High Resolution, High-Throughput Amide Deuterium Exchange-Mass Spectrometry (DXMS) Determination of Protein Binding Site Structure and Dynamics: Utility in Pharmaceutical Design

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Abstract   Mass spectrometry-based peptide amide deuterium exchange techniques have proven to be increasingly powerful tools with which protein structure and function can be studied, and are unparalleled in their ability to probe sub-molecular protein dynamics. Despite this promise, the methodology has remained labor-intensive and time consuming, with substantial limitations in comprehensiveness (the extent to which target protein sequence is covered with measurable peptide fragments) and resolution (the degree to which exchange measurements can be ascribed to particular amides). I have developed and integrated a number of improvements to these methodologies into an automated high throughput, high resolution system termed Deuterium Exchange Mass Spectrometry (DXMS). With DXMS, complete sequence coverage and single-amide (amino acid) resolution are now rapidly accomplished. DXMS is designed to work well with large proteins and when only small amounts of material are available for study. Studies can be performed upon a receptor-ligand pair as they exist on or within a living cell (in vivo) without prior purification, allowing effective in situ study of integral membrane protein receptors. We have ambitious initiatives underway to make DXMS widely available both for basic academic research studies and commercial drug discovery efforts. In this paper I present an overview of DXMS technology and highlight some of the benefits it will provide in drug discovery and basic proteomics research. J. Cell. Biochem. Suppl. 37: 89–98, 2001.   © 2002 Wiley-Liss, Inc.

Key words: proteomics; protein structure; protein function; protein dynamics; drug discovery combinatorial chemistry; hydrogen exchange; deuterium exchange; mass spectrometry

Peptide amide proton exchange has proven to be an increasingly powerful method by which protein dynamics, structure, and function can be studied. It provides information that amplifies and refines inferences drawn from high-resolution structural studies, and can provide unique insights when reliable structural information is unavailable. Tandem liquid-chromatography-mass spectrometry-based deuterium exchange methodologies, developed over the past 10 years, presently provide the most effective approach to study proteins larger that 20–30 kd in size. Proteolytic and/or collision-induced dissociation (CID)-based fragmentation of deuterium-exchanged protein allows exchange behavior to be roughly mapped to subregions of the studied protein. There is every expectation that deuterium-exchange-based studies performed with such methods will play central roles in deciphering proteomic structure, function, and dynamics over the next decade. Despite this promise, mass spectrometry-based peptide amide proton exchange has remained a labor-intensive and time consuming technique, with substantial limitations in comprehensiveness (the extent to which target protein sequence is covered with measurable peptide fragments) and resolution (the degree to which exchange measurements can be ascribed to particular amides).

Building upon the pioneering work of Water Englander, David Smith, and co-workers, we

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have developed and implemented a number of improvements to these methodologies and experimental equipment collectively termed Deuterium Exchange Mass Spectrometry (DXMS). With DXMS, complete sequence coverage, and single-amide resolution are now rapidly accomplished. I will first present an overview of DXMS technology, and then highlight some of the benefits it will provide in drug discovery efforts and basic proteomic studies.

PEPTIDE AMIDE HYDROGEN EXCHANGE

Study of Protein Submolecular Thermodynamics by Hydrogen Exchange

For more than 30 years, hydrogen-exchange techniques have been employed to study the thermodynamics of protein conformational change and to probe the processes by which polypeptide chains fold upon themselves to form structured proteins (reviewed in [Englander et al., 1997; Engen and Smith, 2001]). "Hydrogen-exchange" reflects the fact that many hydrogens (so-called acidic hydrogens such as OH, NH_2, SH, and peptide amide hydrogen) are not permanently attached to the protein, but continuously and reversibly interchange with hydrogen present in water. Most acidic hydrogen exchanges too rapidly to be experimentally useful. An important exception is the more slowly exchanging peptide amide hydrogen (main chain amide hydrogen) present in every amino acid residue except proline.

The hydrogen exchange reaction can be experimentally followed by using tritiated or deuterated water. The chemical mechanisms of the exchange reactions are understood, and several well-defined factors can profoundly alter exchange rates. One of these factors is the extent to which a particular exchangeable hydrogen is exposed (accessible) to water. The exchange reaction proceeds efficiently only when a particular peptide amide hydrogen is fully exposed to solvent water. In a completely denatured (unfolded) polypeptide chain, all peptide amide hydrogens are maximally accessible to water and exchange at their maximal possible rate, with a half-life in the range of 1 sec at 0°C and pH 7.0. In a folded protein, most peptide amide hydrogens are not efficiently exposed to solvent water and exchange rates are slower, often much slower, than this maximal exchange rate. The ratio of exchange rates for a particular amide hydrogen before and after

protein folding is referred to as the exchange protection factor, and directly reflects the free energy change in the atomic environment of that particular hydrogen upon folding. In this sense, amide hydrogens can be used as atomic scale sensors of highly localized free energy changes.

Walter Englander and colleagues pioneered a "medium resolution" tritium exchange method that has proven to be a powerful tool to study protein folding and allostery in simple model proteins [Englander and Englander, 1978; Englander and Kallenbach, 1984; Englander et al., 1985; Englander and Englander, 1994; Bai et al., 1995]. He also devised an approach by which deuterium exchange in protein–protein interactions could, to a limited degree, be followed if the proteins were amenable to NMR analysis. More recently, David Smith and colleagues pioneered a method whereby mass spectrometry is employed to localize exchanged deuterium in proteins [Zhang and Smith, 1993; Dharmasiri and Smith, 1996; Smith et al., 1997; Deng and Smith, 1998; Deng et al., 1999; Engen and Smith, 2001]. Both approaches accomplish localization by the use of the enzyme pepsin to fragment exchange-labeled peptides that have had exchange slowed (quenched) by low pH and temperature, allowing rough localization of label to any of the 10–25 possible amide positions within each pepsin-generated peptide. For years, attempts to improve the localization of label within the pepsin-generated peptides had uniformly met with failure. Considerable difficulty has also been encountered in identifying fragment peptides that substantially represent the complete amino acid sequence of target proteins. For these and other reasons hydrogen exchange study has remained a labor-intensive, time-consuming approach with limited application to studies of protein–protein binding interactions beyond simple model systems.

Adaptation of Medium Resolution Techniques to Study of Receptor Binding Surfaces

A decade ago, I set out to improve, simplify, and automate hydrogen exchange techniques with the aim of developing a robust tool with which to study large protein–protein binding interactions at high resolution, most particularly the structure and dynamics of the ligand binding sites of integrin adhesion receptors. These remarkable cell-surface proteins have several properties (large size, conformational

flexibility, membrane integration) that make them particularly refractory to conventional structural analysis techniques.

The result has been the development of an automated hydrogen exchange-liquid chromatography-mass spectrometry-based technology (DXMS) that is well suited to the high throughput-high resolution study of protein–protein binding interactions, as well as studies of isolated proteins. Many of the improvements have focused on the protein chemistry employed. Improved quench-compatible methods of denaturation and disulfide bond reduction were developed and additional proteinases identified that work robustly under quench conditions, work in solid phase, and fragment protein substrates with patterns distinct and complementary to those produced by the action of pepsin [Woods, 1997, 2001]. Other improvements, such as extensive use of solid-state enzymes, perfusive column chromatography, and robotics that rapidly manipulate the temperatures and composition of samples, have resulted in an integrated apparatus that performs virtually all of the sample manipulation and data acquisition automatically. Much of the proof-of principle studies performed to validate these improvements were funded by University of California BioStar Technology transfer grants.

DXMS TECHNOLOGY

Overview

The technique has two distinct steps. There is an initial functional labeling step, performed under entirely physiologic conditions of pH, ionic strength, and buffer salts (Fig. 1); and a subsequent localization step, performed under very non-physiologic conditions (Fig. 2). The initial functional labeling step can be performed in a number of ways; in one commonly used mode, deuterated water is added to a solution of the receptor protein. During this *on-exchange* incubation, deuterium exchanges onto the amides of the receptor protein, including the surface of any potential binding region. Ligand is then added, trapping some of the receptor-bound deuterium between the receptor and ligand interaction surfaces. The receptor-ligand complex is then transferred to, and incubated in a solution without deuterated water, during which time deuterium *off-exchanges* from the receptor protein just as fast as it originally
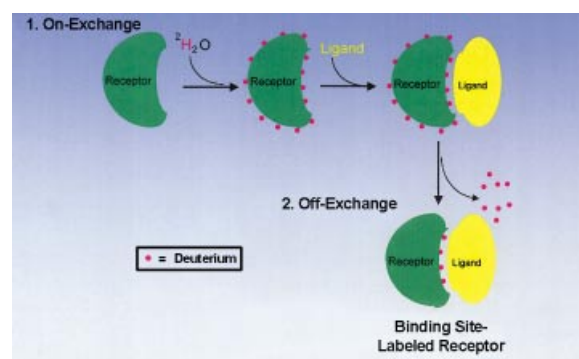

Fig. 1. Functional labeling with deuterium.

exchanged on, except for deuterium that is trapped between the receptor-ligand interface that is no longer solvent water molecule-accessible. After a long period of off-exchange, the result is a receptor protein that has deuterium attached only to each of the amino acids that make up its interaction surface with the ligand (Fig. 1).

In the second step, we determine the precise locations of attached deuterium label within the known amino acid sequence of the receptor (Fig. 2). This is done by first shifting the labeled protein to conditions that simultaneously denature and reduce it and dramatically slow the rate of exchange of all peptide amide hydrogens, effectively locking in place the attached deuterium label. The protein is then proteolyzed by pepsin into fragments of ∼10–20 amino acids in size. The digests are subjected to rapid high performance liquid chromatography (HPLC) separation, and directly analyzed by electrospray-ion trap or time of flight (TOF) mass spectrometry performed under conditions adapted to amide exchange studies. Pepsin-generated peptides that contain deuterium, indicative of prior functional labeling, are identified. This screening process identifies 10–20 residue stretches of primary sequence that contain deuterium that was attached during the initial functional labeling step.

Progressive Proteolysis

High-resolution sublocalization of deuterium in such peptides is then accomplished by progressive proteolysis (Fig. 3) in which the extent of pepsin digestion is finely tuned within the goal of generating multiple overlapping fragments of the initial pepsin-generated peptides. When necessary, additional endoproteinases and carboxypeptidases that work well under slowed exchange conditions are employed to
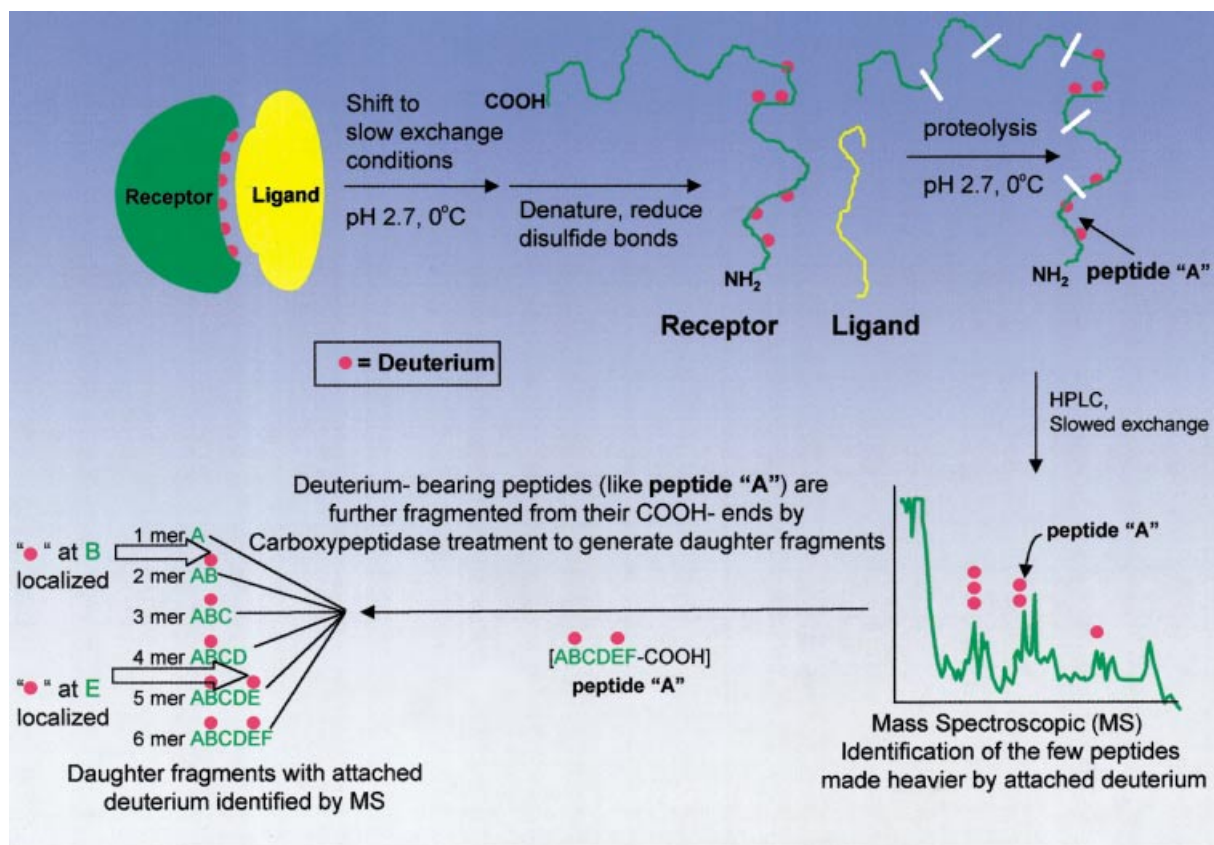
Fig. 2. Deuterium localization.

accomplish such progressive proteolysis. These proteases in general have very little primary amino acid sequence specificity under the conditions we employ them. The pattern and extent of digestion appears to be primarily a function of the precise state of denaturation of the substrate at the time of exposure to the protease. By tuning of the extent and mode of denaturation, we can produce a wide range of fragmentation patterns at will, with each pattern being extremely reproducible, as long as the necessary conditions are held constant. This sub-fragmentation is followed by assessment of deuterium label bound to each generated sub-fragment. These final steps allow the identification of the precise amides within each pepsin-generated peptide that bears deuterium label. We presently perform two to four progressively focused cycles of analysis, each cycle having an initial proteolysis "tuning" step (performed with undeuterated protein) to establish optimal fragmentation conditions for that cycle, and a second step in which the optimal condition is applied to a set of functionally deuterated samples (Fig. 3).

Use of Denaturants to Fine-Tune the Extent of Proteolysis

We have found that, at the cold temperatures required to effectively quench amide hydrogen exchange, many proteins are poorly denatured by simple transition to the acidic conditions employed, at times resulting in total failure of substrate cleavage by pepsin. We have identified quench condition-compatible (acid pH, $0°C$) denaturants that render all proteins tested to date susceptible to pepsin and our other acid-reactive proteases, the most frequently employed one being guanidinium hydrochloride (GuHCl). We have further found that: 1) the type of denaturant, 2) it's concentration, 3) the duration of substrate denaturation prior to proteolysis, and 4) the duration of exposure to solid state protease, behave as independent variables with respect to the resulting protein substrate fragmentation pattern. Manipulation of these variables allows the relatively non-specific sequence specificity of pepsin-mediated proteolysis to be kinetically tuned to desired ends. With suitable tuning of these variables,
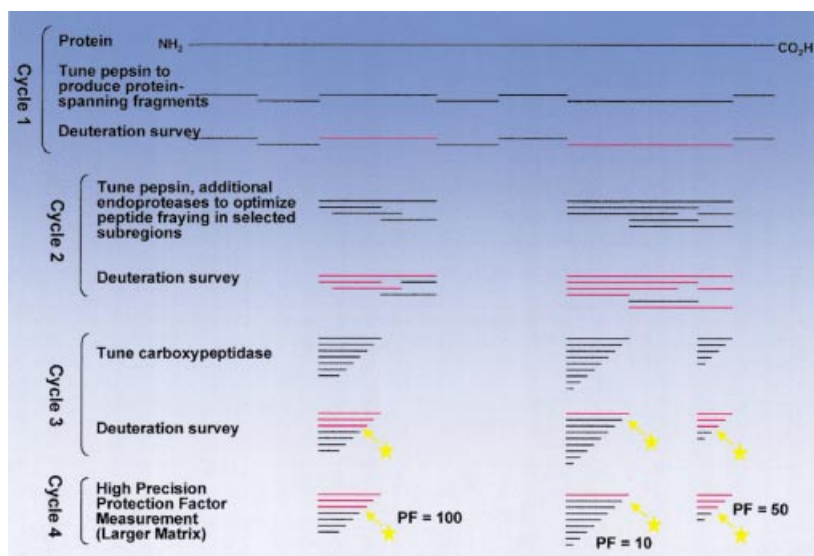
Fig. 3. DXMS progressive proteolysis.

progressive enzymatic "fraying" (generation of closely-spaced, overlapping sets of peptide fragments) can be optimized for selected regions of the target protein, increasing the resolution of exchanged deuterium localization.

Use of Additional Quench-Compatable Proteinases

We have identified additional proteases, both endoproteases and carboxypeptidases, that are as active as pepsin is under slowed exchange conditions, but that have very different cleavage patterns from pepsin and each other. As with pepsin, fragmentation with these enzymes can be tuned with the judicious use of denaturants. This allows us to fine tune the size and sequence of receptor fragments produced, and to compensate for occasional gaps in cleavage fragment coverage. We exclusively employ solid-state proteolysis in DXMS, utilizing small (66 μl) HPLC columns filled with enzymes that have been coupled to perfusive support material. When needed, we can perform sequential proteolysis by rapidly flowing sample over a series of enzyme columns, and can further proteolyze such peptides on-line as they emerge (partially separated) from the reversed phase HPLC column.

Use of Affinity Columns in Exchange Manipulations

While we can perform exchange reactions in a number of ways, we prefer to dilute the first protein in a 20 × excess of deuterated water to initiate on-exchange, and when on-exchange is complete, pass the mixture over an affinity column (60 μl bed volume) bearing covalently-bound solid-state ligand, followed by washing the column (bearing ligand-bound protein) with deuterium-free buffer to initiate off exchange. At the end of off-exchange, an acidic buffer is used to elute the protein from the column, and simultaneously shift it to acidic slowed exchange (quench) conditions. The principal advantages of this approach are: (1) a very small amount of solid state ligand is recycled and suffices for many hundreds of exchange reactions; (2) only eluted receptor, and not solid-state ligand, is subsequently proteolysed, resulting in a much less complex peptide mixture to analyze; (3) only binding-competent receptor is subjected to analysis; and (4) these column manipulations are readily automated.

Disulfide Reduction

Most of the receptors we are interested in studying contain numerous disulfides. These disulfides can be expected to limit the action of denaturants and proteases, and to confound the identification of peptides that remain linked to each other through them. Conventional disulfide-exchange mediated reduction does not occur at the acid pH required for quenched hydrogen-exchange. We have developed recipes that allow certain phosphines, tris-2-carboxyethyl phosphine (TCEP) in particular, to rapidly reduce disulfides under such conditions.

Automation

We have developed an apparatus that automates performance of both the deuterium-exchange functional labeling and subsequent proteolysis and MS-based deuterium localization procedures. This automation robustly integrates the several above-noted improvements in our ability to manipulate progressive proteolysis under quench conditions, allowing rapid testing of variety of proteolysis-tuning conditions, and identification of those that are optimal for a particular protein target and hydrogen-exchange experimental goal. Once identified, these precise conditions are automatically applied to the analysis of deuterated samples of the protein target.

The DXMS apparatus consists of a number of pumps, high pressure switching valves, and electric actuators, along with plumbing, mixing tees, and one way flow check valves that direct the admixture of reagents and their flow over the several columns. The timing and sequence of operation of pumps and valves is controlled by a computer running a LabView program, with interface provided by digital input−output (DIO) boards and solid state relays. Precise temperature control is achieved by enclosing the valves, columns, connecting plumbing in a high thermal capacity refrigerator kept at $3.8°C$ (the freezing point of deuterated water), and components that have no contact with pure deuterated water are immersed in melting ice.

With the apparatus, functional deuteration can be performed in an entirely automated manner, including the steps of deuterium on-exchange of receptor, ligand binding, off exchange, and quench. Alternatively, a sample set (10−50 samples) can be prepared manually in autosampler vials, quenched, and samples frozen at $−80°C$, conditions under which the prepared samples are stable for weeks. A component of the DXMS apparatus is capable of holding the samples at these ultra-low temperatures until a selected sample is withdrawn with a robotic arm, the sample rapidly melted at $0°C$ and then injected onto the protease column. This capability allows samples to be manually prepared at a distant site, and then shipped frozen to the DXMS facility for later automated analysis.

Regardless of the manner of sample preparation, quenched samples are then automatically directed over a battery of protease columns, with the resulting peptide fragments being collected on a small reversed-phase HPLC column. This column is then gradient-eluted, with optional additional post-LC on-line proteolysis. The effluent is then directed to the electrospray head of the mass spectrometer (a Finnegan ion trap or a Micromass Q-TOF) which protrudes into a hole drilled in the side of the refrigerator.

Data Reduction and Analysis

At present, the rate-limiting step in performing DXMS studies is the computational extraction of peptide sequence-specific deuterium incorporation information from the tremendous quantity of data contained in the multidimensional LC-MS data files. While many LCMS data analysis capabilities are highly developed and available, those facilitating the analysis of *isotope distribution data*, the fundamental "information-unit" of deuterium-exchange study, are conspicuously lacking. Until recently, it was necessary for a human operator to "manually" extract such information, in a manner that typically required two week's work to process data that had been acquired in a few hours. In a collaborative effort with Dr. David Stranz (Sierra Analytics, LLC. Modesto, CA), we have developed algorithms and software that now allow us to efficiently reduce DXMS data in a matter of hours, greatly speeding both progressive proteolysis optimization and calculation of site-specific deuteration levels. This software development effort is funded by a University of California Life Sciences Informatics (LSI) Technology transfer grant.

UTILITY OF DXMS IN DRUG DISCOVERY

Many of the insights into protein structure, function, and dynamics afforded by DXMS are of obvious applicability to pharmaceutical design efforts. One such use of DXMS particularly interests us. There has been limited success in the development of orally active (small molecule) drugs that can target integral membrane protein receptors. Recent studies indicate that many proteins bind each other primarily through small thermodynamically dominant subregions (hot spots) of their otherwise large binding surfaces. This insight may allow a solution to this drug design problem: focus development efforts to the small identified energetic "hot spot" regions that mediate high affinity binding between a receptor and its

naturally occurring protein ligand. Unfortunately, there has been no practical technology available that can efficiently identify hot spot regions in most receptor targets. DXMS can now provide a rapid method by which hot spots can be identified, and as leads are developed, rapidly determined if they are actually targeting the hot spots.

Protein Interaction Surfaces are Too Large a Target for Conventional Small Molecule Drug Design Approaches

There is considerable commercial potential to the development of small molecule (orally active) drugs that can mimic the therapeutic actions of the growing number of clinically important protein ligands (cytokines, growth hormones/factors, adhesion proteins, monoclonal antibodies, etc.) that bind to cellular receptor proteins. Unfortunately, conventional structure-based approaches to pharmaceutical design have had difficulty in producing small molecule leads that can successfully modulate the binding interactions that take place between proteins. These types of protein−protein interactions are mediated by large binding surfaces, typically involving 10−30 contact amino acids on each side. Based on this observation, it has been assumed that interaction surfaces of this large size are required to assure sufficient binding affinity and specificity. This is much larger than the size of the typical small molecule drug.

Binding Surface Thermodynamics

The functional activity that results from protein−protein interactions (binding, inhibition, and agonist activity) is completely determined by the detailed thermodynamic interactions that occur between the various components of their interacting contact surfaces. The standard method for obtaining such information is to alter the chemical structure of a subregion of a protein's surface by mutation and then measure how this effects the global binding affinity between the receptor and the mutant binding protein. This approach is time consuming, technically demanding, and provides an unreliable measure of the thermodynamic contribution of individual binding site amino acids. Mutagenesis of individual amino acids does not simply alter side chains of the protein but can often produce unpredictable local and perhaps more distant structural

perturbations in the mutant protein. The structural changes may dramatically effect overall binding affinity, and lead to considerable ambiguity in interpretation of results.

"Hot Spots" in the Thermodynamic Fine-Structure of Protein Binding Surfaces

Recent discoveries indicate that many protein-binding surfaces contain a small number of amino acid residues that predominantly contribute to the binding energy (tightness or strength of interaction) between receptor and ligand. This was first demonstrated by Wells and collaborators in studies of the complex formed between human growth hormone and its cellular receptor protein [Clackson and Wells, 1995; Wells, 1996]. X-ray crystallographic studies of the complex demonstrated that approximately 30 amino acids from each protein make contacts with each other across the interaction surfaces. Extensive mutagenesis data, despite its considerable error, demonstrated that a small number of amino acid residues in each protein's binding surface accounted for most of the binding free energy of the hormone-receptor interaction. Within each of these "hot-spots", 2−3 critical amino acids were surrounded by a larger number of binding surface amino acid residues that contributed at lesser levels. Most importantly, these studies indicated that the functionally predominant residues on the hormone-binding surface directly contacted corresponding hot spots on the receptor's binding surface. Thus, only a small and complementary set of contact residues contributed most of the binding affinity between the receptor and ligand. Subsequent studies have supported Wells' assertion that this may be a general property of protein−protein binding interfaces [Smith-Gill, 1995; Wells, 1996].

If proteins interact with each other through functionally dominant "hot-spots" that are significantly smaller than the entire interaction surface, then the task of designing small molecules that can interact with receptor surfaces with high affinity and selectivity is simplified: design small compounds that efficiently target these thermodynamic hot-spot regions. The ability to rapidly and precisely deduce the thermodynamic fine structure of pharmaceutically interesting receptor-ligand interaction surfaces will be critical to such efforts. Unfortunately, mutagenesis is not precise enough to

provide a practical guide, though it was instrumental in discovery of the existence of binding surface hot spots.

While I devised DXMS to study the structure of protein receptor-ligand binding sites, it can also be used to precisely measure the protection factors that individual binding surface amides experience upon receptor-ligand complex formation. This can be done by systematically measuring label at each amide position as a function of varying on-exchange duration to determine amide exchange rates as they exist in the native, uncompelled proteins, and make measurements as a function of varying off-exchange duration to determine exchange rates in the receptor-ligand complex. The ratio of these two rates is calculated to establish the protection factor for each amide.

The peptide amide hydrogen exchange protection factors that are deduced by this technique directly report the free energy changes of each amide hydrogen upon formation of the receptor-ligand complex [Englander and Kallenbach, 1984]. The portions of the binding surface with the highest protection factors may therefore be related to the thermodynamic "hot-spots" as conceived by Wells. In effect, this approach allows each of the 20–40 peptide amide hydrogens in the studied receptor-ligand binding surface to serve as a precise sub-molecular sensor of localized binding-induced free energy change, resulting in high resolution localization of "hot spots", without mutation-induced ambiguities.

I have proposed the following general scheme for one application of DXMS to drug discovery, which in essence allows a naturally occurring, high affinity protein ligand for a target receptor to function as an immediately available guide to the design of high affinity, hot-spot-targeting small molecule leads: (Fig. 4):

1. DXMS is first used to identify the receptor's "hot spots" in its binding interaction with its naturally occurring high affinity (protein) ligand, as well as the features of the protein ligand that dock to these "hot spots".
2. Next, a focused library of small molecules is generated that mimic the structure of the DXMS-deduced relevant natural ligand peptidic features.
3. This library is screened for binding to the receptor in conventional high-throughput binding assays (HTS).

4. *All* binders (not just those with the highest affinity for receptor) are then screened against the receptor in additional high-throughput *deuterium-exchange* studies that identify the few binding library components that interact precisely with the receptor's hot spot (identified in step 1), and these few selected for further iterative combinatorial chemistry development, regardless of their initial binding affinity for receptor.

It is anticipated that this approach will result in the progressive shaping of small molecule structure to that optimally capable of binding to the receptor's natural ligand-defined binding hot spot. DXMS also detects and localizes any receptor conformational or oligomerization changes that a ligand may induce when it binds. This information may prove useful in understanding precisely how some natural ligands induce receptor activation.

SUMMARY AND FUTURE DIRECTIONS

DXMS, because of its analytical power and high throughput, is now a powerful tool with which to determine protein structure, function, and dynamics rapidly, with high precision and on a large scale. Beginning 6 months ago, we began to make DXMS available to UCSD researchers for collaborative studies. Investigators with abiding interests in particular proteins have brought us their most pressing experimental questions-along with small amounts of the relevant proteins. Focused DXMS experiments have then been performed by us, and data reduction and analysis carried out primarily by the collaborating investigators. We have in progress 10 studies with 7 investigators, applying DXMS variously to issues of protein structure, protein–protein and protein–small molecule interactions, protein allosteric (conformational) change, and protein folding. Our collaborators include Dr. Susan Taylor, Dr. Palmer Taylor, Dr. Patricia Jennings, Dr. Joseph Adams, and Dr. Shu Chien (UCSD); Robert Stroud (UCSF), and S. Walter Englander (U. Penn). One of our more ambitious collaborations (Dr. Phillip Bourne, Lynn TenEyck; UCSD and David Wemmer; UC Berkeley) aims to establish the optimal methods by which high resolution protein structure prediction can be facilitated by DXMS studies.
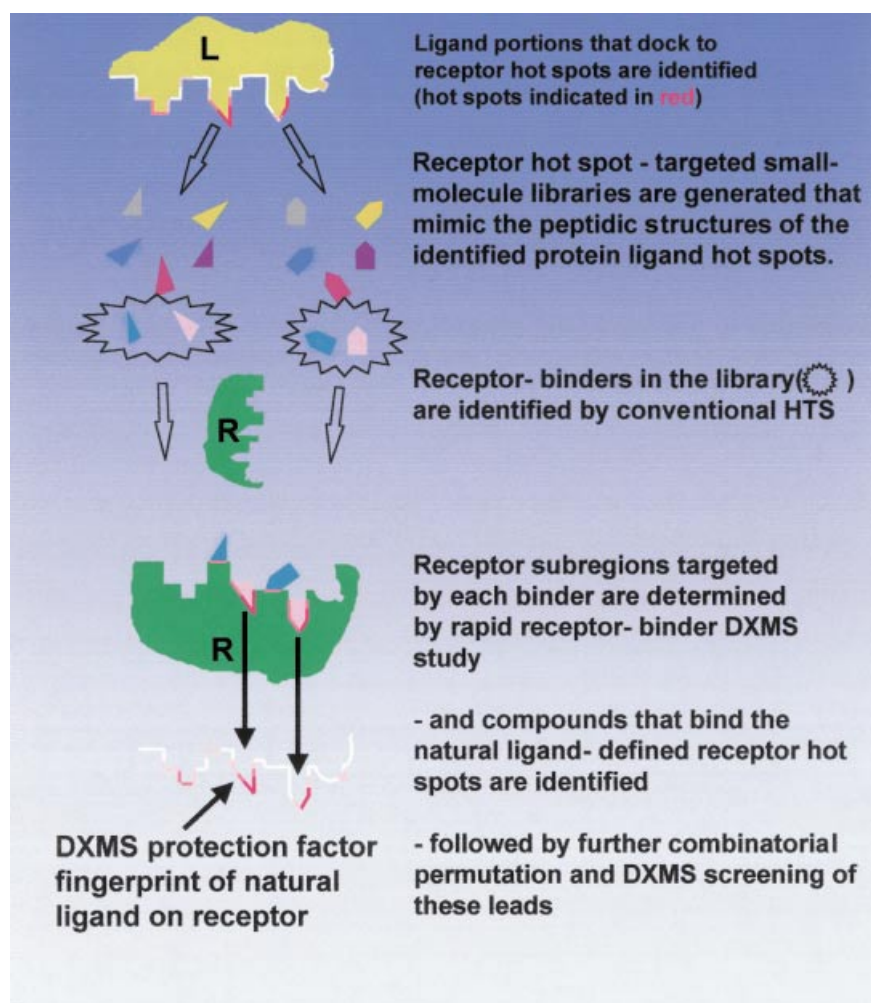
Fig. 4.   DXMS-guided drug design.

A startup biotech company, Carta Proteomics, Inc., Princeton NJ, has recently been formed to make DXMS available for commercial drug design and development purposes. Many of the luminaries of amide hydrogen exchange theory and practice are consultants to Carta, including Dr. David Smith, Dr. Walter Englander, and Dr. David Wemmer, and the author is one of it's scientific founders. Carta Proteomics aims to provide "turn-key" DXMS to the pharmaceutical industry, through provision of a new generation of DXMS instrumentation and methodologies they are developing, based on the present generation of DXMS technology licensed and assigned to them from the University of California, The

University of Nebraska, and Case Western Reserve University.

Proteomics, and both he and Dr. Hamuro have financial interest in the company.

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