# Application of Amide Proton Exchange Mass Spectrometry for the Study of Protein-Protein Interactions

Amide proton exchange experiments have traditionally been performed to study protein folding and unfolding by measuring the solvent exchange rates of amide protons that participate in hydrogen bond formation (Englander et al., 1997). In studies of protein-protein interfaces, the hydrogen bonds examined are usually those that exhibit decreased solvent accessibility arising from factors such as side chain interactions and decreased loop mobility, because hydrogen bonds rarely form across interfaces. Amide groups on the interior of a protein exchange protons with solvent more slowly than do amide groups on the surface, and it is almost always the more rapidly exchanging surface amides that require monitoring in protein-protein interface studies. Typically, amides on the surface of a protein exchange protons on a time scale ranging from seconds to minutes. The corresponding exchange rate is slower than that for amides in unstructured peptides (Bai et al., 1993), but relatively faster than that for amide groups residing within the core of a protein (Dharmasiri and Smith, 1996).

Early amide proton exchange experiments relied on tritium labeling and separation by chromatography; however, these early methods have been supplanted in recent years by NMR and mass spectrometry, both of which measure deuterium incorporation (Rosa and Richards, 1979; Englander and Mayne, 1992; Zhang et al., 1996). NMR has the advantage of being able to detect single amide groups. Among the disadvantages are that the protein must be of a size that is tractable for NMR spectroscopy (typically $<$40 kDa), that it must be expressible, and that it must be isotopically labeled with $^{15}N$ so that resonance assignments for the amide protons can be made. In contrast, mass spectrometric measurements avoid the need for isotopic labeling of the protein and can be performed on proteins of any size (*UNIT 17.6*). The disadvantage of mass spectrometry is that, at present, single-amide resolution is not possible. Mass spectrometric measurements rely on proteolytic digestion of the protein and analysis of the resulting peptide fragments. Thus, the resolution is limited by the sizes of these peptide fragments, although it can be improved if many overlapping fragments are obtained. Because the methodology of measuring amide proton exchange by mass spectrometry is already covered in *UNIT 17.6*, this unit focuses on the particulars of experiments used to study protein-protein interactions.

## STRATEGIC PLANNING

The protein-protein binding equilibrium constant plays an important role in determining whether interface protection is observed. If the proteins in a complex are dissociating and reassociating rapidly, then the amide protons will have time to exchange with solvent protons while the proteins are dissociated, and interface protection will not be observed. To address this issue, some knowledge of the binding equilibrium constant is required. Titration calorimetry (*UNIT 20.4*) is an excellent way to obtain the binding constant, and information on the kinetics of association and dissociation is best obtained from optical biosensor measurements (*UNIT 20.2*).

In the systems the authors have studied by off-exchange, there are amides at the protein-protein interface that are completely protected from exchanging protons with solvent as long as the two proteins remain bound (Baerga-Ortiz et al., 2004; Mandell et al., 2001).

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*Current Protocols in Protein Science* (2005) 20.9.1-20.9.20

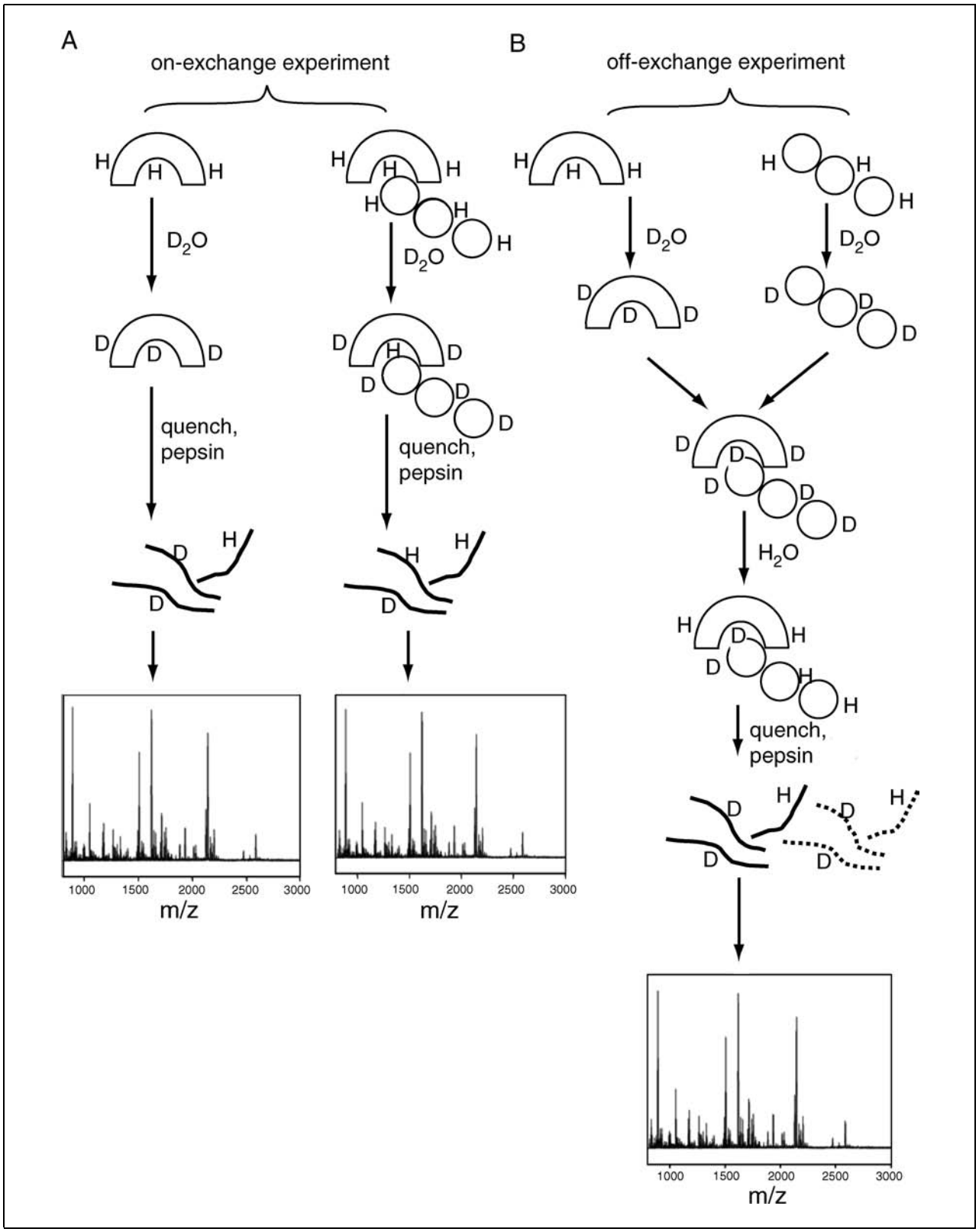


**Figure 20.9.1** Legend at right.

For these completely solvent-excluded amides, the observed $^1H/^2H$ exchange rate is a function of the protein-protein dissociation and association rates, as well as the intrinsic rate of hydrogen exchange ($k_{ex}$):

$$[R_D \cdot L] \underset{k_a}{\overset{k_d}{\rightleftharpoons}} R_D + L \overset{k_{ex}}{\rightarrow} R_H$$

where $R_H$ is the protonated receptor, $R_D$ is the deuterated receptor, L is the ligand, $k_{ex}$ is the intrinsic proton exchange rate (in $min^{-1}$) for amides in the uncomplexed receptor, $k_d$ is the rate of dissociation of the complex (in $min^{-1}$), and $k_a$ is the rate of association of the proteins undergoing complexation (in $M^{-1} \cdot min^{-1}$). $^1H/^2H$ exchange at a protein-protein interface could occur because of solvent accessibility to the interface, or it could occur because the complex has dissociated. A theoretical analysis shows that under the conditions of the $^1H/^2H$ exchange experiment presented in this unit (protein concentration, 3 μM), if $K_d$ (which equals $k_d/k_a$) is <2 nM, a 1:1 ratio of ligand to receptor is sufficient to ensure that essentially 100% of the receptors are bound to ligand throughout the experiment. If $K_d$ is between 10 and 100 nM, then higher ratios of ligand to receptor are required to study $^1H/^2H$ exchange kinetics at the interface in the bound complex, and the interplay between binding kinetics and exchange kinetics becomes important (Mandell et al., 2001). In these cases, full knowledge of the binding kinetics is required prior to quantitative interpretation of the data. For protein-protein interactions that are in rapid equilibrium, the amides excluded at the interface should not show pH-dependent exchange rates (Mandell et al., 2001).

This unit contains two protocols for studying protein-protein interfaces by monitoring amide proton exchange using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (*UNIT 16.2*, *UNIT 16.3*, and *UNIT 16.4*). The simplest protocol, known as the "on-exchange" experiment (see Basic Protocol 1), is used to determine the location of the protein-protein interface. In this experiment, a comparison is made between surface amide deuteration in the uncomplexed proteins and surface amide deuteration in the protein-protein complex (Fig. 20.9.1A). The part of each protein that shows less deuteration in the complex than in the uncomplexed state is the interacting surface, or interface. On-exchange data, in combination with computational docking, have been used by Anand et al. (2003) to build a model of the protein kinase A holoenzyme complex.

The "off-exchange" experiment (see Basic Protocol 2) yields interface protection data that pertain more to conformational changes (Fig. 20.9.1B). In this experiment, by first monitoring on-exchange (i.e., replacement of hydrogen atoms with deuterium atoms) in the separate proteins, one can assess which regions are highly solvent accessible prior to complexation; these regions are likely to be on the surface of the protein. If any of these highly accessible regions are subsequently found to be protected when the deuterated proteins are allowed to complex and off-exchange (i.e., replacement of deuterium atoms with hydrogen atoms) is measured, it is safe to assume that they are at the protein-protein interface, as it is unlikely that a highly accessible surface region will fold into the protein core so as to become completely inaccessible upon complex formation.

**Figure 20.9.1** *(at left)* Flow chart diagram of the two types of amide proton exchange experiments used to study protein-protein interfaces. (**A**) In the on-exchange experiment, the protein-protein complex shows a region in which less deuterium is incorporated when compared with control samples in which each protein is deuterated separately. (**B**) In the off-exchange experiment, each protein is allowed to incorporate deuterium separately, the deuterated proteins are allowed to complex, and then deuterium atoms are off-exchanged with hydrogen atoms by dilution in $H_2O$; residues located at the protein-protein interface are characterized by the retention of deuterons in the protein-protein complex as compared with a lack of retention in control experiments in which only one of the two proteins is present.

The "off-exchange" experiment also allows discrimination between partially inaccessible and completely inaccessible regions of the interface. The ability of solvent $H_2O$ to penetrate a protein-protein interface, as well as the idea that $H_2O$ molecules are released from some portion of a protein's surface when that protein interacts with another one, is a topic of current research interest in biophysics (Baerga-Ortiz et al., 2004; Mandell et al., 2001).

***BASIC PROTOCOL 1***

## IDENTIFICATION OF THE PROTEIN-PROTEIN INTERFACE VIA THE ON-EXCHANGE METHOD

The simplest protocol used to study protein-protein interfaces by monitoring amide proton exchange using MALDI-TOF mass spectrometry is the "on-exchange" experiment, which is used to find the location of the protein-protein interface. In this experiment, a comparison is made between surface amide deuteration in the uncomplexed proteins and surface amide deuteration in the protein-protein complex. The part of each protein that shows less deuteration in the complex than in the uncomplexed state is the interacting surface, or interface. Thus, the experiment involves separately deuterating the individual proteins and the protein complex by incubation in $D_2O$ buffer. After quenching of the exchange reaction and proteolytic digestion of each sample, mass spectrometry is used to reveal regions in the protein complex that were protected from exchange (Fig. 20.9.1A). In order to interpret the results of the "on-exchange" experiment, it is necessary to assume that protection occurs because of interface formation, and not because of conformational changes. If the structures of the interacting proteins are known, it will be readily apparent as to whether this assumption is valid.

Prior to carrying out an amide proton exchange experiment, it is necessary to determine the pepsin digestion conditions that result in the greatest yield of peptide fragments from the protein under study, and it is also necessary to identify the peptides that are obtained from pepsin digestion. Determination of optimal digestion conditions and identification of peptide fragments only need to be performed once for a particular protein and should be performed under conditions identical to those that will be used in the actual amide proton exchange experiment.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (*UNIT 16.2*, *UNIT 16.3*, and *UNIT 16.4*) is used in this protocol. The methodology for measuring amide proton exchange by MALDI-TOF mass spectrometry is relatively simple, and no modification of commercially available instruments is required (Mandell et al., 1988a,b). Mass spectrometric measurement of amide proton exchange was pioneered by David Smith in the early 1990s, using electrospray ionization (Zhang and Smith, 1993). Amide proton exchange results identical to those obtained using Basic Protocol 1 can be achieved using an electrospray mass spectrometer. Furthermore, high-resolution sequencing information similar to that obtained using a MALDI-TOF mass spectrometer can also be obtained using a tandem quadrupole time-of-flight mass spectrometer.

### *Materials*

10× low-salt buffer (e.g., 25 mM Tris·Cl, 25 mM NaCl, 50 mM Na/$KHPO_4$)
Purified receptor and ligand proteins
0.1% and 2.0% (v/v) trifluoroacetic acid (TFA; highest purity possible)
2 to 3 mg/ml immobilized pepsin in 6% (w/v) cross-linked beaded agarose (Pierce Biotechnology)
Matrix solution for MALDI-TOF mass spectrometry (see recipe)
Peptide mass standard mixture (e.g., Sequazyme Calibration Mixture 2; Applied Biosystems)
Deuterium oxide ($D_2O$; 99.996% purity; Cambridge Isotope Laboratories)

pH meter with InLab 423 electrode (Mettler Instruments)
Cold box, 4°C (Isotemp Chromatography Refrigerator; Fisher Scientific)
Microcentrifuge, 4°C (in cold box)
0.5-ml thin-walled microcentrifuge tubes (USA/Scientific)
MALDI target plates, 4°C (in cold box; chilled overnight in a plastic case to prevent absorption of atmospheric $H_2O$)
Vacuum desiccator (with liquid nitrogen trap) located adjacent to MALDI-TOF mass spectrometer
MALDI-TOF mass spectrometer (*UNIT 16.2*, *UNIT 16.3*, and *UNIT 16.4*)
$C_{18}$ analytical column
Additional reagents and equipment for reversed-phase high-performance liquid chromatography (HPLC; *UNIT 8.7*) and post-source decay sequencing (*UNIT 16.1*), carboxypeptidase Y C-terminal sequencing (*UNIT 11.8*), or electrospray ionization tandem mass spectrometric (ESI MS/MS) sequencing (*UNIT 16.10* and *UNIT 16.11*)

*NOTE:* The amide proton exchange reaction is highly sensitive to temperature; exchange is rapid at room temperature but slows dramatically when the reaction sample is chilled to 4°C. Thus, all sample manipulations subsequent to the timed incubation of the receptor, ligand, or receptor-ligand complex in $D_2O$ must be performed at 4°C. The simplest experimental setup that makes this possible involves having a cold box in the same room as the mass spectrometer.

***Optimize pepsin digestion conditions***

1. In 10× low-salt buffer, prepare a 100 pmol/µl sample of one of the two proteins under study. Transfer a 1.2-µl aliquot of this sample to each of several 0.5-ml microcentrifuge tubes, and dilute each aliquot with 12 µl $H_2O$ to yield a sample that is ~1× with respect to the buffer solution.

   *To screen digestion conditions, four to ten separate samples will be required.*

2. Dilute each protein sample (protein in ~1× buffer, from step 1) 10-fold by adding 120 µl of 0.1% TFA, and measure the pH of the resulting solution. For each sample, use 2% TFA to adjust the pH to match the original pH of the added 0.1% TFA solution (pH 2.0 to 2.5), making note of how much TFA was required, and then chill the sample to 4°C in the cold box.

   *When preparing on-exchange samples in subsequent steps, the volume of 2% TFA needed to achieve the desired (optimum) pH will be known, making it unnecessary to monitor the pH of each experimental sample while adding 2% TFA.*

3. Immediately before use, transfer immobilized pepsin (25 µl of pepsin beads, in the form of 50 µl of the 1:1 pepsin bead slurry supplied by the manufacturer) to each of several 1.5-ml microcentrifuge tubes. Wash the immobilized pepsin twice, each time by adding 1 ml chilled 0.1% TFA, pH 2.0 to 2.5, per tube; vortexing; microcentrifuging 2 min at 7000 × *g*, 4°C; and then removing all supernatants.

   *Pepsin is used to cleave proteins into fragments of 8 to 20 amino acids (Zhang and Smith, 1993). Pepsin beads can be washed and reused ~5 times, but they begin to lose their activity 4 hr after acidification.*

   *The pH of the pepsin digestion is controlled by the pH of the 0.1% TFA used to wash the pepsin beads in this step. The washing pH for each tube in this step should match the pH of a sample from step 2.*

4. Add each protein sample (step 2) to a separate tube of immobilized pepsin (washed at the pH of that sample) and incubate at 4°C, using each sample to test a different combination of incubation time and pH.

*The completeness and specificity of pepsin cleavage depend on incubation time and pH; therefore, it is necessary to vary these two parameters to find the conditions that lead to optimal pepsin digestion product yields. The optimal incubation time typically ranges from 1 to 10 min, and the optimal pH typically ranges from 2.0 to 2.5.*

5. Microcentrifuge the digested protein samples 1 min at 14,000 × *g*, 4°C. For each sample, transfer 20-µl aliquots of the supernatant to separate 0.5-ml thin-walled microcentrifuge tubes and immediately freeze in liquid nitrogen. Store aliquots up to 2 weeks at −80°C.

   *In experimental runs, on-exchange samples should be quenched (i.e., treated with TFA), digested, and frozen in <15 min total, since the median time for amide deuteron back exchange under quench conditions is ∼15 min.*

6. In the following way, obtain a MALDI-TOF mass spectrum of at least one 20-µl aliquot of digest mixture obtained under each distinct set of digestion conditions.

   a. Quickly defrost the frozen digestion sample by warming the sample tube by hand, with periodic flicking of the tube to help break up/liquefy the sample.

      *Defrosting should be completed within 30 sec of removing the sample from frozen storage.*

   b. Once the sample has been defrosted, transfer the tube to a prechilled microcentrifuge tube rack sitting in the cold box.

   c. Transfer 5 µl of sample to a clean, prechilled microcentrifuge tube, add an equal volume of prechilled matrix solution, and mix.

   d. Spot 1 µl of the resulting mixture onto a prechilled MALDI target plate, and then immediately transfer the target plate to a vacuum desiccator and apply moderate vacuum.

      *If many samples are to be analyzed, it will be necessary to have at least two additional target plates (i.e., aside from the one in use) being chilled in the cold box at any given time, so that new samples can be prepared without having to wait for a target plate to cool to the appropriate temperature.*

      *A SpeedVac evaporator can be used in place of a vacuum desiccator to dry the target plate. The strength of the vacuum should be such that it takes ∼1 min for the spot to dry. Slow drying of the spot under moderate vacuum or by cycling the vacuum on and off results in higher-quality sample analysis, presumably because it leads to improved crystal growth.*

   e. Once the plate has dried, transfer it as quickly as possible (i.e., within 10 sec) to the mass spectrometer and acquire a MALDI-TOF spectrum in accordance with the manufacturer's instructions.

7. Based on the MALDI-TOF data obtained in step 6, identify the pepsin digestion conditions that result in the highest product yield.

   *It is important that the conditions used in the actual amide proton exchange experiment be identical to those used in the optimized pepsin digestion so that proteolysis products can be accurately identified.*

***Identify pepsin proteolysis products by mass spectrometry***

8. Thaw another aliquot of the digest mixture produced under the optimized proteolysis conditions, combine the aliquot with a peptide mass standard mixture, and analyze by MALDI-TOF mass spectrometry (step 6; add mass standard to digest mixture before combining a 5-µl aliquot with matrix solution). Calibrate the mass spectrum using the masses of the standards.

   *A typical mass standard mixture will require 1:300 dilution before being added to the pepsin digest mixture for MALDI-TOF MS analysis.*

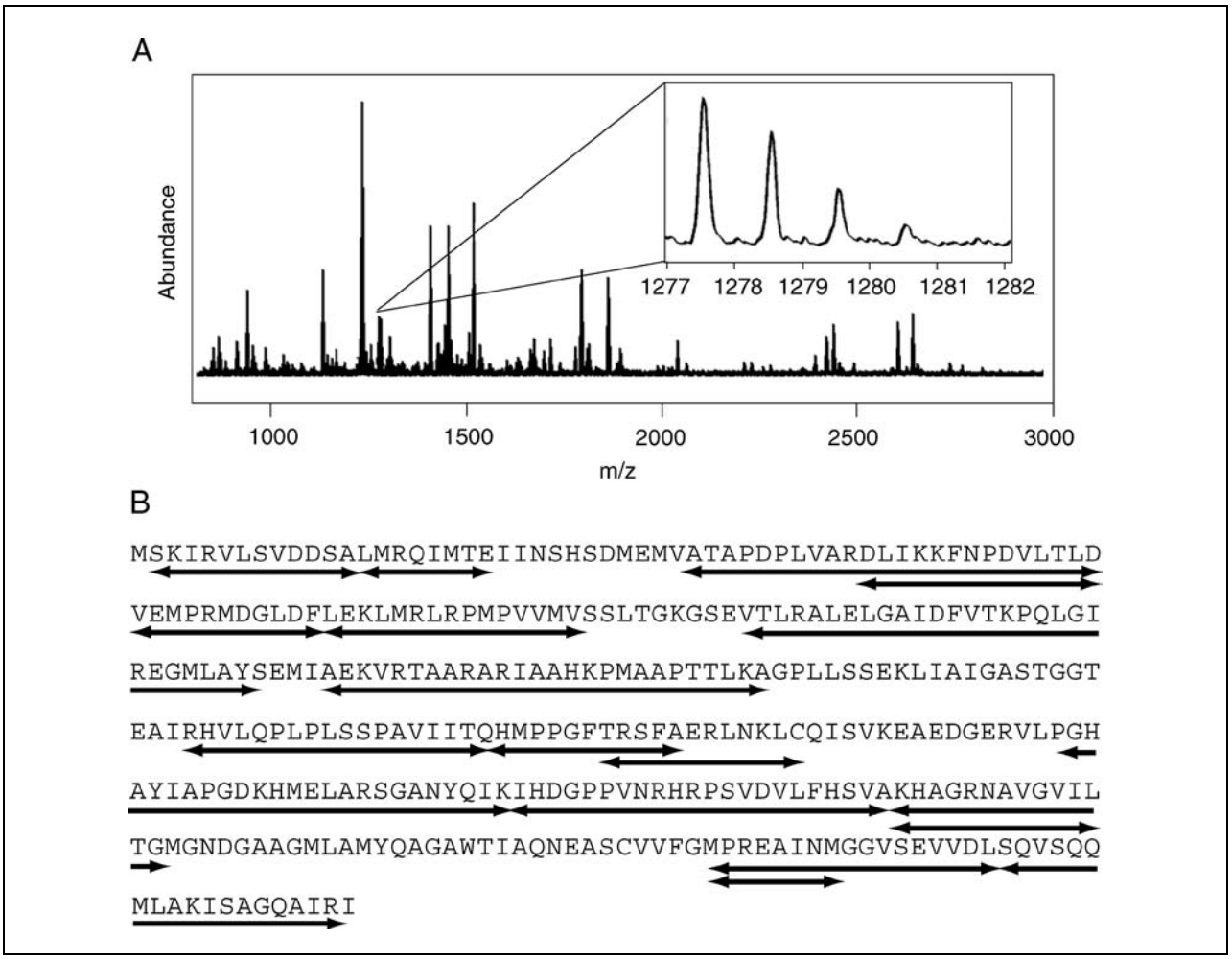


**Figure 20.9.2** (**A**) Example of a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of pepsin digest products. The inset shows that the high resolution of the time-of-flight mass analyzer allows baseline resolution of the mass envelope for each peptide. Each peptide's mass envelope results from the natural abundance of $^{13}C$ within that peptide, such that the peak representing the fraction of peptide molecules containing one $^{13}C$ atom appears one mass unit to the right of the monoisotopic peak, the peak representing the fraction containing two $^{13}C$ atoms appears two mass units to the right of the monoisotopic peak, and so on. (**B**) Sequence coverage map for the protein whose peptide digest mass spectrum is shown in panel A. Each of the lines under the sequence represents a peptide fragment whose mass matches that of a peak observed in the mass spectrum.

*The MALDI-TOF mass spectrum acquired in this step contains all pepsin digest products that will be observed for this protein in subsequent deuterium exchange experiments (Fig. 20.9.2A). If a typical, well-tuned MALDI-TOF instrument is used, the masses of the peptides should be accurate to within 20 ppm (tolerance of 0.02 for a peptide of mass 1000), provided that internal standards are used.*

9. From the calibrated spectrum, determine the $MH^+$ masses of all observed pepsin digest products. Using any of a number of search engines available on the Internet, search the entire sequence of the protein of interest and identify all potential peptide fragments whose masses match (to within 20 ppm) an $MH^+$ mass that appears in the calibrated spectrum.

*For some of the observed $MH^+$ masses, there will be only one peptide fragment that falls within the 20-ppm tolerance window, and so a peak assignment can be made unambiguously. Once at least two peptide fragments are identified unambiguously in this manner, they should be used as internal calibration standards in all subsequent mass spectra.*

10. In the following way, identify all peptide fragments in the digestion mixture that have not yet been identified unambiguously.

    a. Thaw another five 20-µl aliquots of the digest mixture produced under the optimized proteolysis conditions and pool them. Separate the peptides in the pooled mixture using a C-18 analytical column and reversed-phase HPLC (*UNIT 8.7*), and collect each peak fraction.

        *Each peptide fragment that is responsible for a peak in the mass spectrum will be found in one of the HPLC-separated fractions and can be readily sequenced from that purified fraction.*

    b. Sequence each previously unidentified peptide obtained in substep a by mass spectrometric methods.

        *HPLC separation of ~1 nmol of protein will provide sufficient peptide for post-source decay sequencing (UNIT 16.1) or carboxypeptidase Y C-terminal sequencing (UNIT 11.8), both of which are MALDI-based methods, or for ESI MS/MS sequencing (UNIT 16.10 and UNIT 16.11) using an ion trap or tandem quadrupole-time-of-flight mass spectrometer.*

        *It is important to note that while pepsin cleavage is not predictable, it is completely reproducible. Therefore, the sequencing of peptide fragments resulting from the digestion of a particular protein need only be carried out once prior to a set of amide proton exchange experiments.*

11. Map all identified peptide fragments back onto the sequence of the protein to determine sequence coverage (Fig. 20.9.2B).

    *If there are regions of the protein that are not covered by the peptide fragments found in the mass spectrum, it may be necessary to change the pepsin digestion conditions, perhaps by including up to 1 M urea as a denaturant (if the protein is particularly stable) or by using a different protein concentration. Buffers can also play a role, with low salt concentrations (<10 mM) generally being preferable for all types of mass spectrometry; thus, reducing the salt content in the initial protein solution often improves sequence coverage.*

12. Repeat steps 1 to 11 using the other of the two proteins being studied.

13. Prepare a 100 pmol/µl sample of the receptor protein in 10× buffer, and then prepare a separate 100 pmol/µl sample of the ligand protein in 10× buffer. In each of several 0.5-ml microcentrifuge tubes, mix 0.6 µl of the receptor protein solution and 0.6 µl of the ligand protein solution and then dilute with 12 µl $H_2O$. Using the resulting samples, repeat steps 2 to 11 to identify the optimal digestion conditions for the receptor-ligand complex.

***Perform amide proton exchange experiments to determine time course of deuteration for each protein***

14. In the following way, prepare several microcentrifuge tubes (one for each on-exchange time point—0, 1, 2, 5, 10, and 20 min, typically) containing the quench solution for one of the two proteins being studied.

    a. To each tube, add 120 µl of chilled 0.1% TFA (4°C) at the optimal digestion pH for the protein in question.

    b. To each tube, add the appropriate volume of 2% TFA for maintaining the optimal digestion pH after the addition of a sample of the protein (as determined in step 2).

    c. Chill the tubes at 4°C in the cold box.

15. For each tube from step 14, prepare a tube of immobilized pepsin beads as described in step 3, making sure to wash at the optimal digestion pH.

16. In 10× buffer, prepare a 100 pmol/μl sample of one of the two proteins under study. Transfer a 1.2-μl aliquot of this sample to each of several 0.5-ml microcentrifuge tubes, and dilute each aliquot with 12 μl $D_2O$. Incubate each of the resulting samples for a different length of time (ranging from 0 to 30 min, except for one sample, for which incubation should last 24 hr) at the temperature of interest (typically 25° to 37°C).

    *The sample that is incubated for 24 hr is subsequently used to correct experimental results for the effects of back exchange (Support Protocol 1, step 5).*

17. At the end of each sample's incubation period, quench on-exchange by adding the contents of one tube of quench buffer to the sample, transfer the resulting mixture to a tube of immobilized pepsin beads, and incubate for the optimal length of time (as identified in step 7) at 4°C. When digestion is complete, centrifuge, aliquot, freeze, and store the digest mixture as described in step 5.

18. Repeat steps 14 to 17 for the other of the two proteins being studied.

***Perform amide proton exchange experiment to identify protein-protein interface***

19. Prepare several microcentrifuge tubes (one for each on-exchange time point) containing the quench solution for the receptor-ligand complex (see step 14).

20. For each tube from step 19, prepare a tube of immobilized pepsin beads as described in step 3, making sure to wash at the optimal digestion pH for the receptor-ligand complex.

21. Prepare a 100 pmol/μl sample of the receptor protein in 10× buffer, and then prepare a separate 100 pmol/μl sample of the ligand protein in 10× buffer. In each of several 0.5-ml microcentrifuge tubes, mix 0.6 μl of the receptor protein solution and 0.6 μl of the ligand protein solution and then dilute with 12 μl $D_2O$. Incubate each of the resulting samples for a different length of time (ranging from 0 to 30 min, except for one sample, for which incubation should last 24 hr) at the temperature of interest.

    *Alternatively, a 100 pmol/μl sample of the protein complex in 10× buffer can be prepared in advance and used in place of the separate receptor and ligand solutions that are mixed together in this step.*

    *The sample that is incubated for 24 hr is subsequently used to correct experimental results for the effects of back exchange (Support Protocol 1, step 5).*

22. At the end of each sample's incubation period, quench on-exchange by adding the contents of one tube of quench buffer to the sample, transfer the resulting mixture to a tube of immobilized pepsin beads, and incubate for the optimal length of time (as identified in step 7) at 4°C. When digestion is complete, centrifuge, aliquot, freeze, and store the digest mixture as described in step 5.

***Obtain mass spectra of amide proton exchange samples***

23. Obtain mass spectra of the digested amide proton exchange samples (steps 17, 18, and 22) as described in step 6, substeps a to e. Analyze all mass spectrometric data as described in Support Protocol 1.

    *Amide proton exchange samples for the receptor, ligand, and receptor-ligand complex should all be analyzed on the same day using the same batch of matrix solution, as doing so produces more consistent results. In addition, only a single block of mass spectrometer time will need to be reserved if all spectra are obtained on the same day.*

    *For each protein, the interface region is identified as the area that shows decreased solvent accessibility in the complex as compared with the uncomplexed state (Fig. 20.9.3).*

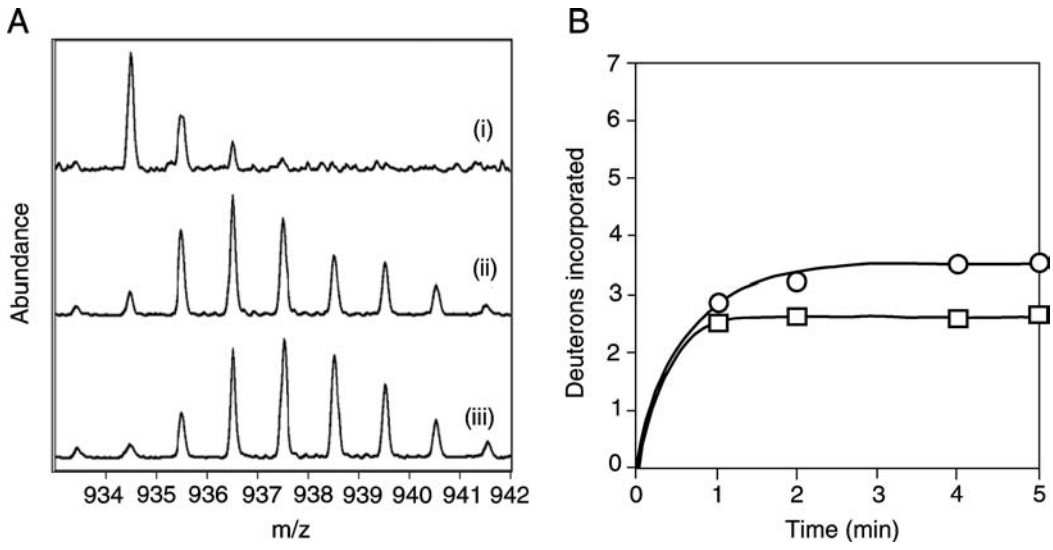


**Figure 20.9.3** In the on-exchange experiment, the interacting proteins, both separately and in the complexed state, are incubated in deuterated buffer for varying lengths of time prior to quenching, pepsin digestion, and mass spectrometric analysis. (**A**) Example of a peptide mass envelope resulting from pepsin digestion of (i) nondeuterated CheB protein; (ii) a deuterated protein complex involving CheB; and (iii) CheB alone after being subjected to the same deuteration period as the complex in (ii). The mass envelope broadens somewhat upon deuteration, and there is less deuteration of the peptide in the protein-protein complex than in the protein alone. (**B**) Graph of number of deuterons incorporated into the peptide shown in panel A versus deuteration time. Circles, uncomplexed CheB; squares, complexed CheB.

***SUPPORT PROTOCOL 1***

## ANALYSIS OF EXPERIMENTAL DATA FROM THE ON-EXCHANGE EXPERIMENT

### *Materials*

MALDI-TOF mass spectra of pepsin digest products from all receptor, ligand, and receptor-ligand complex on-exchange samples (Basic Protocol 1)

MALDI-TOF mass spectra of pepsin digest products from nondeuterated receptor, ligand, and receptor-ligand complex samples (Basic Protocol 1)

Software for displaying multiple mass spectra in stacked layout (provided by manufacturer of the mass spectrometer)

CAPP software package (Mandell et al., 1988b)

KaleidaGraph 3.0 (Synergy Software) or other nonlinear fitting software

1. Display the mass spectra from Basic Protocol 1, step 23, using software that allows the stacking of plots, which will facilitate accurate assignment of monoisotopic peaks (Fig. 20.9.3).

   *Even in the mass spectra of nondeuterated proteins, a "mass envelope" will be observed for each peptide fragment. Within each mass envelope, the lowest-mass peak (termed the $MH^+$ peak) corresponds to the version of the peptide fragment in which all carbons are $^{12}C$ atoms, and the next highest–mass peak corresponds to a version of the peptide fragment in which one $^{13}C$ atom is present. It is possible to calculate the expected relative heights of the peaks in a mass envelope based on the natural abundance of $^{13}C$ and on the number of carbon atoms in the peptide fragment.*

   *Because the mass of $^2H$ is exactly one mass unit greater than that of $^1H$, the presence of a $^2H$ atom in a peptide fragment will shift the entire mass envelope for that fragment by one mass unit. Usually, $^2H$ incorporation also leads to broadening of the mass envelope, although this depends on the distribution (across individual molecules) of the number of $^2H$ atoms incorporated.*

2. Stack the mass spectrum of each deuterated sample on top of the mass spectrum of the corresponding nondeuterated sample. Use the known masses of the peptide fragments in the nondeuterated sample (see Basic Protocol 1, step 9) to calibrate the mass spectra of the corresponding deuterated samples.

   *For some deuterated peptide fragments, a monoisotopic peak will not be present in the mass envelope, and so higher-mass peaks within the same envelope will need to be used. If necessary, these peaks can be identified in the uncalibrated (other than instrument calibration) stacked plots, and since they differ from their corresponding $MH^+$ peaks by an exact integer number of mass units, they can be used for internal calibration.*

3. Calculate the average mass of each peptide fragment in each deuterated sample and each nondeuterated sample by determining the centroid of the fragment's mass envelope using CAPP software (Mandell et al., 1998b). Determine the raw amount of deuterium present in each peptide fragment at each on-exchange time point by calculating the difference between that fragment's average mass in each $D_2O$ labeling sample and its average mass in the nondeuterated sample in which it appears.

   *CAPP software is available free of charge from the author (ekomives@ucsd.edu).*

   *The difference between the average mass of a peptide fragment in a deuterated sample and the mass of that same fragment in the nondeuterated sample equals the raw amount of deuterium that has been incorporated. However, when using MALDI-TOF $^1H/^2H$ exchange data, two corrections (steps 4 and 5) are required to convert the raw result into the actual number of deuterons that have exchanged positions with protons.*

4. In the following way, correct each raw result (step 3) for deuterium content at the rapidly exchanging sites in each peptide due to the presence of a residual amount of deuterium in the MALDI-TOF sample.

   a. Based on the extent to which $D_2O$ was diluted during labeling, pepsin digestion, and subsequent MALDI-TOF sample preparation, calculate the percentage of deuterium present in the MALDI-TOF sample spot.

      *For example, if the experiment involved mixing 100% $D_2O$ with a concentrated protein solution in a 90:10 ratio (for protein labeling), diluting the resulting mixture by a factor of 10 with TFA (for pepsin digestion), and then diluting the digest mixture by a factor of 2 with matrix solution (for MALDI-TOF sample preparation), then the residual deuterium content in the sample spot would be 90% × 1/10 × 1/2 = 4.5% (0.045).*

   b. Calculate the total number of rapidly exchanging proton positions in the peptide fragment of interest (3 at the N-terminus and 1 at the C-terminus, plus 1 for every Asp, Glu, Cys, His, Ser, Thr, Tyr, or Trp residue; 2 for every Asn, Gln, or Lys residue; and 4 for every Arg residue).

   c. Multiply the proportion of residual deuterium present in the MALDI-TOF sample spot (substep a) by the number of rapidly exchanging proton positions in the peptide fragment of interest (substep b), and subtract the resulting quantity from the raw result obtained in step 3.

5. In the following way, correct all non–24-hr incubation results from step 4 for back exchange to determine the true amount of deuterium incorporation during the labeling period.

   a. For each 24-hr labeling sample, using the results from step 4, determine what percentage of potential amide proton exchange sites in each peptide fragment are actually deuterated. In each sample, identify the peptide fragment containing the highest percentage of deuterated sites.

*The percentage of nondeuterated sites in the most deuterated peptide fragment is taken to be the percentage of sites at which back exchange occurred in each corresponding sample during analysis. For example, if the most deuterated peptide fragment in the 24-hr labeling sample shows deuteration at 71% of all possible amide proton exchange sites, then the overall back exchange frequency is calculated to be 100% − 71% = 29%.*

*On-exchange will have run its complete course in some, but not all, regions of a globular protein by 24 hr.*

b. Obtain fully corrected results by dividing each non–24-hr incubation result from step 4 by the percentage of deuterated sites in the most deuterated peptide fragment from the corresponding 24-hr labeling sample.

*For example, if a peptide fragment obtained from a receptor labeling sample was calculated (up to this point) to contain an average of 5 deuterons per molecule, and the maximum percentage of deuterated sites in any peptide fragment obtained from the 24-hr receptor labeling sample was 71%, then the actual amount of deuterium incorporation into the fragment of interest would be 5/0.71 = 7 deuterons per molecule.*

*Since all peptide fragments are analyzed simultaneously in the MALDI-TOF experiment, the same back exchange correction term is used for all fragments obtained from the same type of sample (receptor, ligand, or receptor-ligand complex).*

6. Using the corrected results obtained in step 5, construct a plot of deuterium incorporation versus labeling time for each peptide fragment yielded by each type of sample. Use KaleidaGraph 3.0 (Synergy Software) or any other appropriate nonlinear fitting program to fit the data in each plot to the following two-exponential model, which accounts for positions at which exchange is very rapid (i.e., amides that are fully solvent accessible) and positions at which exchange occurs more slowly (i.e., amides with reduced solvent accessibility in the folded protein):

$$D = N_{\text{fast}}\left(1-e^{-k_{\text{fast}}t}\right) + N_{\text{slow}}\left(1-e^{-k_{\text{slow}}t}\right)$$

where $D$ is the total number of deuterons that have undergone on-exchange by time $t$; $N_{fast}$ is the number of deuterons exchanging at the faster rate, $k_{fast}$; and $N_{slow}$ is the number of deuterons exchanging at the slower rate, $k_{slow}$.

*All instances of rapid exchange will have occurred by the first time point, so $k_{\text{fast}}$ can be fixed to improve fitting accuracy. Other floating parameters ($N_{\text{fast}}$, $N_{\text{slow}}$, and $k_{\text{slow}}$) are completely insensitive to changes in the value of $k_{\text{fast}}$ over a range from 10 $\text{min}^{-1}$ to 100 $\text{min}^{-1}$, and so $k_{\text{fast}}$ can be set to 30 $\text{min}^{-1}$, the median amide proton exchange rate for rapidly exchanging amide protons in the P13 domain of E. coli mannose permease (Gemmecker et al., 1993).*

*Depending on the range of incubation times sampled and the number of data points, a single exponential fit will sometimes be sufficient to describe the data obtained. In such a case, both the number of deuterons undergoing on-exchange and the rate of on-exchange should be fitted parameters.*

*The plots obtained from analysis of the mass envelope in Figure 20.9.3A are shown in Figure 20.9.3B. In this example, the peptide fragment being analyzed corresponds to an interface region. It is clear from the raw data that this region is protected in the complex (Fig. 20.9.3A), and this protection is confirmed at various on-exchange time points out to 5 min (Fig. 20.9.3B).*

## DETERMINATION OF THE SOLVENT ACCESSIBILITY OF A PROTEIN-PROTEIN INTERFACE VIA THE OFF-EXCHANGE METHOD

The rate of off-exchange of deuterons from a protein-protein interface can provide additional information about the solvent accessibility of the interface (Mandell et al., 2001). The protocol described here includes measurement of the on-exchange of deuterium into each of the proteins involved in an interaction. After on-exchange is measured, a suitable set of on-exchange time points are chosen, and the protein samples corresponding to those time points are then combined to allow complex formation, after which the complex is diluted in $H_2O$ for the off-exchange portion of the experiment (Fig. 20.9.1B). With both the on-exchange and off-exchange properties of the interface available, the effects of conformational changes and the effects of interface protection can more easily be distinguished.

***Additional Materials*** *(also see Basic Protocol 1)*

$H_2O$ buffer: 10 mM Tris·Cl (*APPENDIX 2E*), pH 6.6 to 7.9, as appropriate

1. Determine optimal digestion conditions for the separate proteins of interest and the complex and obtain on-exchange data for each of the separate proteins (but not the complex) by performing Basic Protocol 1, omitting steps 19 to 22 (Fig. 20.9.1).

   *On-exchange data provide information about the solvent accessibility of various surface regions on the proteins of interest. In addition, 24-hr on-exchange data are used to obtain back exchange correction factors for use in off-exchange data analysis.*

2. For each off-exchange sample that is to be generated, prepare a tube of immobilized pepsin beads as described in Basic Protocol 1, step 3, making sure to wash at the optimal digestion pH.

3. Prepare several on-exchange samples of the receptor protein and the ligand protein as described in Basic Protocol 1, step 16, and incubate these samples at 25°C for a sufficient length of time to deuterate the surface of the protein (i.e., at least 10 min).

4. At the end of the on-exchange incubation period, combine a 6-µl aliquot of each receptor sample with a 6-µl aliquot of a ligand sample in the same 0.5-ml microcentrifuge tube, mix, and incubate 2 min at 25°C to allow complex formation.

5. Initiate off-exchange by adding 120 µl $H_2O$ buffer to each sample tube, and then incubate at 25°C for times ranging from 1 to 30 min.

   *A typical off-exchange experiment involves testing of five to eight different time points.*

6. At the end of each off-exchange sample's incubation period, quench the sample by plunging the tube into ice water and rapidly adding a predetermined amount of 2% trifluoroacetic acid (~6 µl) to bring the pH of the sample to the optimal digestion pH.

   *The appropriate amount of 2% TFA to add is determined ahead of time, as in Basic Protocol 1, step 2, but with $H_2O$ buffer taking the place of 0.1% TFA in the sample containing the protein complex.*

7. Using the pepsin beads prepared in step 2, perform protein digestion (under the optimal conditions identified in step 1) followed by mass spectrometric analysis of each off-exchange sample (see Basic Protocol 1, steps 4 to 6).

8. Perform control experiments in which each protein is analyzed separately for off-exchange by repeating steps 2 to 7, using $D_2O$ in place of the other protein in step 4.

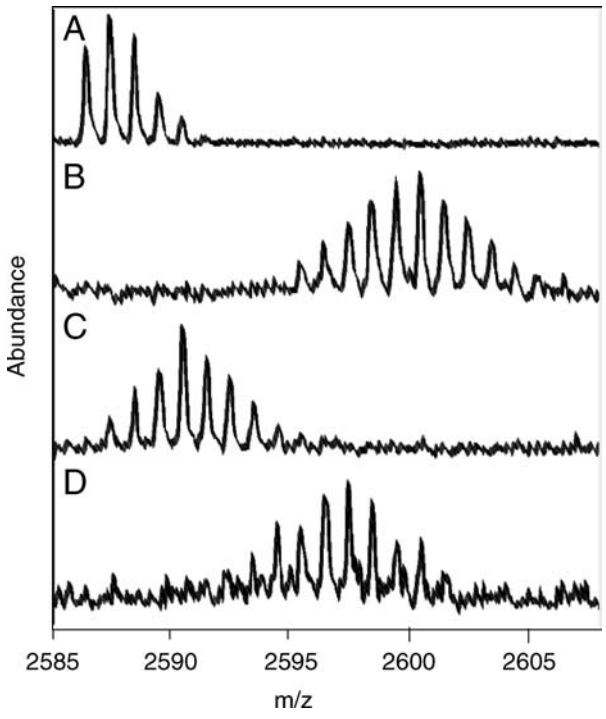


**Figure 20.9.4** Sample off-exchange data. A mass envelope for a single peptide fragment at a single off-exchange time point is shown; however, data from several different time points should be collected to check for consistency in the data. (**A**) Nondeuterated peptide fragment from the uncomplexed protein. (**B**) Peptide fragment from the uncomplexed protein after on-exchange for 10 min. (**C**) Peptide fragment from the uncomplexed protein after on-exchange for 10 min followed by off-exchange for 10 min. Some residual deuteration is seen, due to the fact that some $D_2O$ remains in the $H_2O$ buffer used for off-exchange. (**D**) Peptide fragment from the complexed protein after on-exchange (performed on each component of the complex separately) for 10 min followed by off-exchange (performed on the bound complex) for 10 min. In the bound complex, the peptide retains deuterium throughout off-exchange, whereas in the uncomplexed state, it does not. This indicates that the peptide is probably part of the protein-protein interface region.

*Comparison of receptor-ligand complex data with control data will reveal interfaces as sites at which deuterium (from the on-exchange portion of the experiment) is retained in the complex but not in the uncomplexed protein (Fig. 20.9.4).*

9. Analyze off-exchange data as described in Support Protocol 2.

*ALTERNATE PROTOCOL*

## DETERMINATION OF THE SOLVENT ACCESSIBILITY OF A PROTEIN-PROTEIN INTERFACE VIA THE OFF-EXCHANGE METHOD USING AN AGAROSE-BOUND INTERACTION PARTNER

When both proteins involved in the interaction of interest are large (>40 kDa each), it may be necessary to remove one of them from the mixture prior to mass spectrometric analysis so that good sequence coverage from the observed pepsin fragments can be obtained. In the example presented here, removal of an antibody prior to analysis of the epitope-containing protein is required for successful epitope mapping (Baerga-Ortiz et al., 2002). In such a case, the protein to be removed is covalently linked to agarose beads and eventually separated from its interaction partner by microcentrifugation.

***Additional Materials*** *(also see Basic Protocol 1)*

Antibody of interest
Protein G-agarose beads (Sigma)

20 mM dimethylpimelimidate (Pierce Chemical)
Epitope-containing protein of interest

1:1 mixture of 1-propanol/0.1% (v/v) trifluoroacetic acid (TFA; pH 2.25), chilled to 4°C

Additional reagents and equipment for buffer exchange (*UNIT 4.4*)

***Perform on-exchange experiment for epitope-containing protein***

1. Determine optimal digestion conditions and obtain on-exchange data for the epitope-containing protein by performing Basic Protocol 1, omitting steps 12 to 13 and steps 18 to 22.

***Prepare antibody beads***

2. Using the guidelines provided by the manufacturer, bind the antibody of interest to Protein G-agarose beads and perform cross-linking using 20 mM dimethylpimelimidate. Wash the beads, perform buffer exchange (*UNIT 4.4*), and then store the beads in 30-µl aliquots according to the manufacturer's instructions. Immediately before use, exchange the antibody beads into deuterated buffer (10× protein buffer added to $D_2O$ to a final buffer concentration of 1×).

   *See Basic Protocol 1 for indications regarding appropriate 10× protein buffers.*

3. Perform control experiments to ascertain nonspecific binding of the epitope-containing protein to the Protein G-agarose beads and to determine the binding capacity of the antibody beads.

   *These control experiments entail performing the procedure that follows (steps 4 to 10) using a number of different samples. To test for nonspecific binding, samples containing different amounts of Protein G beads (not linked to the antibody of interest) should be used, and to test for binding capacity, samples containing different amounts of antibody-cross-linked beads should be used. The amount of beads that yields a reproducible mass spectrometer signal while minimizing nonspecific binding should be used in all subsequent experiments.*

***Perform off-exchange experiment***

4. For each sample that is to be generated, prepare a tube of immobilized pepsin beads as described in Basic Protocol 1, step 3, making sure to wash at the optimal digestion pH.

5. In several separate 0.5-ml microcentrifuge tubes, add 1 µl of 100 pmol/µl epitope-containing protein to 9 µl deuterated buffer and incubate 10 min at 25°C to allow deuteration of surface amides. Beginning at the same time, resuspend 30-µl aliquots of antibody beads in 270 µl deuterated buffer and incubate 10 min at 25°C.

6. Microcentrifuge the antibody bead suspensions 1 min at 14,000 × *g*, 25°C. Remove and discard 270 µl of supernatant from each, and add a 10-µl deuterated epitope-containing protein sample to the remaining 30-µl aliquot of antibody beads.

7. Initiate off-exchange by diluting each sample with an additional 360 µl of $H_2O$. Immediately centrifuge each diluted sample 1 min at 14,000 × *g*, 25°C, and then discard 360 µl of supernatant. Resuspend each of the remaining 40-µl aliquots in 360 µl $H_2O$, and then incubate each for a different length of time (0 to 30 min) before centrifuging once more for 1 min at 14,000 × *g*, 25°C, and discarding as much supernatant as possible (leaving a volume of ~30 µl, most of which should be antibody beads, in each tube).

   *If the final centrifugation is performed immediately after resuspension of the antibody beads in $H_2O$, then the time from initiation of off-exchange to completion of the final centrifugation should be ~2 min. During these 2 min, off-exchange of deuterons from the*

*complex is occurring. Longer off-exchange time points are acquired by allowing additional time to pass between resuspension of the beads and centrifugation of the resulting sample.*

8. For each sample, quench the reaction by adding 30 µl of a chilled 1:1 mixture of 1-propanol and 0.1% TFA (pH 2.25). Centrifuge each quenched sample 1 min at 14,000 × *g*, 4°C.

   *The 1:1 1-propanol/0.1% TFA solution not only stops off-exchange but also elutes the binding protein from the antibody, which is covalently attached to the beads.*

9. Add each supernatant from step 8 (30 µl) to a tube of pepsin beads prepared in step 4 and incubate under the optimal digestion conditions for the epitope-containing protein. Process all digestion samples as described in Basic Protocol 1, step 5.

10. Obtain mass spectra of all digestion samples as described in Basic Protocol 1, step 6, substeps a to e.

***Perform control experiments and analyze data***

11. Perform the control experiment (no beads) in the following way.

    a. In each of several 0.5-ml microcentrifuge tubes, add 1 µl of 100 pmol/µl binding protein to 9 µl deuterated buffer and incubate 10 min at 25°C.

    b. Initiate off-exchange by diluting each sample with an additional 90 µl of $H_2O$, and incubate each sample at 25°C for a length of time corresponding to one of the experimental off-exchange time points. When incubation is complete, transfer 10 µl of the sample to a tube containing 30 µl of a chilled 1:1 mixture of 1-propanol and 0.1% TFA (pH 2.25) to quench the reaction.

    c. Prepare an immobilized pepsin bead slurry. Add 100 µl of pepsin slurry to each quenched sample and incubate under the optimal digestion conditions for the epitope-containing protein. Process all digestion samples as described in Basic Protocol 1, step 5.

    d. Obtain mass spectra of all digestion samples as described in Basic Protocol 1, step 6, substeps a to e.

       *In control samples, $D_2O$ is diluted 10-fold in $H_2O$ during off-exchange, while in experimental samples, $D_2O$ is diluted by a factor of ~100. Despite this difference, retention of deuterium in the complex as compared with the uncomplexed protein should be observed. Differences in $D_2O$ dilution are corrected for during data analysis.*

12. Analyze off-exchange data as described in Support Protocol 2.

***SUPPORT PROTOCOL 2***

## ANALYSIS OF OFF-EXCHANGE DATA

An analogous procedure to the one used to analyze on-exchange data (see Support Protocol 1), including corrections for back exchange and residual deuteration, should be used for off-exchange data. The kinetics of off-exchange typically fit best to either a biexponential or a triexponential model. The rate constants in the triexponential model correspond to amides that are not protected from solvent in the complex and remain susceptible to rapid exchange (rate constant set at 30 $min^{-1}$; see Support Protocol 1), amides that are partially protected and thus undergo proton exchange at an intermediate rate (typically 0.1 to 0.5 $min^{-1}$), and amides that are completely protected and thus undergo proton exchange at a slow rate (typically <0.04 $min^{-1}$), respectively. The biexponential model is used when the triexponential model fails to lead to any improvement in the fitting of the experimental data. In such cases, typically, there are no sites at which exchange occurs at an intermediate rate. The triexponential equation is as follows:

$$D = N_{\text{fast}} e^{-k_{\text{fast}} t} + N_{\text{inter}} e^{-k_{\text{intermed}} t} + N_{\text{slow}} e^{-k_{\text{slow}} t}$$

where $D$ is the total number of deuterons that have undergone off-exchange by time $t$; $N_{fast}$ is the number of deuterons exchanging at the fast rate, $k_{fast}$; $N_{inter}$ is the number of deuterons exchanging at the intermediate rate, $k_{intermed}$; and $N_{slow}$ is the number of deuterons exchanging at the slow rate, $k_{slow}$.

## REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

### *Matrix solution for MALDI-TOF*

5 mg/ml $\alpha$-cyano-4-hydroxycinnamic acid (Sigma; recrystallized once from ethanol) in 1:1:1 (v/v/v) acetonitrile/ethanol/0.1% (v/v) trifluoroacetic acid
Adjust pH to 2.2 with 2% (v/v) trifluoroacetic acid
Chill on ice for at least 2 hr prior to use
Prepare fresh before each use

## COMMENTARY

### Background Information

#### *Identification of pepsin digest products*

It is important to determine the identity of the pepsin digest products in a reliable manner. Several bioinformatics tools and mass spectrometry data analysis software packages provide the option of entering peptide fragment masses along with a protein sequence and purport to identify pepsin digest peptides based on this information. These algorithms are only correct some 85% of the time, as they are based on the most common, but not the only, pepsin cleavage sites. Therefore, in order to determine peptide identities reliably, the approach described in Basic Protocol 1 must be used.

#### *Requirement for structural information*

It is important to note that the results of these types of experiments are extremely difficult to interpret in the absence of a structure of at least one of the proteins present in the complex. If a structure is available, then one can "map" the results onto the structure and observe where the changes in proton exchange behavior are occurring. If the segments found to be protected from solvent in the complex all coalesce to a single surface of the protein, then this surface is very likely to be the protein-protein interface. If the segments found to be protected from solvent are on different parts of the molecule, with regions in-between that do not show protection, it is likely that a conformational change is occurring upon protein-protein complex formation.

### Critical Parameters and Troubleshooting

Amide proton exchange rates are strongly dependent on pH, and therefore, control of the pH throughout the exchange experiments is critical for obtaining reproducible, high-quality data. Measurements of pH should be made on nondeuterated mock solutions to avoid electrode isotope effects. Even when deuteration is to be performed on 10-µl protein samples, prior to the first experiment, five 10-µl mock samples should be pooled so that the pH of the mock solution (and, thus, the pH of the actual deuteration solution) can be determined exactly. Measurements of solutions of >50 µl can be accurately made using an In-Lab 423 pH electrode (Mettler Instruments). In the authors' experience, however, probes that purport to measure pH in smaller volumes of solution are very inaccurate. Deuterated buffer solutions should be prepared from 1 M stock buffers (in $H_2O$) in exactly the same manner as the mock solutions.

### Anticipated Results

#### *Finding the solvent-inaccessible "core" of the interface*

The first step in finding the solvent-inaccessible "core" of the interface is to ensure that the complex remains 100% bound throughout the experiment. In one case, the authors studied the binding of two proteins for which the $K_d$ was 120 nM. This interaction showed increasing amounts of deuterium retention at interface peptides as the ratio of one protein to the other was increased from 2.6:1 to 7:1, corresponding to an increase from 98.77% bound complex to 99.78% bound complex (Fig. 20.9.5). The change in the amount of bound complex was highly significant, because at the 2.6:1 ratio, the interacting proteins were apart for 1.5 sec during the 2 min off-exchange period, compared with 0.3 seconds

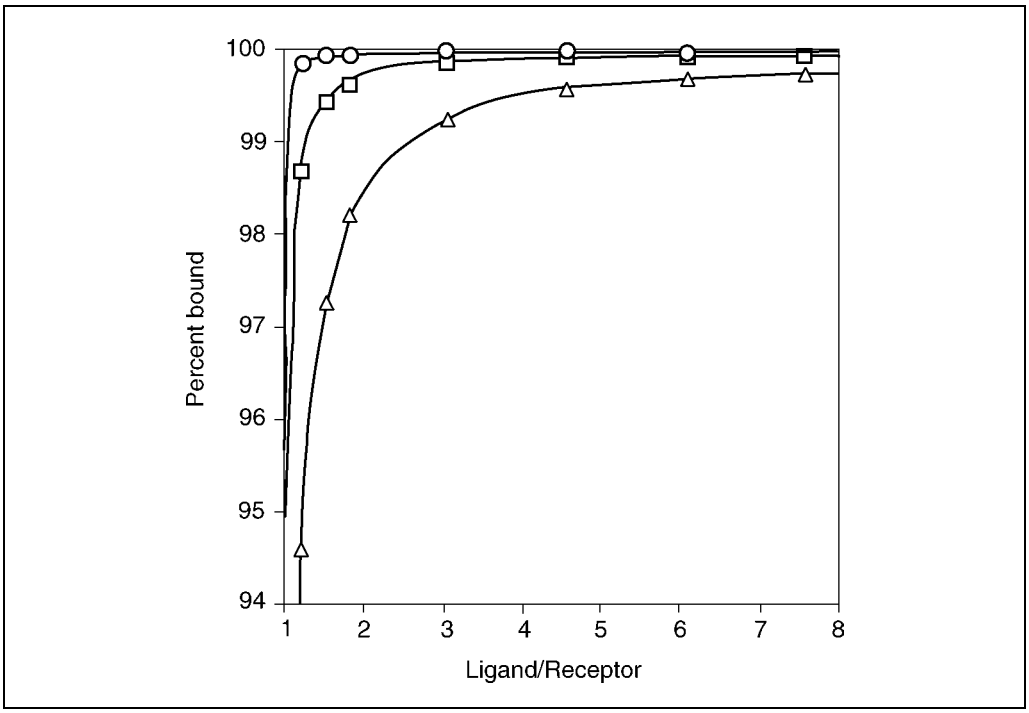


**Figure 20.9.5** Plot showing the relationship between ligand concentration and percentage of receptor molecules bound to the ligand in a typical amide proton exchange experiment. To ensure that 100% of receptor molecules are bound, ligand-to-receptor ratios > 1:1 are required when $K_d \geq 10$ nM. Theoretical curves for $K_d = 1$ nM (circles), $K_d = 10$ nM (squares), and $K_d = 50$ nM (triangles) are shown. Actual experimental data from an interaction for which $K_d = 120$ nM most closely resembled the $K_d = 50$ nM curve.

when a 7:1 ratio was used, and this difference was enough to decrease the observed amount of deuteration. Thus, to ensure that the observed $^1H/^2H$ exchange rate was influenced by amides at the interface of the protein complex and not by complex dissociation, a ratio of 7:1 was used for the quantitative measurement of the exchange rate. The possibility of nonspecific binding arises with high ratios of ligand to receptor, and so comparisons should be made using lower ligand-to-receptor ratios as well; the same surface regions should show slowed exchange in the complex at ratios of 7:1 and 2.6:1, consistent with a lack of nonspecific binding.

Once conditions are found in which the complex remains 100% bound during the experiment, it is sometimes possible to observe amide deuterons that do not off-exchange with solvent $H_2O$ protons. Such peptides become deuterated quickly during on-exchange but then retain their deuterons throughout the off-exchange period (Mandell et al., 2001). An important check on this result involves changing the pH, because the off-exchange reaction (at physiological pH) is a base-catalyzed reaction of $OH^-$ with an amide deuteron. Thus, by changing the pH from 7.4 to 8.4, the concentration of $OH^-$ is increased 10-fold, and therefore, the rate of this base-catalyzed reaction is also increased 10-fold. If exchange occurs more rapidly at the higher pH, then the amides in question must be at least partially accessible to solvent. On the other hand, if these amides only undergo exchange when the complex dissociates, then pH will have no effect on the exchange rate, since this rate will be limited by the rate of dissociation. Examples of the kinetic plots obtained from a solvent-inaccessible region and a partially solvent-accessible region of the thrombin-thrombomodulin complex are shown in Figure 20.9.6.

### *Differentiating conformational changes from interface protection*

It is often difficult to differentiate between changes in solvent accessibility that arise from conformational changes and those that arise from interface protection. Sometimes when proteins interact, a region of the protein becomes *more* accessible to solvent in the complex, and this can readily be interpreted as resulting from a conformational change. An example of this type of result is seen in the regulatory subunit of protein kinase A (Anand et al., 2002).

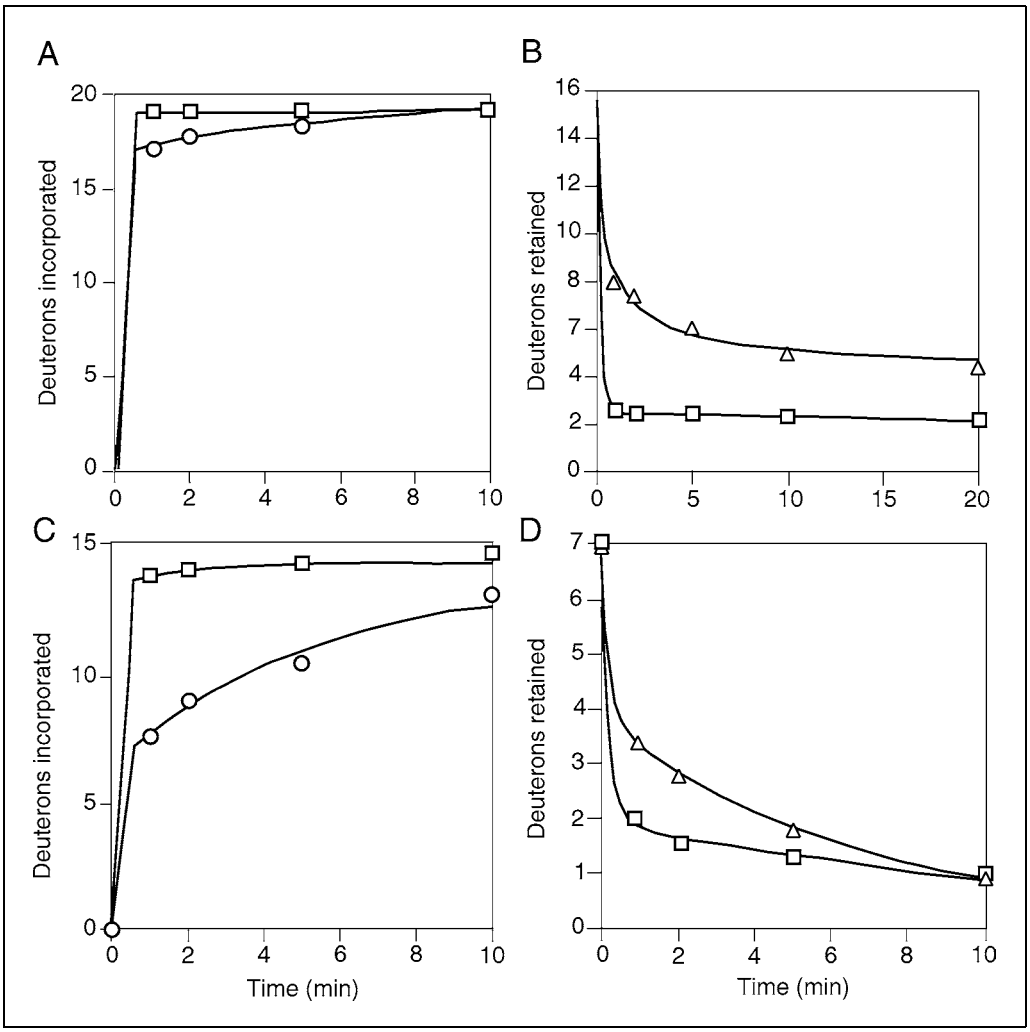


**Figure 20.9.6** Kinetic plots of amide deuteration after data analysis to correct for back exchange and residual side-chain deuteration. (**A**) On-exchange data for a solvent-accessible region of an uncomplexed protein at two different pH values, 6.5 (circles) and 7.9 (squares). (**B**) Off-exchange data for the same solvent-accessible region in the uncomplexed protein (squares) and in the bound complex (triangles). The data obtained from the uncomplexed protein show that off-exchange occurs as rapidly as on-exchange did, and the difference between the two curves indicates that amides are protected from off-exchange in the complex. Identical data were obtained at both pH 6.5 and pH 7.9. (**C**) On-exchange data for a partially solvent-accessible region of the uncomplexed protein at two different pH values, 6.5 (circles) and 7.9 (squares). (**D**) Off-exchange data for the same partially accessible region indicate that at pH 6.5, some deuterium is retained in the bound complex (triangles), but only for a short time. No deuterium retention was observed at pH 7.9 (data not shown).

For regions that show "protection" from amide proton exchange in the bound complex, it is important to map the observed peptide fragments back onto the structure of the protein. This will reveal whether the region corresponding to the peptides in question is on the protein's surface. In the example of thrombomodulin, one of the peptide fragments found to be fully protected in the complex was completely exposed to solvent and susceptible to rapid on-exchange in the control experiment involving the uncomplexed protein. Such data are the most rigorous indicator of the location of the interface that can be obtained from amide proton exchange experiments, and regions that exhibit such behavior can unambiguously be assigned to the interface.

If a peptide fragment exhibiting protection in the complex runs from an interior region of the uncomplexed protein to a surface region, then assignment of the fragment to the interface can sometimes be made by interpreting the time courses of on-exchange and off-exchange in the uncomplexed protein at two

different pH values (for example, 7.4 and 8.4). If the off-exchange kinetics are insensitive to pH, then it is likely that the peptide is located at the interface. However, if protection is only observed when the amides are deuterated at the higher of the two pH values, and not at the lower pH, then it is likely that the amides being deuterated at the higher pH are those located on the interior. This would indicate the possibility that a conformational change is occurring in the complex, since the amides in question are not on the surface of the protein. Finally, if a region incorporates deuterium slowly at both pH levels, any observed protection in the complex should be interpreted with caution.

## Time Considerations

Because amide proton exchange experiments are time sensitive, it is important to set up all materials (e.g., chilled solutions, tubes) ahead of time so that once deuteration is begun, samples can be processed rapidly and consistently. It is best to do one experiment at a time, as this leads to better temporal reproducibility. The number of separate experiments required and the range of deuteration times explored will depend on the protein-protein interaction of interest, but five to six time points (range, 1 to 20 min) typically are sufficient for sampling the changes that occur on a protein's surface.

The time required for a typical experiment varies greatly depending on the instrumentation available and the proteins being studied. In particular, there are large variations in the length of time required to accurately determine the identities of the pepsin digest products, which can range from a few days to weeks. The rest of the protocol, including planning (1 day), pilot studies (1 day), execution of the actual experiment (2 to 3 days), and data analysis (2 to 3 days), typically takes 1 to 2 weeks.

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