# Painting Proteins with Covalent Labels: What's In the Picture?

Michael C. Fitzgerald and Graham M. West
Department of Chemistry, Duke University, Durham, North Carolina, USA

Knowledge about the structural and biophysical properties of proteins when they are free in solution and/or in complexes with other molecules is essential for understanding the biological processes that proteins regulate. Such knowledge is also important to drug discovery efforts, particularly those focused on the development of therapeutic agents with protein targets. In the last decade a variety of different covalent labeling techniques have been used in combination with mass spectrometry to probe the solution-phase structures and biophysical properties of proteins and protein–ligand complexes. Highlighted here are five different mass spectrometry–based covalent labeling strategies including: continuous hydrogen/deuterium (H/D) exchange labeling, hydroxyl radical-mediated footprinting, SUPREX (stability of unpurified proteins from rates of H/D exchange), PLIMSTEX (protein–ligand interaction by mass spectrometry, titration, and H/D exchange), and SPROX (stability of proteins from rates of oxidation). The basic experimental protocols used in each of the above-cited methods are summarized along with the kind of biophysical information they generate. Also discussed are the relative strengths and weaknesses of the different methods for probing the wide range of conformational states that proteins and protein–ligand complexes can adopt when they are in solution. (J Am Soc Mass Spectrom 2009, 20, 1193–1206) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Proteins fold into elaborate three-dimensional structures and, under native solution conditions, they spend a large fraction of time in highly compact structures. However, the solution-phase structures of proteins are not static. Proteins sample a wide range of conformational states in solution. The conformational changes that proteins undergo in solution can be as dramatic as a global unfolding event in which all higher-order structure is lost or as subtle as a breathing motion in which a specific element of secondary structure is partially unfolded in a more local unfolding event (see Figure 1). Knowledge about the structures, kinetics, and thermodynamics involved in the conformational changes that proteins undergo in solution and as they interact with ligands is crucial for understanding the fundamental biological processes in which proteins participate. It is also important to drug discovery efforts, particularly those focused on the development of therapeutic agents with protein targets.

Mass spectrometry (MS) has become an increasingly useful analytical tool for acquiring biophysical information about the conformational properties of proteins in solution. A common strategy that has emerged for acquiring such information is to use matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) MS to read out the results of solution-phase reactions that introduce covalent modifications into proteins. Two general types of MS-based covalent labeling strategies have proven useful for probing the biophysical properties of proteins. One type of strategy has relied on the use of chemical crosslinking reagents to "trap" specific conformational state(s) of a protein and generate structural information about the different states [1–3]. A second type of strategy has involved measuring the rate at which a specific covalent labeling reaction proceeds to ascertain information about the structure and other biophysical properties of protein conformations. The discussion in this work is focused on this second strategy and, more specifically, on the use of protein amide hydrogen/deuterium (H/D) exchange and protein oxidation reactions to probe the conformational properties of proteins and protein–ligand complexes.

In the last decade, protein amide H/D exchange and oxidation reactions have been exploited in a number of different MS-based studies to characterize the conformational properties of proteins and protein–ligand complexes. Our aim is not to provide a comprehensive summary of these studies or to provide an in-depth explanation of the methodology. Such summaries and explanations can be found in recent reviews [4–7]. Our goal is to summarize the different types of experimental

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Address reprint requests to Dr. Michael C. Fitzgerald, Duke University, Department of Chemistry, Science Drive and Towerview Road, Box 90346, Durham, NC 27708-0346. E-mail: michael.c.fitzgerald@duke.edu

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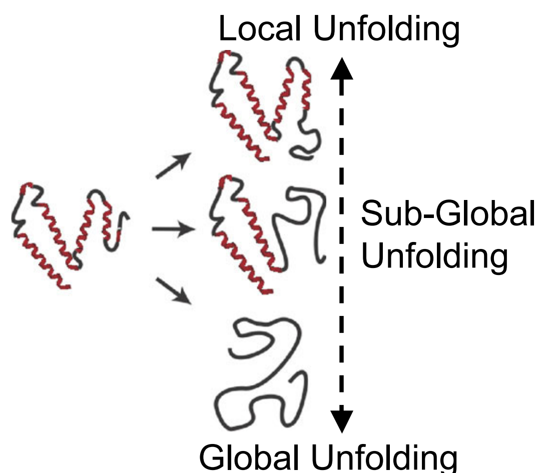



**Figure 1.** Schematic representation of the range of conformational changes that proteins can undergo in solution.

methods that have been developed in this area and to discuss the kind of information that they yield.

Highlighted here are five different H/D exchange- and protein oxidation–based methods that have been developed for use in MS-based studies of protein folding and ligand binding. These methods include continuous H/D exchange labeling [8–16], hydroxyl radical-mediated footprinting [17–23], SUPREX (stability of unpurified proteins from rates of H/D exchange) [24–32], SPROX (stability of proteins from rates of oxidation) [33], and PLIMSTEX (protein–ligand interaction by mass spectrometry, titration, and H/D exchange) [34–38]. All of these methods have advantages over the spectroscopic, calorimetric, and X-ray crystallographic techniques that have long been used for the characterization of protein structure and function. One important advantage is that these covalent labeling methods can be performed on proteins when they are in solution at a wide range of different concentrations. X-ray crystallographic data are collected on protein samples when they are confined in the solid state of a crystal, and thus offer little insight into the conformational dynamics of proteins when they are dissolved in solution. Nuclear magnetic resonance (NMR) analyses of protein structure are performed in the solution phase, but they require highly concentrated protein samples—which can sometimes cause nonspecific aggregation—raising questions about their physiological relevance. Also, whereas many proteins can be purified in high yield, solubilized for NMR analyses, and crystallized for X-ray crystallographic analyses, there are also many proteins that have limited solubility and are not easily crystallized. In such cases, MS-based covalent labeling experiments provide a unique means by which to study the structural and biophysical properties of protein folding and ligand binding.

Several of the methods highlighted here (e.g., SUPREX, SPROX, and PLIMSTEX) are directly analogous to spectroscopy-based methods. The SUPREX and SPROX methodologies are analogous to circular dichroism- and fluorescence-based chemical denaturation methods that have been used for decades to measure the thermodynamic properties of protein-folding reactions. Similarly, the PLIMSTEX methodology is analogous to the titration experiments and Scatchard plot analyses that have long been performed using various optical spectroscopy instrumentation to monitor conformational changes upon ligand binding and measure protein–ligand binding affinities. The MS readouts in SUPREX, SPROX, and PLIMSTEX require significantly less material than the spectroscopy-based chemical denaturation and titration methods they parallel. In addition, the MS-based methods are more general in the sense that they are amenable to the analysis of a wide range of binding affinities and ligand classes and that they do not require the protein target and/or ligand to have specific optical properties. In addition, the MS-based approaches are better suited than their spectroscopy-based counterparts to the analysis of proteins in multicomponent protein mixtures.

The covalent labeling methods highlighted here are all similar in that they involve the use of MALDI and/or ESI MS to read out the rate of a covalent labeling reaction. However, there are important differences among the methods. The continuous H/D exchange labeling experiment, SUPREX, and PLIMSTEX all involve a labeling reaction in which amide protons in the protein are exchanged with solvent deuterons. The labeling reaction in SPROX involves the specific oxidation of methionine amino acid side chains and the labeling reaction in hydroxyl radical-mediated footprinting involves the more nonspecific oxidation of a number of different amino acid side chains (see the following text). In continuous H/D exchange labeling and hydroxyl radical-mediated footprinting methods the rate of the covalent modification reaction is monitored as a function of time; in SUPREX and SPROX the rate of the covalent modification reaction is monitored as a function of chemical denaturant concentration; and in PLIMSTEX the rate of the chemical modification reaction is monitored as a function of ligand concentration.

The above-cited differences in the nature of the covalent labeling reaction and in the way the reaction rate is monitored give the different MS-based covalent labeling strategies highlighted here their own strengths and weaknesses for gleaning different kinds of information about the structure, kinetics, and thermodynamics of protein folding and ligand binding. For each of the five methodologies discussed here we provide an overview of the experimental protocols they use and the kind of information they generate. Also, discussed are the relative strengths and weaknesses of the different methods for probing the wide range of conformational states that proteins and protein–ligand complexes can adopt when they are in solution.

# 1. Continuous H/D Exchange Labeling

## The Experiment

The basic protocol for continuous H/D exchange labeling experiments is outlined in Figure 2. The labeling reaction is initiated upon dilution of the fully protonated protein or the fully protonated protein–ligand complex into a $D_2O$-containing buffer. The buffer conditions are generally chosen such that the protein is under native solution conditions. The rate at which the labile protons in the protein are exchanged with solvent deuterons is determined by monitoring the weight gain of the protein as a function of time in mass spectral analyses of either the intact protein or proteolytic peptides of the protein. The hydrogen atoms in proteins that can exchange with solvent deuterium include those at the amide positions and those in the amino acid side chains of Arg, Asn, Asp, Glu, Gln, Lys, and Trp. However, only the deuterium atoms incorporated at amide positions are preserved in the MS readout. In continuous labeling experiments involving H/D exchange the reaction is typically monitored from several seconds to many hours.

The solvent deuterons that exchange into the amide positions of the protein can be localized to different regions of the protein's polypeptide chain using specialized liquid chromatography (LC)-MS-based peptide mapping and sequencing strategies in which the deuterated state of the protein is largely preserved [39]. The LC-MS-based peptide mapping and sequencing strategies currently used to dissect the results of continuous H/D exchange labeling experiments employ acid proteases, low temperatures, and rapid chromatographic methods to minimize the back exchange of deuterons with solvent protons during the protein digestion, peptide separation, and mass analysis steps. With good

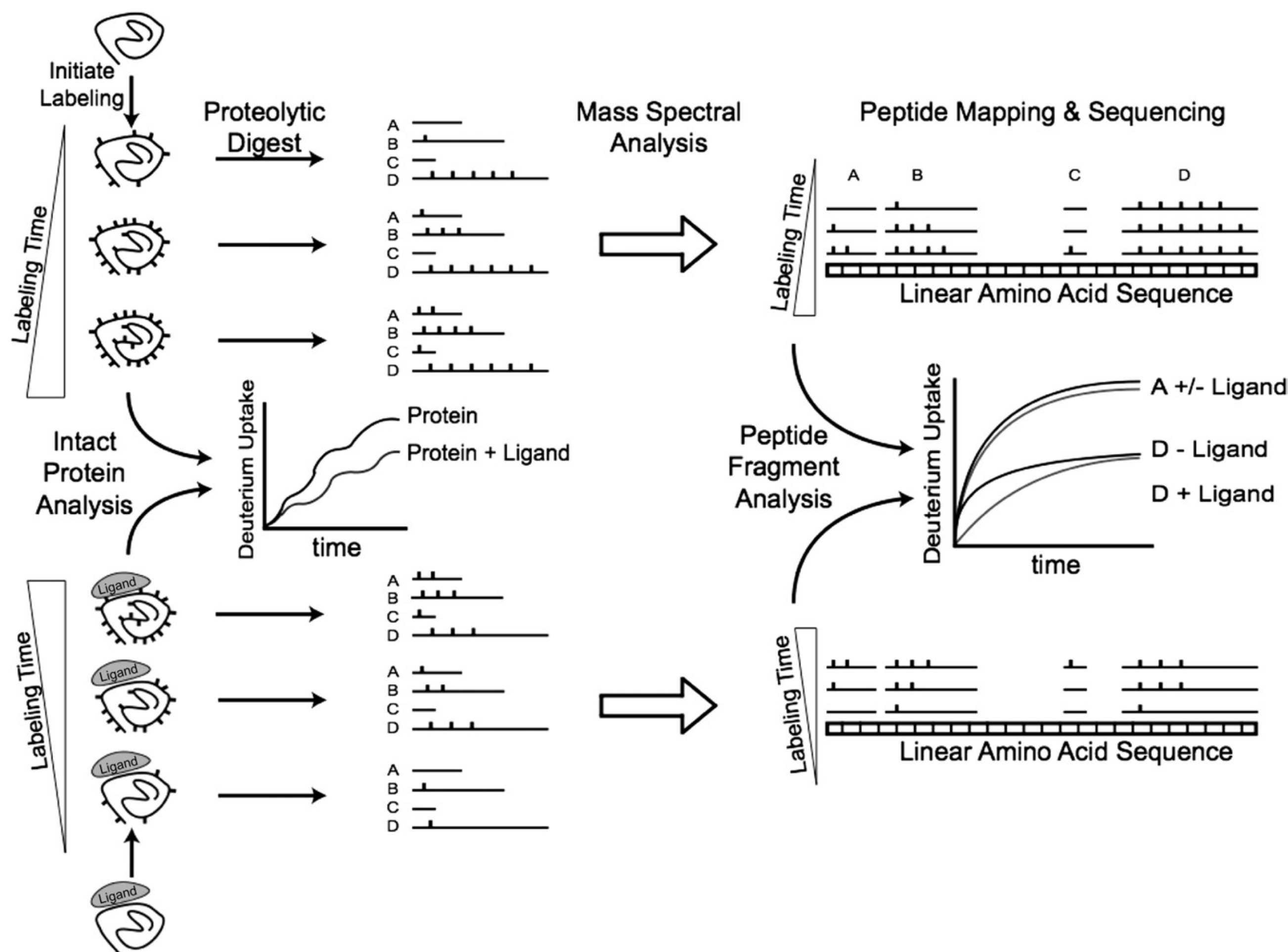
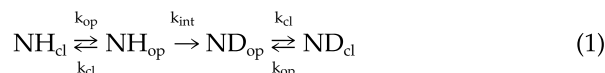



**Figure 2.** The basic protocol used in continuous H/D exchange labeling experiments. The protein or protein–ligand complex is incubated in $D_2O$-containing buffers for various amounts of time before the reaction is quenched. Mass spectral analyses of samples taken at the various time points are used to follow the rate of deuterium uptake for the entire protein or the protein can be digested to follow the rates of deuterium uptake at different regions of the protein sequence. Peptides are typically sequenced and mapped back to specific regions of the protein primary structure. The experiment can be performed in the absence and in the presence of a ligand (see the top half and bottom half of the schematic) and the data from each experiment are compared (see middle of the schematic).

peptide coverage and careful attention to minimize back-exchange, the deuterium uptake in proteins and, ultimately, the structural properties of protein conformational changes, can often be mapped to a resolution of about 10 amino acids.

### *The Information*

The generally accepted mechanism for amide hydrogen exchange in proteins is outlined in the following equation [16]:



$$NH_{cl} \underset{k_{cl}}{\overset{k_{op}}{\rightleftarrows}} NH_{op} \xrightarrow{k_{int}} ND_{op} \underset{k_{op}}{\overset{k_{cl}}{\rightleftarrows}} ND_{cl} \quad (1)$$

In eq 1, $NH_{cl}$ and $NH_{op}$ represent amide protons in their closed and open forms, respectively. In their closed form, amide protons are protected from exchange because they are involved in hydrogen-binding interactions and/or buried in solvent-inaccessible regions of a protein's three-dimensional structure. In their open form, amide protons are exposed to solvent and are free to exchange with solvent deuterons. In eq 1, $k_{op}$ and $k_{cl}$ are the rate constants associated with the protein's opening and closing reactions and $k_{int}$ is the intrinsic exchange rate of an unprotected (i.e., solvent exposed) amide proton.

There are two kinetic limits in the H/D exchange mechanism outlined earlier. In one limit, which is the so-called EX1 exchange condition, the closing reaction is much slower than the exchange rate of an unprotected amide proton (i.e., $k_{cl} \ll k_{int}$). Under EX1 exchange conditions every opening event results in quantitative exchange of the newly exposed proton amides with solvent deuterons. Mass spectra collected under EX1 conditions show a bimodal distribution of the protein (or peptide) ion signals. One mode represents the protein population that has not exchanged because it has not experienced an unfolding event. The other mode represents the protein population that has been subject to unfolding events and the newly exposed amide protons have quantitatively exchanged. Under EX1 exchange conditions the overall rate at which deuterium is incorporated into the protein (i.e., $k_{ex}$) is related to the opening rate, $k_{op}$. In the so-called EX2 exchange condition, the closing reaction is much faster than the exchange rate of an unprotected amide proton ($k_{cl} \gg k_{int}$). Under EX2 exchange conditions many opening and closing events must occur before a protected amide within the protein is quantitatively exchanged. Mass spectra collected under EX2 exchange conditions show one ion signal (or one isotope distribution) for the protein as it gradually increases in mass. Under EX2 exchange conditions $k_{ex}$ is related to the equilibrium constant for the opening and closing reactions.

There is some primary amino acid sequence context to the exchange rates of amide protons in the polypeptide backbone of proteins (e.g., $k_{int}$ values at a given pH and temperature vary over an order of magnitude depending on the chemical functionality in the flanking amino acid side chains) [40]. However, the most significant differences in amide exchange rates measured in a continuous H/D exchange labeling experiment arise from the different degrees of protection that amide protons experience in the protein's three-dimensional structure. This differential protection occurs as a result of the different opening and closing rates of the protein's different conformational changes.

As illustrated in Figure 1 there can be a wide variety of opening and closing events in proteins. The different opening and closing events also occur on a wide range of timescales. The global unfolding/refolding reactions are generally the slowest/fastest and the more local unfolding/refolding reactions are the fastest/slowest. This means that H/D exchange occurring as a result of global unfolding/refolding reactions generally occurs in the EX2 regime and the H/D exchange that occurs as a result of more local unfolding/refolding reactions often occurs in the EX1 regime.

In a continuous H/D exchange labeling experiment the time course of deuterium incorporation is typically multiphasic. The different phases and their amplitudes result from the different amide protons in a protein being subjected to the solvent for different fractions of time, depending on the relative rates of the different opening and closing reactions that give rise to their solvent exposure. In theory, the continuous H/D exchange labeling experiment can be used to ascertain the pseudo-first-order rate constants associated with the exchange of the different amide protons involved in a protein's different opening and closing reactions. Under EX2 exchange conditions the observed rate constants can be related to the equilibrium constant for the opening and closing reaction as described earlier. One complicating issue in such quantitative analysis of H/D exchange data is that a given amide proton may participate in some opening and closing events that are in the EX2 regime and some that are in the EX1 regime. If the event occurs in the EX1 regime then the overall rate of H/D exchange is related to the rate of the unfolding reaction. This complication and the fact that it can be a challenge to resolve the multiphasic H/D exchange behavior of many proteins in the continuous labeling reaction has limited such *quantitative* determinations of thermodynamic and kinetic parameters in the continuous labeling experiment.

The weight gain (i.e., deuterium uptake) versus time data collected in continuous labeling experiments is mostly used in a *semi-quantitative* manner. The relative rates of deuterium incorporation are compared for a given protein under different sets of conditions, most frequently with and without ligand. Differences in the relative rates of deuterium incorporation can be detected at the protein level or at the peptide level after the protein is subject to a protease digestion (see Figure 2). Increased protection observed in H/D exchange studies of protein–ligand binding can come from two sources, including: (1) the direct protection by the

ligand at the binding site and/or (2) protein conformational changes that are induced by ligand binding, but removed from the binding site. A common misinterpretation of H/D exchange results obtained in the presence and absence of ligand is to assume that the regions of the polypeptide chain that experience increased protection are those at the location of the ligand-binding site. In the continuous H/D exchange labeling experiment it is in fact not possible to differentiate the above two sources of increased protection. Amide protons in specific regions of a protein's structure can also experience decreased protection in H/D exchange studies of protein–ligand binding. Such decreased protection is generally attributed to ligand-induced conformational changes that are removed from the binding site.

### *The Advantages and Disadvantages*

The main advantage of continuous labeling H/D exchange is that a wide range of different solution-phase conformations can be probed, from more local fluctuations in structure to more global changes in structure provided that the labeling reaction is monitored for sufficient time. The ability to map such conformational changes at the peptide level is also a major strength of the continuous H/D exchange labeling experiment. The experiment can provide a comprehensive picture of the conformational changes in a protein that are induced by mutation [41, 42], aggregation [43, 44], and/or ligand binding [8, 11, 45]. Often, very subtle conformational changes in different ligand-binding modes are resolved in the relatively large picture that is painted in these experiments [8, 11]. However, continuous H/D exchange labeling experiments are best suited for investigating the detailed conformational properties of relatively small sets of target ligands to specific protein targets.

The primary disadvantage to continuous H/D exchange labeling experiments is that they are time consuming and not inherently high-throughput. Efforts to optimize the throughput of these methods have demonstrated screening rates of up to 10 ligands per day using the continuous H/D exchange labeling experiment [46]. Although such rates are significantly faster than those that can be achieved using other techniques for the structural analysis of protein–ligand binding (e.g., X-ray crystallography and NMR spectroscopy), they are not generally fast enough to be useful in high-throughput screening (HTS) applications where large numbers (i.e., >thousands) of ligands need to be screened for binding. The methodology is also not easily adapted to proteins in complex protein mixtures. Although good peptide coverage (>80%) of single proteins has been achieved in a number of studies using these strategies, the analysis of proteins in multicomponent mixtures using this methodology is challenging. Proof-of-principle results on a four-component protein mixture have been reported [47] and a specialized approach for the analysis of a target protein in a complex mixture has been described [48]. However, it is difficult to envision applications of the methodology to the complex protein mixtures routinely analyzed in proteomic studies. The rapid, low-temperature separations and the need for pepsin, a relatively nonspecific protease, in the digestion reaction would likely limit the number of protein identifications. Another challenge to proteomic applications of continuous H/D exchange labeling experiments is obtaining the extensive peptide coverage that is key to the success of these experiments. Peptide coverage in conventional MS-based proteomic analyses of complex mixtures is typically low, with many protein identifications being made using only two to three peptides. In the future, it is conceivable that alternative chromatographic methods such as supercritical fluid chromatography [49] and orthogonal gas-phase separation methods such as ion mobility mass spectrometry could be used in the continuous H/D exchange labeling experiment to afford the analysis of more complex mixtures.

## 2. Hydroxyl Radical-Mediated Footprinting

### *The Experiment*

The protocol used in hydroxyl radical-mediated footprinting experiments is very similar to that used in continuous labeling H/D exchange experiments. However, more elaborate means than just sample dilution are required to initiate the labeling reaction. The labeling reaction in footprinting experiments is typically initiated with hydroxyl radicals generated by Fenton chemistry [19], by the radiolysis of water [7, 17, 21–23, 50], by electrochemistry [18], or by the UV photolysis of hydrogen peroxide [20, 51]. These different methodologies produce different concentrations of the hydroxyl radical on different timescales.

The exposure times used in footprinting experiments can range from microseconds to hours, depending on how the hydroxyl radicals were generated. Synchrotron radiolysis of water generates a relatively high yield of hydroxyl radical and uses very short labeling times (10–200 ms). Experiments generating hydroxide radicals from Fenton chemistry use longer exposure times (5–30 s) because they produce hydroxyl radicals at slower rates. Electrochemical methods and $\gamma$-rays emitted from $^{60}$Co or $^{137}$Cs particles also produce small amounts of hydroxyl radical and thus require longer exposure times from seconds to many minutes. The exposure times used for footprinting experiments with hydroxyl radicals generated using UV photolysis of hydrogen peroxide vary, depending on the light source. They can be nearly microseconds using laser sources [52, 53] or as long as several hours using a UV lamp [20]. The results of oxidative chemical modification from hydroxyl radical exposure is generally thought to be independent of the technique used for radical production [5, 54, 55], although complications arising from

the chemical reactivity of various reaction by-products have been noted in several methods [56].

The intrinsic chemical reactivity of the hydroxyl radical with the functional groups found in proteins has been well studied over the years [23, 57–59]. Studies have shown that hydroxyl radicals can react with 14 of the 20 naturally occurring amino acid side chains with rate constants ranging from $1.7 \times 10^7$ to $3.5 \times 10^{10}$ $M^{-1}$ $s^{-1}$ [23]. The oxidation products are numerous and have been reviewed elsewhere [5]. Hydroxyl radicals can also lead to nonspecific cleavage of protein backbones. However, these backbone cleavage reactions occur at rates 10–1000 times slower than side-chain oxidation [23, 57, 59]. Most hydroxyl radical-mediated footprinting experiments are conducted under conditions that maximize side-chain modification events and minimize backbone cleavage events. The 14 most commonly modified residues in hydroxyl radical-mediated footprinting experiments are Cys, Met, Trp, Tyr, Phe, His, Leu, Ile, Arg, Lys, Val, Ser, Gln, and Glu.

Hydroxyl radical-mediated footprinting experiments all rely on high-resolution peptide-mapping experiments to identify the specific sites of modification in a protein. This is accomplished by the LC-MS analysis of proteolytic digests of the protein. In these analyses tandem MS experiments on the detected peptides are then used to locate side-chain–specific modification. The time course of the oxidation reaction in hydroxyl radical-mediated footprinting experiments is generally followed by monitoring the disappearance of unmodified peptide ions as well as the appearance of ions from modified peptides in the LC-MS readout. The fraction of unmodified peptide is determined and plotted as a function of time. The data obtained for a given peptide are fit to a first-order rate equation to extract a pseudo-first-order rate constant for the modification reaction of the peptide.

### The Information

A major difference between the continuous H/D exchange labeling experiment and the hydroxyl radical-mediated footprinting experiment is that the oxidation reactions in footprinting experiments are generally probed for a much shorter time than the H/D exchange reaction is probed in the continuous labeling experiment. The short labeling time and fast reaction kinetics in protein oxidation experiments with hydroxyl radicals mean that the sites of modification are those that are solvent exposed or in regions of the protein structure that are highly flexible (e.g., the more local unfolding reactions in Figure 1). In theory, long exposure times could be used in the hydroxyl radical-mediated footprinting experiments to probe the more global conformational changes in proteins. In practice, the use of long labeling times in the footprinting experiment often results in oxidative unfolding of the protein, which in itself can be interesting [60], although it is of limited use for probing the conformational properties of native proteins.

When the timescale of the hydroxy radical-mediated labeling reaction is short (e.g., less than several hundred milliseconds) the measured oxidation rate of a given peptide largely depends on two factors, including: (1) the intrinsic reactivity of the amino acid side chains and (2) the solvent exposure of the amino acid side chains in the protein's folded three-dimensional structure. The rate constants obtained on the different peptide fragments generated in a hydroxyl radical-mediated footprinting experiment are generally used to identify the regions of a protein's polypeptide chain that are either buried or exposed in the protein's three-dimensional structure. For example, the relative rates of two peptides with similarly reactive amino acid side chains can be used to compare the relative surface accessibility of the two regions from which the peptides were derived. The oxidation rate data obtained in footprinting experiments can also be used together with X-ray crystallographic data to obtain unique information about the conformational dynamics of certain systems. For example, peptides from regions of a protein's structure that appear buried in the X-crystallographic data, but that do not have reduced oxidation rates, can be identified as being highly dynamic.

### The Advantages and Disadvantages

A drawback to hydroxyl radical-mediated footprinting experiments is that oxidative covalent labeling of protein side chains can significantly alter the conformational properties of the protein. This is most problematic at long labeling times when the more global fluctuations start to expose buried side chains to hydroxyl radical-enriched solvent. The oxidation of buried side chains through such a mechanism complicates the interpretation of the hydroxyl radical-mediated footprinting experiment. It is for this reason that the oxidation time in hydroxyl radical-mediated footprinting experiments is kept to a minimum. On the one hand, short labeling times in footprinting experiments limit the range of solution-phase conformations that can be probed. On the other hand, the short labeling times account for a unique strength of the experiment—an improved sensitivity to low-amplitude motions that occur on fast timescales.

As in the H/D exchange experiment, LC-MS-based peptide mapping and sequencing strategies provide very useful data on the location of oxidized residues. Hydroxyl radical-mediated footprinting experiments have the technical advantage over continuous H/D exchange labeling experiments in that they do not require specialized LC-MS-based strategies to preserve the label. This means that chromatography conditions can be optimized for maximum peptide resolution in the LC-MS experiment. The stability of the covalent modification in the hydroxyl radical-mediated footprinting experiment also means that the site of modifi-

cation can be readily determined in a tandem MS experiment. The gas-phase scrambling of peptide protons and deuterons is problematic for tandem MS analyses in H/D exchange experiments.

Compared to the continuous H/D exchange labeling experiment, the footprinting experiment also requires the resolution and detection of many more peptide species in the LC-MS readout. This is because the radical oxidation of any given peptide often yields a number of different reaction products, with varied molecular weights and retention times in the LC-MS readout. Thus, like the continuous H/D exchange labeling experiment, the hydroxyl radical-mediated footprinting experiment is largely restricted to the analysis of proteins in relatively simple mixtures.

## 3. SUPREX

### *The Experiment*

The basic SUPREX protocol is outlined in Figure 3. The experiment begins with the distribution of a protein or protein–ligand complex into a series of deuterated H/D exchange buffers that contain increasing concentrations of a chemical denaturant such as guanidinium chloride (GdmCl) or urea. The protein samples in the series of exchange buffers are allowed to undergo H/D exchange for a specified amount of time. After this specified H/D exchange time (a time that is the same for each protein-containing exchange buffer in the series), the mass of each deuterated protein sample is determined using MALDI-MS. Ultimately, the deuterium content (i.e., ΔMass value) is determined for the protein in each denaturant-containing H/D exchange buffer; these values are then used to generate a SUPREX curve (i.e., a plot of ΔMass versus [denaturant]) at a specific exchange time (see Figure 4).

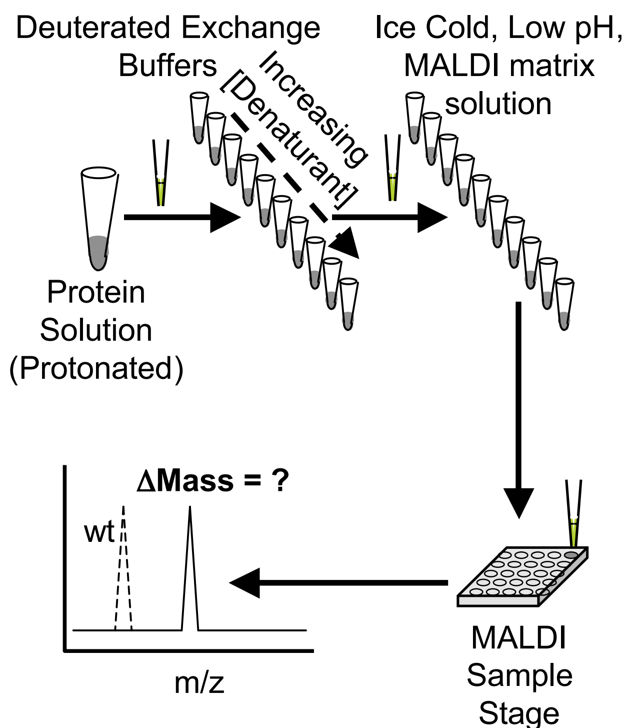



**Figure 3.** The basic protocol used in SUPREX experiments. The H/D exchange reaction is allowed to proceed for a specific time in a series of deuterated buffers with increasing concentrations of denaturant. After the reactions are quenched, the samples are prepared for MALDI analysis and the ΔMass value is calculated by subtracting the mass of the unmodified protein from the mass determined in the MALDI analysis.

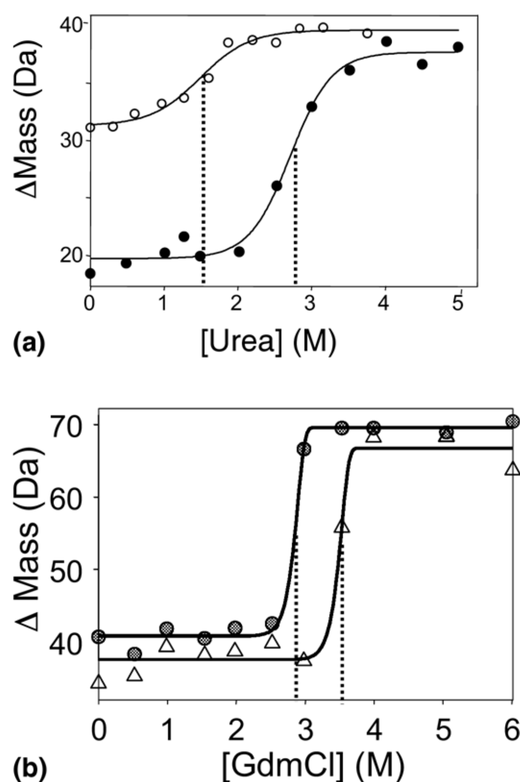



**Figure 4.** SUPREX data generated for two model proteins with and without ligand. The dotted lines mark the denaturant concentration at the transition midpoint of each curve shift (i.e., the $C^{1/2}$ value). (**a**) SUPREX data obtained for an SH3 domain in the presence and the absence of a peptide. In this case the ligand-binding event results in a measurable degree of amide protection, as evidenced by the different pretransition baselines of the two curves. (**b**) SUPREX data for tryptophan repressor in the presence and the absence of tryptophan. Although there is little to no amide protection as a result of this ligand-binding event, a measurable shift in the $C^{1/2}$ value is observed in the SUPREX experiment. Data in (**a**) and (**b**) are taken from Powell et al. ([63] and [27], respectively).

SUPREX experiments can also be performed using a strategy that incorporates a protease digestion step into the basic SUPREX protocol [61]. This protocol significantly expands the application of SUPREX to large multidomain protein systems. The SUPREX protease digestion protocol is similar to the basic SUPREX protocol (see Figure 3), except that it contains a protease digestion step *after* the H/D exchange step in the deuterated buffers and *before* the protein is transferred to the MALDI matrix solution. The purpose of the

protease digestion step is to *partially* digest the protein and generate peptide fragments from the various domains.

Useful peptide fragments for the SUPREX with protease digestion protocol are those that include buried regions of a specific domain. Such fragments, which are globally protected from H/D exchange in the context of the individual domain and subglobally protected in the context of the intact protein, will have a denaturant dependence on their H/D exchange behavior (i.e., have a SUPREX transition). Peptide fragments that are not useful in the protease digestion protocol include those fragments from regions of the protein that are subject to local fluctuations in structure and/or that are solvent accessible. Such peptide fragments exchange their amide protons for solvent deuterons, but their H/D exchange rate is not denaturant dependent (i.e., a SUPREX curve would not be generated).

### The Information

As in the time-dependent H/D exchange experiments outlined earlier, all of a protein's opening and closing reactions provide a mechanism for deuterium incorporation into the protein in a SUPREX experiment. However, the H/D exchange rates of the more locally protected and fast exchanging amide protons in a protein are not nearly as sensitive to the denaturant concentration as the exchange rates of the more globally protected and slow exchanging amide protons in a protein. Therefore, the denaturant in SUPREX selectively accelerates the H/D exchange rates of the globally protected amide protons in a protein. Thus, the denaturant dependence on the ΔMass values in a SUPREX curve (i.e., the transition region of the curve) is exclusively defined by the H/D exchange properties of the globally protected amide protons. Thus, SUPREX is not a useful probe of the more local conformational changes in proteins. More local conformational changes are best probed using the continuous H/D exchange labeling experiment. The SUPREX experiment is designed to evaluate the thermodynamic properties of the global unfolding/refolding reactions in proteins.

In cases where the global unfolding/refolding reaction of a protein is well modeled by a two-state transition (i.e., partially folded intermediate states of the protein are not populated), SUPREX provides reasonably accurate $\Delta G_f$ and *m*-values for the transition [24, 27, 31, 32]. Such SUPREX-derived $\Delta G_f$ and *m*-values are especially useful for the quantitation of protein–ligand binding affinities. SUPREX measurements of a protein's $\Delta G_f$ and *m*-value made in the presence and in the absence of a ligand can be used to calculate a $\Delta\Delta G_f$ value between the protein and protein–ligand complex (i.e., the binding free energy) [27, 31, 32, 62, 63].

In the case of proteins that have a cooperative unfolding/refolding transition, albeit non-two-state (i.e., partially folded conformations of the protein are in equilibrium with the folded and unfolded states), the SUPREX experiment can still yield useful thermodynamic information. SUPREX-derived $\Delta G_f$ and *m*-values on non-two-state folding proteins are not meaningful in themselves. However, the values can be used in a relative sense (i.e., to calculate $\Delta\Delta G_f$ values). For example, the $\Delta G_f$ values extracted from SUPREX analyses performed on proteins in the absence and in the presence of a target ligand can be used to calculate a binding free energy for the ligand [30, 31].

In the case of proteins that do not have cooperative unfolding/refolding reactions (e.g., the different domains of a protein do not fold/unfold in a concerted manner) the basic SUPREX protocol does not produce useful information. Such protein systems must be analyzed by the SUPREX with protease digestion protocol outlined earlier [61]. The SUPREX behavior of peptide fragments generated in the SUPREX with protease digestion protocol can be used to report on the biophysical properties of the individual domains from which they were derived. In the SUPREX with protease digestion protocol, any peptide that can be either entirely or partially mapped to solvent-inaccessible (i.e., "buried") regions of a particular domain can be used to report on the domain's thermodynamic properties. For an individual domain's unfolding/refolding reaction to be effectively probed using the SUPREX with protease digestion protocol it must undergo a relatively large amplitude motion (i.e., involve the exposure/burial of a significant amount of hydrophobic surface area). Peptides generated from regions of a protein's structure that undergo relatively small amplitude motions (i.e., involve only very local unfolding reactions) will not show a denaturant dependence on their H/D exchange behavior in the SUPREX with protease digestion experiment [40, 64].

SUPREX experiments using either the basic protocol or the protease digestion protocol provide very little information about the detailed structural changes that proteins may undergo upon ligand binding. The thermodynamic parameters extracted in the SUPREX experiment are those that describe the more global changes induced by ligand binding. For example, in cases where the binding interaction results in the burial of a large amount of hydrophobic service area (e.g., about hundreds of angstroms) then a change in *m*-value may be observed for the protein when it is complexed with ligand. The magnitude of the *m*-value change can be used to estimate the amount of hydrophobic surface area buried [65]. Ligand-binding events that bury additional amide protons can also be detected by the decrease in the pretransition baseline of a protein's SUPREX curve upon ligand binding (see Figure 4). However, such increased protection and/or hydrophobic surface area burial is not a requirement for the detection and quantitation of protein–ligand binding by SUPREX (see Figure 4). Protein–ligand binding detection and quantitation in SUPREX is accomplished by recording the transition midpoint shift of a protein's SUPREX curve to higher denaturant concentrations in

the presence of ligand; the shift in the transition midpoint is then used to quantify the ligand-binding affinity, which is the primary use of SUPREX-derived thermodynamic parameters.

### The Advantages and Disadvantages

One advantage of SUPREX is that unpurified proteins can be analyzed so long as other components in the mixture do not suppress the protein's ion signal in the MALDI readout. The ability to analyze unpure proteins is experimentally convenient because it eliminates the often time-consuming task of preparing highly purified protein. It also creates the opportunity to study proteins and protein–ligand interactions in a more biologically relevant context. The SUPREX experiment has been effectively used to study the thermodynamic properties of multicomponent protein complexes, the thermodynamics of protein folding in vivo [66], and the strength of protein–ligand binding interactions under the ex vivo conditions of cell lysates [32, 67]. An important caveat to the SUPREX analysis of proteins in complex mixtures is that the ion signals of the proteins of interest must be identifiable in the MALDI readout. This is an important constraint for applications of SUPREX to proteins in exceedingly complex mixtures, such as crude cell lysates, for example, in which the protein ion signals may be poorly resolved or difficult to assign based solely on an intact molecular weight analysis. Nonetheless, there are several examples in the literature where selected proteins from cell lysates have been successfully analyzed. These examples have included cases in which the protein was overexpressed in *E. coli* [66, 68] and in which the protein was at endogenous levels [32, 67].

Probably the biggest advantage of SUPREX over the other methodologies highlighted here is that it is the most amenable to high-throughput analyses. The results of a recent HTS experiment conducted in our laboratory demonstrated the speed and efficiency with which SUPREX can be used to screen large numbers of ligands for binding to a target protein [69]. This work used a single-point SUPREX protocol [70] in which binding events were detected by measuring the target protein's mass change after H/D exchange in a single SUPREX buffer containing a specific chemical denaturant concentration. Screening of an 880-member library was accomplished at a rate of 3 min/ligand using a conventional MALDI-time of flight mass spectrometer. One of the bottlenecks in HTS screening experiments with SUPREX is the rate at which MALDI spectra can be accumulated. The use of high-throughput MALDI mass spectrometers equipped with fast sample positioning mechanisms and high repetition rate lasers has the potential to increase speed of HTS by SUPREX to less than 20 s/ligand.

The speed and efficiency of SUPREX in HTS applications is competitive with many existing technologies for the detection of protein–ligand binding interactions in HTS platforms. The assays currently used in HTS platforms typically rely on radiometric, spectroscopic, or cell-based readouts. Advantages of the SUPREX assay over many existing assays are that it can be performed on protein–ligand complexes directly in solution without immobilization or labeling of the target or library compounds. Many existing HTS assays also require that the protein target already have a known ligand or substrate. The SUPREX assay is especially well suited for protein targets with no known ligands, or where the goal of the HTS project is to discover new ligands to a target protein that have novel binding properties. Ligand selection in more typical assays that require a known ligand or substrate is limited to those ligands that target a specific protein site and/or enzymatic activity. It is perhaps an advantage or disadvantage (depending on the goal of the HTS project) that the SUPREX assay selects for ligands that directly interact with the target protein regardless of their binding site and/or binding mode.

The speed of SUPREX analyses comes at the expense of structural resolution. SUPREX provides very little structural information about protein folding and ligand binding. This is in contrast to the continuous H/D exchange labeling and hydroxyl radical-mediated footprinting methodologies, which are much more time consuming to perform attributed in large part to the peptide-mapping steps, but generate much higher structural resolution of ligand-binding–induced changes. The SUPREX with protease digestion protocol does provide some structural resolution to the conformational changes detected, but the information is confined to the level of individual domains. It is important to emphasize that the peptide fragments generated in the SUPREX with protease digestion protocol *are not* used to map the specific sites of amide H/D exchange in the intact protein, as is often done in the continuous H/D exchange labeling experiment [16, 39, 71–73]. Thus, in contrast to those experiments, it is not necessary to perform the challenging and time-consuming task of obtaining a detailed peptide map of the target protein. Rather, it is important to generate only at least one appropriate peptide (see earlier text) from a given domain. This requires only a minimal amount of peptide mapping. Thus, even the SUPREX with protease digestion protocol has considerably lower resolution than that of the continuous H/D exchange labeling experiment.

## 4. PLIMSTEX

### The Experiment

In PLIMSTEX [34–36, 38, 74] the target protein is equilibrated with increasing concentrations of ligand in aqueous buffer to create a series of samples with varying [ligand]/[protein] ratios. Each sample is diluted into a deuterated buffer under native solution conditions and the protein is allowed to exchange for a set time. The time, which is generally determined from a previously

performed continuous labeling H/D experiment, is set such that there is a maximum difference between the deuterium uptake of the protein when it is alone in solution and when it is complexed with ligand. After the H/D exchange reaction proceeds for the specified time, the deuterium content of the protein in each sample is measured in a mass spectrometry readout. LC-MS readouts of the intact protein and of proteolytic peptide fragments generated from the protein have been successfully used in PLIMSTEX analyses [34]. Ultimately, the deuterium uptake in the protein and/or in specific peptides from the protein is plotted as a function of the [ligand]/[protein] ratio. The resulting data are fit to appropriate equations to generate ligand-binding constants [36].

## *The Information*

The PLIMSTEX experiment is primarily designed to measure the binding affinities of protein–ligand complexes. The models that have been developed to analyze the raw titration data in PLIMSTEX experiments [36] require only two assumptions, including: (1) that the ligand bind in a stepwise fashion and (2) that the H/D exchange of each amide hydrogen is independent. The values of three unknown parameters are extracted from the raw titration data. One parameter is the overall binding constant of the ligand. The other two parameters are deuterium shifts associated with the protein. One deuterium shift is defined by the number of deuterons that exchange into the apo-protein during the specified H/D exchange time (see $D_0$ in Figure 5). The other deuterium shift is defined by $\Delta D$, the number of deuterons that are either protected or exposed upon ligand binding.

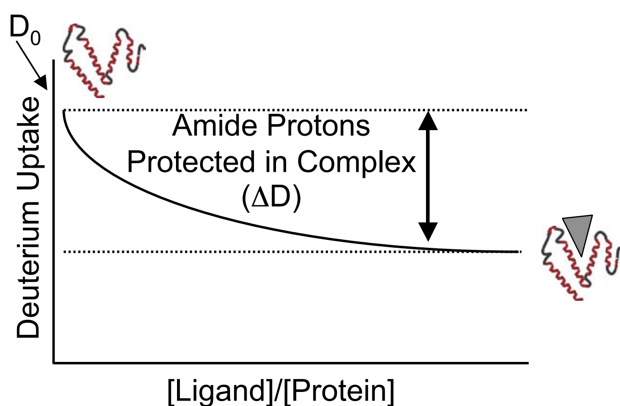



**Figure 5.** Schematic representation of data obtained in a PLIMSTEX experiment. The $D_0$ value represents the total deuterium uptake of the protein in the absence of ligand. The $\Delta D$ represents the number of amide protons in the protein that are protected from exchange when the protein is complexed with ligand.

## *The Advantages and Disadvantages*

PLIMSTEX generates much of the same thermodynamic information about the strength of protein–ligand binding affinities as does SUPREX. One advantage of PLIMSTEX over SUPREX for measuring protein–ligand binding affinities is that PLIMSTEX does not involve the use of denaturant. In some cases the chemical denaturant can alter or even preclude a ligand-binding interaction. For example, the use of guanidinium chloride in the SUPREX experiment precluded the binding of DNA to a transcription factor [62], although the SUPREX analysis of protein–DNA interactions can be effectively probed using urea as the denaturant [62]. Another important advantage of PLIMSTEX over SUPREX is that the modeling methods in PLIMSTEX permit the evaluation of multiple macroscopic binding constants, provided each binding event results in a measurable amount of either increased or decreased amide protection. In SUPREX the overall thermodynamics associated with the binding of ligand to multiple sites in the protein can be quantified in the measured binding free energy. In theory, the ligand concentration dependence to such SUPREX-derived binding free-energy measurements could be used to ascertain the relative binding affinities of multiple ligand-binding sites in a protein. In practice, the accuracy of SUPREX-derived binding free energies is not currently good enough to make such determinations. SUPREX-derived binding free energies can be converted into accurate dissociation constants only when the binding stoichiometry is 1:1. Unlike PLIMSTEX, it cannot be used to determine the binding stoichiometry. However, the use more accurate and higher-resolution mass spectrometers (e.g., Fourier transform ion cyclotron resonance instruments) in the SUPREX experiment may make such determinations possible.

The primary disadvantage to the use of PLIMSTEX is that it is limited to the analysis of ligand-binding interactions in proteins that produce a measurable change in the number of protected amide protons (i.e., the $\Delta D$ value in Figure 5). The $\Delta D_1$ value is what gives rise to the amplitude of the titration curve. Therefore, a critical first step in PLIMSTEX is to identify an H/D exchange time in which there is a measurable difference in the deuterium uptake of the protein alone and the protein complexed with ligand. Such a measurable difference is easy to measure when the protein–ligand binding event protects new amide protons in the protein. However, our experience with SUPREX indicates that there are a number of protein–ligand binding events that do not protect additional amide protons, but rather just increase the protection of already globally protected amide protons (e.g., the ligand-binding data in Figure 4b in which there is no shift in the pretransition baseline of the protein's SUPREX curve upon ligand binding). Such increased global protection may not produce measurable $\Delta D$ values on the timescale of PLIMSTEX experiments. This is a potential drawback to

the use of PLIMSTEX in HTS screening applications where it would be unknown, irrespective of whether the ligand was missed because of a lack of binding or because of the lack of increased protection in the binding event. Also, the throughput of PLIMSTEX for such HTS applications remains untested. The need to analyze multiple [ligand]/[protein] ratios is more time and material intensive than the single-point SUPREX protocol, although a "single-point" PLIMSTEX protocol is possible and could mitigate this latter issue.

## 5. SPROX

### *The Experiment*

The SPROX experiment is very similar to the SUPREX experiment. In SPROX the target protein is diluted into aqueous buffers containing increasing amounts of denaturant and a constant amount of hydrogen peroxide. The oxidation reaction conditions (i.e., the time and $H_2O_2$ concentration) are tuned such that the primary site of oxidation in the protein target is the thioether group in the side chain of methionine residues. The $H_2O_2$ concentration and the reaction time are chosen such that the pseudo-first-order oxidation reaction of an unprotected methionine residue will proceed for 2–3 half-lives. After the oxidation reaction proceeds for the specific time, it is quenched (e.g., by the addition of catalase and/or free methionine) and submitted to either a MALDI or ESI mass spectral analysis to determine a weight-averaged ΔMass value ($\Delta Mass_{wt,av}$ value) of the protein at each denaturant concentration. The $\Delta Mass_{wt,av}$ value is determined using MALDI- or ESI-generated ion signal intensities from the unoxidized and oxidized protein. Ultimately, a SPROX curve is generated by plotting the $\Delta Mass_{wt,av}$ values of the protein versus the denaturant concentration; $\Delta G_f$ and *m*-values are then extracted from the $\Delta Mass_{wt,av}$ versus [Denaturant] data using data analysis methods [33] that are analogous to those used to analyze SUPREX data.

### *The Information*

All of the methionine residues in a protein are susceptible to oxidation in the SPROX experiment. Solvent-accessible methionine residues are readily oxidized to the sulfoxide in the SPROX experiment. However, the buried methionine residues are oxidized at a rate related to the opening and closing rates of the large-amplitude global unfolding/refolding events. The oxidation reaction mechanism for the globally protected methionine residues in the SPROX experiment is directly analogous to the H/D exchange mechanism of the globally protected amide protons in the SUPREX experiment (see eq 1). Therefore, just like the H/D exchange rates of the globally protected amide protons in SUPREX, the oxidation rates of the globally protected methionine residues in SPROX are denaturant dependent.

The intrinsic rate at which unprotected methionine residues are oxidized in a SPROX experiment (e.g., the pseudo-first-order rate constant is 0.0014 $s^{-1}$ when the $H_2O_2$ concentration is 0.1 M [33]) is relatively slow compared with the intrinsic rate at which amide protons are exchanged for solvent deuterons in the SUPREX experiment (e.g., the pseudo-first-order rate constant is 0.04 $s^{-1}$ at pH 7.4 and room temperature [68]). This means the SPROX experiment, like the SUPREX experiment, is performed in the so-called EX2 regime. Thus, analogous to the EX2 regime in the continuous labeling H/D exchange experiment, the rate of the covalent labeling reaction in SPROX can be related to the equilibrium constant of the protein's opening and closing reactions. Also, as in the SUPREX experiment, the denaturant in the SPROX experiment ensures that the opening and closing reactions being probed are the more global unfolding/refolding reactions of the protein. The measured oxidation rates of the globally protected methionine residues in SPROX are ultimately used to derive the same thermodynamic parameters ($\Delta G_f$-, *m*-, and $K_d$-values) that the H/D exchange rates of the globally protected amide protons in SUPREX are used to derive.

### *The Advantages and Disadvantages*

SPROX and SUPREX share many of the same advantages and disadvantages. Although neither technique provides site-specific information about the conformational changes in proteins that are induced by ligand binding, both techniques do provide a quantitative measure of the $\Delta G_f$ and *m*-values associated with the global unfolding/refolding reactions in proteins and protein–ligand complexes. The accurate determination of $\Delta G_f$ and *m*-values by both techniques also requires that the rate of the modification reaction be slower than the refolding reaction and that the denaturant-induced equilibrium unfolding/folding reaction of the protein under study be well modeled by a reversible, two-state process (i.e., partially folded intermediate state(s) are not significantly populated). In the case of non-two-state folding proteins both techniques also have the potential to measure accurate binding free energies, even though the $\Delta G_f$ and *m*-values generated by the techniques are not meaningful.

The SPROX technique does have several additional caveats. The SPROX experiment requires that the protein or protein domain under study have at least one globally protected methionine. The frequency of methionine residues noted in several proteomes (e.g., 2.5% in *E. coli* [75]) suggests that protein domains, which typically range in size from 50 to 150 amino acids, are expected to contain 1 to 3 methionine residues on average. Limiting the covalent labeling reaction in SPROX to a single, low-frequency amino acid residue in proteins has the advantage that it minimizes potential structural perturbations that may be caused by the label. On the other hand, it has the disadvantage that it

provides only a relatively low resolution probe for studying conformational changes in proteins.

Of all of the techniques discussed within this review, SPROX holds the most promise for the analysis of proteins in exceedingly complex mixtures such as those that are routinely analyzed in proteomic applications. We envision the use of SPROX to generate thermodynamic information about the protein folding and ligand-binding properties of proteins in complex mixtures such as cell lysates. The experiments we envision involve subjecting the intact proteins in a cell lysate or other biological mixture to oxidation at different chemical denaturant concentrations, quenching the oxidation reaction in each denaturant-containing SPROX buffer, and ultimately submitting the proteins in each denaturant-containing SPROX buffer to a conventional proteomics platform to quantify the denaturant dependence of the oxidation reaction in each protein (i.e., following the appearance of oxidized methionine-containing peptide and/or the disappearance of unoxidized methionine-containing peptides). Label loss during the conventional gel-based and/or LC-MS-based chromatographic separations used in proteomics platforms would not be an issue and it would not be necessary to generate extensive peptide maps of each protein. In theory, thermodynamic data could be generated on each protein in the mixture that was identified with a methionine-containing peptide in the proteomics experiment. Similar to the peptides generated in the SUPREX with protease digestion protocol described earlier, the SPROX behavior of the detected methionine-containing peptides would report on the thermodynamic properties of the proteins from which they were derived. In this way it would be possible to simultaneously generate thermodynamic information on the many proteins in a complex cell lysate. The results of experiments performed both in the presence and in the absence of specific ligands could then be used to discover the protein targets of the ligand.

## Closing Remarks and Summary

There are general advantages to using the five covalent-labeling MS-based approaches outlined here over the other more conventional spectroscopic or calorimetric techniques that have long been used to characterize the conformations of proteins and protein–ligand complexes. The biggest advantage of the above-cited techniques is arguably their ability to handle small amounts of protein. The techniques outlined earlier also have their own unique advantages and disadvantages. The time-dependent techniques, such as continuous H/D exchange and hydroxyl radical-mediated footprinting, provide the most detailed structural information about protein conformations in solution. However, they are relatively low throughput and not easily used for the analysis of proteins in complex mixtures. SUPREX, PLIMSTEX, and SPROX provide little structural information. A strength of these techniques is their ability to quantify the thermodynamic properties of proteins and protein–ligand complexes, particularly the dissociation constants for protein–ligand complexes. The SUPREX and SPROX methodologies show the most promise of the techniques highlighted here for high-throughput screening applications. In addition to its high-throughput screening potential, the SPROX methodology holds the most promise for studying protein folding and ligand binding on the proteomic scale.

It is important to note that the five covalent labeling MS-based approaches outlined here are at different stages of development. The continuous H/D exchange experiment has been practiced now for well over a decade and it is arguably the most widely practiced and robust of the approaches. The hydroxyl radical footprinting experiment has been the subject of much detailed development over the last decade and it is being used very successfully in an increasing number of "real-world" applications. SUPREX, PLIMSTEX, and SPROX are the youngest of the approaches described here, with SUPREX and SPROX being the most and least mature of these three approaches, respectively. It is only in recent years that the SUPREX and PLIMSTEX experiments have progressed beyond proof-of-principle studies and begun to see use in "real-world" applications. To date, there has been only one publication describing the SPROX methodology. Clearly there is no one perfect technique for painting the picture of a protein—and it is very likely that all five of the approaches outlined here will make important brush strokes in protein paintings of the future.

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