 30 % (v/v) H₂O₂, and the oxidation reaction was allowed to proceed for 3 min. The final concentration of H₂O₂ in each oxidation reaction was 0.98 M and the final concentrations of GdmCl in each reaction were the same as those used in the CPP experiment. The oxidation reaction was quenched by adding a 500 µl aliquot of 500 mM TCEP. For each replicate, equal amounts of the (+) ligand samples were combined as were equal amounts of the (–) ligand samples. The resulting 5 pairs of (+) and (–) ligand samples were subjected to the same iFASP protocol described above in the CPP experiments. Briefly, the combined (+) and (–) samples from each replicate were transferred into a 10 kDa MWCO centrifugal filter unit. Buffer exchange was performed by adding 8 M urea in 0.1 M Tris-HCl pH 8.5 followed by TCEP reduction, MMTS alkylation, digestion with trypsin, and TMT10-Plex labeling according to the manufacturer's protocol. Labeled peptides were centrifuged through the filters. Equal volumes of solution from each TMT 10-Plex labeled sample were combined into one tube. C18 Macropsin column cleanup was performed on the combined, labeled sample followed by enrichment of methionine-containing peptides with the Pi³™ Methionine reagent kit according to manufacturer's protocol. The enriched sample was dried in a speed vac prior to LC-MS/MS analyses.

TPP Analysis

The (+) and (–) ligand samples were subjected to a TPP analysis similar to that previously described [7], with the exception that a one-pot analysis strategy [24, 25] was employed in the mass spectrometry-based proteomics read-out. Aliquots of the (+) and (–) samples were distributed into a series 12 different tubes prior to thermal denaturation which involved heating the protein material in the (+) and (–) ligand samples for 3 min at one of 12 temperatures that were equally spaced at 2 °C intervals between 43 and 65 °C. The total protein concentration in sample was 2 mg/mL and the final CsA concentration in the (+) ligand samples was 120 µM. The protein samples were removed from the heat and equilibrated at room temperature for 3 min prior to placing them on ice. The (+) samples and the (–) ligand samples were combined to generate a single (+) and (–) ligand sample. The

resulting (+) and (–) ligand samples were centrifuged at 48000 rpm for 20 min using a TPA100.1 rotor and a Beckman Optima TL ultracentrifuge. The supernatants were transferred into 10 kDa MWCO centrifugal filter units and buffer exchanged to 8 M urea in 0.1 M Tris-HCl pH 8.5 prior to TCEP reduction and MMTS alkylation. The alkylated proteins were then digested with trypsin, and the peptides were TMT 10-Plex labeled according to the manufacturer's protocol. The labeled peptides were eluted from the filters and equal amounts from each labeled sample were combined into one tube. C18 Macrospin column cleanup was performed on the combined, labeled sample prior to LC-MS/MS analysis.

Quantitative LC-MS/MS Analysis

The LC-MS/MS analyses were performed using a nanoAcquity UPLC system (Waters) coupled to a Thermo Orbitrap Fusion Lumos mass spectrometer systems. The dried peptide material generated from each analysis was reconstituted in 20 μ L (CPP), 15 μ L (TPP), 12 μ L (PP), and 10 μ L (SPROX) of 1% TFA, 2% acetonitrile in H₂O and a 1 μ L aliquot was injected into the UPLC system. The peptides were first trapped on a Symmetry C18 20 mm $\times$ 180 μ m trapping column (5 μ L/min at 99.9/0.1 water/acetonitrile, v/v). The analytical separation was performed using an Acquity 75 μ m $\times$ 250 mm high strength silica (HSS) T3 C18 column with a 1.8 μ m particle size (Waters); the column temperature was set to 55 °C. Peptide elution was performed using a 90 min linear gradient of 3–30 % ACN with 0.1 % formic acid at a flow rate of 400 nL/min.

The MS data was collected using a top 20 data-dependent acquisition (DDA) method which included MS1 at 120k and MS2 at 50k resolution. The MS1 AGC target was 4.0×10^5 ions with a max injection time of 50 ms. For MS2, the AGC target was 1.0×10^5 ions with a max injection time of 105 ms. The collision energy was set to 38 %, and the scan range was 375–1500 m/z. The isolation window was 0.7 and the dynamic exclusion duration was 60 s. The peptide sample generated in each of the four techniques was subjected to 3 LC-MS/MS analyses. The raw MS data generated in this work has been uploaded to the PRIDE database (accession number PXD014309).

Proteomic Data Analysis

Proteome Discoverer 2.2 (Thermo) was used to search the raw LC-MS/MS files against the yeast proteins in the 2017-06-07 release of the UniProt Knowledgebase. The raw LC-MS/MS data generated in the CPP, TPP, and SPROX experiments was searched using fixed MMTS modification on cysteine; TMT 10-Plex labeling of lysine side chains and peptide N-termini; variable oxidation of methionine; variable deamidation of asparagine and glutamine; and variable acetylation of the protein N-terminus. Trypsin was set as the enzyme, and up to two missed cleavages were allowed. For peptide and protein quantification, reporter abundance was set as intensity, and the normalization mode and scaling mode were each set as none. All other settings were left as the default values. Only proteins/peptides with protein/peptide FDR confidence labelled as “high” (i.e., FDR <1%) and with no quantification channels being 0 were used for subsequent analyses. The raw LC-MS/MS data generated in the PP experiment was searched using parameters identical to those described above for the CPP, TPP, and SPROX experiments with the exception that i)

trypsin (semi) was set as the enzyme instead of trypsin and ii) three missed cleavages were allowed instead of 2.

For each biological replicate, a normalization factor was calculated using the ratio of the summed signal intensities recorded in the (+) and (–) ligand samples from each biological replicate; The signal intensities used in the TPP and CPP experiments were the reporter ion intensities from the proteins generated in Proteome Discoverer. The signal intensities used in the SPROX and PP experiments were the reporter ion intensities from the wild-type methionine-containing peptides and the semi-tryptic peptides, respectively. For each identified protein in the CPP and TPP experiment and for each identified methionine-containing and semi-tryptic peptide in the SPROX and PP experiments, respectively, a ratio of the observed reporter ion intensities in the (+) ligand sample to the (–) ligand sample was generated for each biological replicate. The resulting ratio was divided by the normalization factor for each of the 5 replicates. These normalized ratios (fold change) were then log₂-base transformed, averaged, and tested by two-tailed student's t test comparing with a mean of 0.

RESULTS

Experimental Workflow

Shown in Figure 1 is the experimental workflow used for the SPROX, TPP, CPP, and PP experiments performed in this work. A total of 5 biological replicates were analyzed using each technique. Each biological replicate involved the preparation of a yeast cell lysate both in the presence and in the absence of CsA. The (–) and (+) CsA samples in each biological replicate were distributed into a series of buffers containing increasing concentrations of chemical denaturant (SPROX, PP, and CPP) or heated to different temperatures (TPP). After equilibration, the protein samples in the denaturant-containing buffers (SPROX, PP, and CPP) or at the different temperatures (TPP) were modified using reaction conditions previously established for each technique: the SPROX samples were reacted with hydrogen peroxide to selectively oxidize methionine residues [32], the PP samples were digested with thermolysin [6], and the TPP and CPP samples were centrifuged [7, 8]. After the modification reactions were quenched, equal amounts of the (–) ligand samples from a given biological replicate were combined into a single sample. Equal amounts of the (+) ligand samples from a given biological replicate were also combined into a single sample. This ultimately generated 5 pairs of (–) and (+) ligand samples for each technique. These 5 pairs of samples were prepared for a quantitative bottom-up proteomics analysis in which the 10 samples generated for the 5 biological replicates of each technique were labeled with a TMT 10-Plex (Figure 1A).

The TMT reporter ion intensities obtained in LC-MS/MS analyses of the proteomic samples generated for each technique (Figure 1B) were used to identify proteins with CsA-induced stability changes. The TMT reporter ion intensities measured in the LC-MS/MS analyses represent the $F_{\text{avg}(-)}$ and $F_{\text{avg}(+)}$ values (see Figure 1C) for the (–) and (+) ligand denaturation curves in each biological replicate. Proteins with $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios that were significantly and consistently different than “1” in the biological replicates performed with each technique (see hit selection criteria below) were identified as “hit” proteins (i.e., proteins with CsA-induced changes to their denaturation behavior) with each technique.

Proteomic Coverage

The peptides and proteins successfully assayed in the TPP, CPP, PP, and SPROX experiments are summarized in Tables S1–4 provided in the Supplemental Material. The numbers of proteins successfully assayed for CsA binding in each of the four techniques are summarized in Table 1. The protein coverages reported in Table 1 are those resulting from identical LC-MS/MS analyses, performed in triplicate, on the proteomic samples generated for each technique. The same instrument and instrument parameters were used in each case. Not surprisingly, the number of proteins assayed in the TPP and CPP experiments were similar. Both rely on a similar protein precipitation reaction and utilize essentially the same protein centered data analysis. The composition of the proteomic samples (i.e., the tryptic peptide mixtures) submitted to the LC-MS/MS analyses in TPP and CPP were also likely very similar. In contrast to the TPP and CPP techniques, the SPROX and PP techniques rely on peptide centered data analyses involving unique peptide mixtures (i.e., semi-tryptic peptides in PP and tryptic methionine-containing peptides in SPROX). While the number of semi-tryptic peptides detected in PP was greater than the number of methionine-containing peptides in SPROX, the methionine-containing peptides in SPROX mapped to more proteins than did the semi-tryptic peptides in PP (Table 1). In fact, the largest protein coverage was observed for the SPROX experiment. An analysis of the overlapping and unique proteins assayed in the four techniques (Figure 2) revealed that unique proteins were assayed in each of the four techniques with a total of almost 2000 proteins successfully assayed in at least one of the four techniques. The largest number of unique proteins were analyzed by SPROX (Figure 2). The unique proteins identified in SPROX and the other techniques also tended to be the less abundant proteins (Figure 2C).

Hit Identification

Shown in Figure 3 are volcano plots of the p-values and $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios generated for each technique. The CPP and TPP experiments both utilize a protein centered readout in which all the peptides mapping to a specific protein can be used to report on the fraction of protein that remains soluble after the precipitation reaction. Accordingly, the $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios used to generate the CPP and TPP data in Figures 3A and 3B, respectively, were calculated using the TMT reporter ion intensities data generated from all the peptides identified from a given protein. In total, the $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios for 1217 and 1095 proteins were quantified and considered for hit selection in the CPP and TPP experiments, respectively (see Figures 3A and 3B). The SPROX and PP experiments both utilize a peptide centered readout, which requires the detection and quantitation of methionine-containing and semi-tryptic peptides, respectively. The methionine-containing and semi-tryptic peptides detected in the peptide readouts in SPROX and PP (respectively), report on the stability of the protein folding domains to which they map. Because different protein folding domains within the same protein can have different protein folding and ligand binding properties, different methionine-containing and semi-tryptic peptides from the same protein can display different behavior. Accordingly, the $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios used to generate the SPROX and PP data in Figures 3C and 3D, respectively, were calculated using the TMT reporter ion intensities data generated for each identified peptide in the LC-MS/MS readout. In total, the $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios for 3923 methionine-containing and 6435 semi-tryptic peptides were

quantified and considered for hit selection in the SPROX and PP experiments, respectively (see Figures 3C and 3D).

The selection of protein “hits” using each technique was based on the same two criteria including i) the p-value from a two-tailed student’s T-test was less than 0.001 and ii) the average $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratio was either greater or smaller than 3 standard deviations from the mean of all the average $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios determined for each technique. Interestingly, the mean and standard deviation of the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values measured for all the proteins in CPP and TPP and for all the peptides in SPROX and PP were similar (close to 0 and 0.1, respectively). The protein hits selected in each technique are summarized in Table 2. The hit selection criteria used in this work were selected to maximize the detection of known CsA-binding proteins and minimize the selection of false positives. For example, relaxing the p-value constraint to $p < 0.01$ and the standard deviation from the mean to 2, only resulted in the additional selection of either 0 (PP, SPROX) or 1 (CPP, TPP) known CsA binding protein, but it doubled the false positive rate of each technique.

DISCUSSION

Proteomic Coverage

One goal of this work was to directly compare the proteomic coverage obtained in each technique using identical samples, identical LC-MS/MS conditions (e.g., the same instrumental parameters as well as the same number of LC-MS/MS runs), and the same data analysis strategy. The proteomic coverage was largest in the SPROX experiment. This is likely due to the methionine-containing peptide enrichment step used in the SPROX protocol. The unique focus on methionine-containing peptides in the bottom-up proteomics readout in SPROX has a two-fold benefit for increasing the coverage in bottom-up shotgun proteomic analyses. One benefit is that it helps overcome some of the problems associated with the detection of proteins across a wide dynamic range of abundance levels in proteomic samples by limiting the maximum number of potential peptides from a given protein. This helps reduce the extent to which highly abundant proteins can suppress the signals of less abundant proteins in the bottom-up proteomics readout. It also reduces the complexity of the tryptic peptide mixture generated in the bottom-up proteomics analysis prior to LC-MS/MS analysis. It is well established that the increased fractionation of proteomic samples prepared from complex proteomes results in deeper proteome coverage. The incorporation of additional and more conventional fractionation strategies (e.g., HILIC, MudPit strategies) into the SPROX, TPP, CPP, and PP workflows would no doubt further extend the proteomic coverage of these strategies. In fact, such fractionation strategies have been used in combination with TPP to assay up to 5000 proteins in a single TPP experiment [7]. The main drawback to the use of such fractionation strategies is that they significantly increase the instrument time needed for analysis.

An analysis of the proteins successfully assayed in the SPROX, TPP, CPP, and PP techniques under the conditions of this study revealed that the proteins assayed in SPROX included a larger number of less abundant proteins in the yeast proteome (Figure 2B). Interestingly, the unique proteins assayed in each technique were also those of lesser abundance (Figure 2C). However, the numbers of unique proteins detected in the CPP, TPP,

and PP techniques were relatively small (108, 45, and 73, respectively) compared to the over 450 unique proteins detected in the SPROX experiment. The largest amount of overlap in the assayed proteins (70%) was observed with the TPP and CPP techniques. This is not surprising given that these techniques rely on a similar protein precipitation reaction and utilize essentially the same protein centered readout.

False Positive and Negatives

Summarized in Table 2 are all the protein hits and known CsA binding proteins identified in the SPROX, TPP, CPP, and PP experiments performed here. The 9 proteins in Table 2 that are not cyclophilins, are most likely false positives. Consistent with this conclusion is the observation that none of these 9 proteins were identified as a hit in more than one technique, despite each of the 9 proteins being assayed in at least 3 of the 4 techniques. If these 9 proteins are classified as false positives, the false positive rates for the TPP, CPP, PP, and SPROX techniques are similar and close to 0.1% (Table 1). These false positive rates, calculated from the ratio of the total number of peptide (SPROX and PP) or protein (CPP and TPP) hits selected to the total number of peptides (SPROX and PP) or proteins (CPP and TPP) covered, are consistent with the p-value of 0.001 that was used in the hit selection criteria. In this study with CsA, which has well-known protein targets, about one-third of the hits selected using each technique were classified as false positives, with exception of the PP technique in which just over two-thirds of the hits selected were classified as false positives. The differentiation of false positives and true positives in the application of these techniques to ligands with less well-understood protein targets is a significant challenge. Our results suggest that the frequency of false positives in the hits selected using these techniques with the one-pot strategy outlined here was 30–70%. Our results further suggest that the use of multiple techniques can be especially useful for differentiating true positives from false positives. For example, requiring hit proteins to appear in more than one technique brings the false positive rates of the 4 techniques in this study to 0.

False negatives in protein target discovery experiments using the SPROX, PP, CPP and TPP techniques can be divided into two categories. In the first category are potential protein hits that are not included in the assay because they are not successfully detected and quantified in the LC-MS/MS readout. A second category of false negatives includes potential protein hits that do not meet the selection criteria, even though the protein was successfully assayed. The number of false negatives in the first category is impacted by the proteomic coverage. Our results suggest that the use of multiple techniques significantly increases the total proteomic coverage (see Table 1), which reduces the number of false negatives in the first category. The number of false negatives in the second category is impacted by analytical measures such as the accuracy and precision with which $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios can be determined as well as the relative magnitude of the $F_{\text{avg}(+)}/F_{\text{avg}(-)}$ ratios observed for potential protein hits. Interestingly, the mean and standard deviation of the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values measured for all the proteins and peptides in each technique were similar (close to 0 and 0.1, respectively) across the 4 techniques. However, the magnitudes of the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values for the same hits varied across the 4 techniques in this study (Table 3). The average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values for the protein hits in the SPROX and PP techniques were generally 2–3 fold larger than those for the two protein

precipitation-based techniques, CPP and TPP, with one exception (i.e., the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ value for CypD in PP) that is discussed in more detail below. The larger average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values observed for the hit proteins in SPROX and PP make these techniques more sensitive. For example, the larger average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values enabled the selection of CypD as a hit in SPROX but not in TPP or CPP (Table 2).

The peptide-centered readouts in SPROX and PP can generate false negatives. This is because the peptides detected in SPROX and PP only report on the folding properties and ligand binding behavior of the protein folding domain to which they map. Thus, in the case of a large, multi-domain protein only peptides from the ligand-binding domain will display hit behavior. If such peptides from the ligand binding domain are not detected in the LC-MS/MS readout but peptides from other regions of the protein are detected, then the protein can appear as a false positive. It is also possible that the detected methionine-containing or semi-tryptic peptide in SPROX or PP (respectively) does not map to a globally protected region of protein structure (e.g., the peptide maps to a solvent exposed region of protein structure). In such cases there will not be a chemical denaturant dependence to the methionine oxidation reaction in SPROX or the thermolysin digestion in PP. Thus, the abundance of the peptide will not change as a function of the denaturant concentration, and it is not likely to be altered with ligand. Peptides with such behavior are easily identified and removed from analysis in conventional SPROX and PP experiments where the entire denaturation curve is recorded. However, in the one-pot experiment peptides that do not map to globally protected regions of protein structure will not generally display hit behavior, even if a ligand binding interaction occurs with the protein. This is likely the reason why the two semi-tryptic peptides from CypD in the PP experiment appeared as false negatives.

Qualitative vs. Quantitative Protein-Ligand Binding Detection

The results reported here demonstrate that a one-pot protocol can be applied to protein-target discovery applications of SPROX, PP, CPP, and TPP. In each case, the one-pot protocol enabled the detection of known protein targets of CsA. One advantage of SPROX, PP, and CPP over TPP is that the use of chemical denaturant in SPROX, PP, and CPP enables the evaluation of thermodynamic parameters associated with protein folding and ligand binding interactions including folding free energies and the dissociation constants of protein-ligand binding interactions. In theory, the $F_{\text{avg}(-)}$ and $F_{\text{avg}(+)}$ values generated for the SPROX, PP, and CPP protein hits using the one-pot protocol described here can be used to calculate such

G_f and K_d values as described in reference 24 [24]. However, these calculations require knowledge of the relative magnitudes of the pre- and post-transition baselines of a protein's denaturation curve.

The one-pot strategy can be adapted to include measurements of the pre- and post-baseline signals for the (–) and (+) ligand samples. Indeed, such measurements have been incorporated in the one-pot strategies previously reported for PP and TPP [24, 25]. Unfortunately, the incorporation of pre- and post-baseline measurements into the one-pot protocol described here would reduce the number of biological replicates that could be incorporated into a single TMT 10-Plex from 5 to 2. This would undoubtedly reduce the statistical significance of selected hits. The one-pot strategy described here was designed to

maximize the statistical significance of selected hits. Unfortunately, this comes at the cost of making quantitative determinations of binding affinities. However, a qualitative analysis of the relative binding affinities of different protein hits can be accomplished using the one-pot strategy described here if the relative magnitudes of the pre- and post-transition baselines for the different proteins being compared are similar. Provided this is a good assumption for the three cyclophilin hits detected in this work, the $\text{Log}_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values measured for the cyclophilin proteins in the SPROX, PP, CPP, and TPP results (Table 3) are all consistent with CypD, CypC, and CypA binding CsA with increasing affinity, respectively. This ranking is consistent with previously measured K_d values of 90 and 36 nM for CsA binding to CypC and CypA, respectively [33]. Presumably, the one cyclophilin protein, CPR6, that was not selected as a hit in any of the techniques binds CsA with even weaker affinity than CypD.

Unfortunately, the minimum binding affinity that can be detected using the one-pot strategy outlined here is difficult to calculate. As noted above, the $F_{\text{avg}(-)}$ and $F_{\text{avg}(+)}$ values generated for the SPROX, PP, and CPP protein hits using the one-pot protocol can be used to calculate such G_f and K_d values provided additional information is known about the structure of the chemical denaturation curve (e.g., the relative magnitudes of the pre- and post-transition baselines of a protein's denaturation curve). Without this added information, it is not possible to translate the minimum $\text{Log}_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ value of 0.3 used for hit selection in these experiments into a meaningful chemical denaturation curve shift and ultimately a K_d value. However, the one-pot protocol is not expected to be any more sensitive to ligand binding than SPROX, PP, CPP, and TPP experiments conducted using the conventional protocols in which complete denaturation curves are recorded in the presence and in the absence of ligand. Based on the 120 μM free ligand concentration used in this work, the conventional SPROX, PP, and CPP protocols are expected to detect protein-ligand complex with a K_d values up to $\sim 40 \mu\text{M}$ (see Supplementary Text). The use of larger free ligand concentrations would enable the detection of even weaker binding ligands (see Equation S2 in the Supplementary Text). The minimum binding affinity that can be detected using the conventional TPP protocol is more difficult to estimate as binding free energies and/or dissociation constants of protein-ligand complexes cannot be calculated from ligand-induced denaturation curve shifts in TPP.

CONCLUSIONS

Our results indicate that one general feature of the four techniques studied here using the one-pot protocol is that the precision with which ligand binding measurements can be made (i.e., $\text{Log}_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values evaluated) is similar across the four techniques. In the case of other features observed in this model study (e.g., the increased sensitivity of the SPROX and PP techniques), it is difficult to know if the differences between methods are general or specific to CsA's binding properties. Clearly, there are fundamental differences between the different methodologies (e.g., utilization of different denaturants and peptide probes as well as the exploitation of different "modification" reactions) that have the potential to make one or more of the techniques more or less amenable to specific ligand interactions. For example, the SPROX technique not only requires the presence of a methionine residue in the protein folding domain involved in ligand binding, but also that a methionine-containing tryptic peptide from the domain be detected in the bottom-up proteomics readout. This, for

example, eliminated CPR6 from the SPROX assay but not from the CPP, TPP, and PP assays.

The most important finding from this work is that there are benefits to utilizing all four strategies for protein target discovery. The combined use of the SPROX, PP, CPP, and TPP techniques reduces false negatives in protein target discovery efforts by maximizing proteomic coverage, since not all the techniques sample the same proteins in the proteome. Using the one-pot strategy described here the false positive rates associated with the SPROX, PP, CPP, and TPP techniques were similar. However, the combined use of all four strategies in a protein target discovery project can help differentiate true positives from false positives, which can constitute a significant fraction (30–70% in this work) of the selected hits identified using one of the four techniques. Our results also show that the above benefits of using all four strategies can be easily realized using the one-pot strategy described here, as it significantly reduces the reagent cost and instrument time required for each technique.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. (A)

(A) Schematic representation of the experimental workflow used in this work. (B) Schematic representation of raw data (i.e., isobaric mass tag intensities detected in product ion mass spectra of peptides detected in bottom-up proteomics analysis) used to identify protein hits and non-hits. (C) Schematic representation of the denaturation curve behavior and relative $F_{avg(-)}$ and $F_{avg(+)}$ values expected for protein hits and non-hits.

Figure 2.

Summary of proteomic coverage observed in SPROX, PP, CPP, and TPP analyses performed in this work. **(A)** Venn diagram showing overlap of the assayed proteins in each technique.

(B) Distribution of protein expression levels observed for all the proteins assayed in the SPROX, PP, CPP, and TPP techniques. **(C)** Distribution of protein expression levels observed for the unique proteins detected in the SPROX, PP, CPP, and TPP techniques. The protein expression level data in **(B)** and **(C)** were from reference 34 [34].

Figure 3.

Volcano plots of the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values and p-values generated using a two-tailed Student's T-test to analyze the CPP, TPP, SPROX, and PP data generated in this work. The data for the assayed proteins in the CPP and TPP analyses are shown in (A) and (B), respectively. The data for the assayed peptides in the SPROX and PP analyses are shown in (C) and (D), respectively. In each plot the vertical and horizontal dotted lines mark the hit selection criteria cut-off values for the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values and $-\log_{10}(\text{p-values})$, respectively. Note that the X-axes of (d) is expanded and the average $\log_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ cut-off values are similar for each technique (*i.e.*, ~ 0.3). In each plot the green data points indicate true positives (*i.e.*, known CsA binding proteins selected as hits) and the red

data points indicate false negatives (i.e., known CsA binding proteins or peptides that were not selected as hits).

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Table 1.

Summary of proteomic coverages and hits observed in CsA ligand binding experiments using the SPROX, PP, CPP, and TPP techniques.

Technique	Protein (Peptide) Coverage	Protein (Peptide) Hits	Known Protein (Peptide) Targets Detected as Hits	False Positive Rate
CPP	1217(NA ^a)	3 (NA ^a)	CypA (NA ^a); CypC (NA ^a)	0.08% ^b
TPP	1095(NA ^a)	3 (NA ^a)	CypA (NA ^a); CypC (NA ^a)	0.09% ^b
SPROX	1403(4955)	5 (9)	CypA (2); CypC (2); CypD (3)	0.04% ^c
PP	866(6435)	7 (24)	CypA (17); CypC (1)	0.09% ^c
Total	1949	12	CypA; CypC; CypD	

^aNot applicable.

^bThe false positive rate was calculated by dividing the number of protein hits detected with no known CsA binding properties by the total number of proteins assayed (i.e., the protein coverage).

^cThe false positive rate was calculated by dividing the number of unique peptide hits that mapped to proteins with no known CsA binding properties by the total number of unique peptides assayed (i.e., the peptide coverage).

Table 2.

Summary of the protein hits and known CsA binding proteins identified in the CPP, TPP, SPROX, and PP experiments performed in this work. “H” indicates a protein was a hit. “NH” indicates that proteins was assayed but not identified as a hit. “NA” indicates the protein was not assayed.

Protein (Accession #)	CPP	TPP	SPROX	PP
Cyclophilin A (P14832) ^a	H	H	H	H
Cyclophilin C (P25719) ^a	H	H	H	H
Cyclophilin D (P35176) ^a	NH	NH	H	NH
CPR6 (P53691) ^a	NH	NH	NA	NH
GTP-binding protein SAR1 (P20606)	H	NA	NH	NH
Glyceraldehyde-3-phosphate dehydrogenase 3 (P00359)	NH	NH	NH	H
40S ribosomal protein S7-A (P26786)	NH	NH	NA	H
60S ribosomal protein L12-B (P0CX54)	NH	NH	NA	H
40S ribosomal protein S19-A (P07280)	NH	NH	NH	H
Pyruvate Decarboxylase (P06169)	NH	NH	NH	H
Hexokinase-A; Hexokinase-B (P04806; P04807)	NH	NH	H	NH
40S ribosomal protein S15 (Q01855)	NH	NH	H	NH
Cleavage factor IB (Q99383)	NH	H	NH	NH

^aKnown CsA binding protein.

Table 3.

Summary of average $\text{Log}_2(F_{\text{avg}(+)}/F_{\text{avg}(-)})$ values for hit proteins with known CsA binding interactions that were identified in each technique.

Protein (Accession #)	CPP	TPP	SPROX	PP
Cyclophilin A (P14832)	0.75	0.70	1.37 ^a	-1.52 ^a
Cyclophilin C (P25719)	0.43	0.36	0.92 ^a	-1.35 ^a
Cyclophilin D (P35176)	NH (0.28)	NH (0.20)	0.65 ^a	NH(-0.03) ^b

^aReported value is the average value obtained from all peptide hits.

^bReported value is the average value obtained from the two non-hit peptides detected for CypD.