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Experimental Assignment of Disulfide-Bonds in Purified Proteins

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Abstract

The formation of disulfide bonds in proteins is an important post-translational modification that is critical for stabilizing the native structures of proteins, particularly proteins exposed to oxidizing environments. For this reason, most cysteines in secreted proteins or protein domains on the surface of the cell are in disulfides, whereas most cysteines in the cytoplasm are in the unmodified -SH form. Disulfide linkages must be experimentally determined as they cannot be predicted from amino acid sequence. These assignments provide insights into three-dimensional structure and contribute to the understanding structural-functional relationships. This unit details a series of protocols that have been applied successfully to map disulfide bonds in proteins. The general strategy involves chemical or proteolytic cleavage of the protein followed by chromatographic separation of the resultant peptides. Disulfide-containing peptides are identified and the sites of disulfide linkage are determined by mass spectrometry. A partial reduction and alkylation strategy for mapping disulfide linkages in peptides with multiple disulfide bonds is also presented.

Keywords

disulfides; disulfide linkage; chemical cleavage; protease cleavage; HPLC; mass spectrometry

INTRODUCTION

The formation of disulfide bonds in proteins is an important post-translational modification that is essential for stabilizing and maintaining the three-dimensional structure of proteins, a property which is critical for their biological activity. The disulfide linkages in a protein cannot be predicted from its amino acid sequence. However, if disulfide bonds in an evolutionarily-related homolog have been assigned, one can predict that the disulfide bond linkages have also been preserved. Indeed, the most strongly conserved amino acid evolutionarily are cysteines that are in disulfides. For all other cases where disulfide bonds are present, their linkages must be experimentally determined. Determination of disulfide linkages is an important step toward a detailed understanding of the structural properties of a protein. The difficulty of determining disulfide linkages generally increases with the number of disulfides present in the protein; i.e., assignment of two disulfides in a protein domain is often not difficult whereas *de novo* assignment of 10 disulfides is often a substantial research

project. Other features that increase difficulty of disulfide assignments include multiple cysteines closely spaced in the primary sequence, cysteine knots, and nested disulfides.

Although MS-based methods for disulfide reduction and software for assignments are rapidly expanding, direct assignment of disulfides using only mass spectrometry (MS) remains very challenging and is often limited to simple linkage assignment problems. For more complex problems, protein digestion and chemical modification methods as described below need to be combined with final analysis using MS. The primary method (see Basic Protocol) describes disulfide-bond mapping using immobilized trypsin as the cleavage agent for proteins. Disulfide-containing peptides are then identified from chromatographic separations of the tryptic digest under reducing and nonreducing conditions. In Alternate Protocol 1, chemical cleavage of proteins with cyanogen bromide (CNBr) is presented. CNBr cleavage generates larger fragments and is especially suited for use as the first cleavage step for disulfide bond mapping of large proteins (>50 kDa). CNBr is also able to cleave proteins with extensive secondary structure that are resistant to denaturation without disulfide reduction, which makes these domains resistant to most proteolytic cleavage. Alternate Protocol 2 describes a partial reduction and alkylation strategy to determine disulfide linkages of proteins/peptides with multiple disulfide-linked cysteine residues that have no appropriate cleavage sites between half-cystinyl residues or are resistant to cleavage. The protein of interest is converted into a mixture of isoforms that ideally have only one of its disulfide bonds reduced. Subsequent alkylation and analysis of the isoforms allow assignment of the disulfide linkages. In the Support Protocol, a method is presented to determine the number of disulfide bonds present in the protein. The information obtained from this protocol can be used to confirm the completeness of the disulfide bond assignments.

NOTE: The major problem in disulfide bond determination is artifactual disulfide bond scrambling, which is the exchange of partners between thiols and disulfides (see Strategic Planning and Critical Parameters and Troubleshooting). Disulfide scrambling occurs at neutral or alkaline pH and at higher rates at elevated temperatures, especially if free thiols are present after the native structure is disrupted with denaturants or by partial hydrolysis of the protein with proteases or chemical cleavage reagents. Disulfide scrambling can usually be suppressed by the use of low pH. Hence, all procedures should be performed in buffers with pH <7 whenever possible.

STRATEGIC PLANNING

Overall Strategy

The basic strategy for disulfide bond mapping involves four major steps (Fig. 1). First, the protein is cleaved by proteases and/or chemical cleavage reagents under nonreducing and acidic conditions to minimize disulfide bond rearrangement. Second, protein fragments are separated from one another by chromatographic methods. Third, disulfide-containing complexes are identified and confirmed by their ability to be reduced into constituent peptides. Finally, the identified disulfide-linked peptides are characterized by MS (see *UNIT 16.1* for overview). N-terminal Edman sequencing (*UNIT 11.10*) is an older method that was traditionally used to obtain sequence information of reduced peptides. However, this

method has largely been replaced by tandem MS (*UNIT 16.10*). Peptides containing multiple disulfides are subjected to further chemical/protease cleavages or analyzed by partial reduction and alkylation to produce simpler disulfide-linked peptides suitable for unambiguous determination of cysteine residues involved in disulfide bond formation. Figure 2 illustrates the determination of disulfide linkages of a protein (GA733–2) with six disulfide bonds, and also shows the structure of some commonly encountered disulfide-linked peptides.

Improvements in MS technology has led to alternative analysis strategies for disulfide bond mapping (Fig. 1). One MS-based strategy is the top down MS method which involves direct analysis of intact protein by electrospray ionization (ESI) on a high performance mass spectrometer (Tipton et al., 2011). Generally, the top down approach is limited to characterizing proteins less than ~50 kDa, but modifications to MS conditions such as thermal and collisional activation can extend the limit to proteins greater than 200 kDa (Han et al., 2006). An ionized protein can be induced to undergo MS/MS fragmentation, and the accurate mass measurement of fragment ions allows high specificity protein structural characterization, including identification of disulfide bonds (Han et al., 2006). However, the top down MS approach requires more complex instrumentation and specialized techniques, such as ultraviolet photodissociation (Qucik et al, 2018) that are not commonly available in most MS laboratories. The development of electron-transfer dissociation (ETD) as an alternative MS/MS fragmentation method has also simplified the methods for identifying disulfide bonds. Traditional MS disulfide bond mapping uses collision-induced dissociation (CID) or higher energy collision dissociation (HCD) for MS/MS fragmentation, which preferentially fragments peptide backbones at the amide bond. However, ETD preferentially cleaves at the disulfide bonds in a disulfide-linked peptide, resulting in disulfide-cleaved peptides that are further fragmented using CID or HCD for sequence information (Wang et al., 2011; Ni et al., 2013; Liu et al., 2014). An alternative MS-based strategy for dissociating disulfide linked peptides in the instrument uses in-source reduction of disulfide linked peptides rather than comparing unreduced and reduced peptide maps (Cramer et al., 2017; Li et al., 2018).

In general, MS/MS spectra from disulfide-linked peptides have to be manually interpreted because commonly used database searching algorithms, such as Sequest and Mascot, are not capable of identifying these peptides. The availability of new bioinformatics tools, such as MassMatrix, pLink-SS and SlinkS, capable of identifying disulfide-linked peptides directly from a LC-MS/MS analysis has greatly facilitated disulfide bond mapping (Xu et al., 2008; Liu et al., 2014; Lu at al., 2014). In particular, the program pLink-SS is capable of identifying disulfide-linked peptides in simple samples as well as from complex proteomes (Lu at al., 2015). Detailed protocol for using pLink has been published recently (Lu at al., 2015; Fan et al., 2015). As with many other MS database search algorithm, identification of disulfide-linked peptides using these programs are subjected to a false discovery rate control. Therefore, it is useful and necessary (in cases where the MS/MS assignments are ambiguous), to confirm the disulfide bond assignments using the protocol described here. A recent comprehensive review of MS-based techniques summarizes and compares most bottom up techniques and associated software for disulfide bond assignment (Lakbub et al., 2018).

Special Instruments

The procedures listed here require the use of a reversed-phase high performance liquid chromatography (RP-HPLC) system with UV detection (*UNIT 11.6*) and a matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometer (*UNIT 16.2*). Alternatively, a high resolution tandem mass spectrometer with a front end nanocapillary UPLC and with CID or HCD fragmentation capacity (*UNIT 16.10*) will have higher sensitivity and require less of the purified protein of interest. A mass spectrometer with ETD capability will have the additional advantage of preferential fragmentation of disulfide bonds. Availability and expertise with these instruments are essential; otherwise the MS procedures can be performed by utilizing a proteomics core facility or by collaborating with a biochemical research laboratory with the needed resources. To assist in the identification of the numerous peptides generated by chemical/proteolytic cleavage, bioinformatics software capable of correlating masses with peptide sequences is indispensable. The authors' typically use the GPMaw program (www.gpmaw.com), but free web-based analysis software, such as Protein Prospector (Clauser et al., 1999) is also suitable. In addition, database search software capable of identifying disulfide-linked peptides, such as MassMatrix and pLink-SS, are also recommended (Xu et al., 2007; Lu et al., 2015; Fan et al., 2015).

Sample Preparation

The assignment of correct disulfide linkages in proteins containing multiple disulfide bonds is a challenging task and becomes increasingly difficult as the number of cysteine residues increases. In addition, as noted above, artifactual disulfide bond scrambling can contribute to the difficulty of disulfide bond assignment. Very often, a protein requires multiple consecutive or simultaneous cleavages to produce suitable disulfide-linked peptides for unambiguous identification. To ensure the success of the project, a relatively large amount (e.g., low nmole range for the off line HPLC/MALDI MS approach) of the protein of interest in a highly purified, native state is desirable, especially for larger proteins with high disulfide bond content. Newer generation ESI mass spectrometers, such as the Thermo Scientific Q Exactive HF with an online nanocapillary HPLCs, have far better sensitivity and will require less starting sample (i.e., low pmol level). However, sample amount is usually not a problem because disulfide linkages are often determined from purified recombinant proteins with known amino acid sequences. More importantly, protein purification strategies must be modified to avoid potential disulfide bond rearrangements or partial reduction. Whenever possible, buffers used in protein purification should be slightly acidic (pH ~6.5) to minimize disulfide scrambling, particularly during steps that may perturb tertiary structure, and reducing agents should not be used. If proteases will be used for fragmentation of the protein, protease inhibitors should be omitted from the purification (see *UNIT 11.1*) or removed after purification.

Production of Disulfide-Containing Peptides

Cleavage of the protein at sites that will produce simple disulfide-linked peptides is a critical step toward successful assignment of disulfide linkages. Cleavage is often performed in the presence of denaturing agents (urea or guanidine-HCl) to allow optimal access to the

cleavage sites. The choice of protease (*UNIT 11.1*) or chemical cleavage reagent (*UNIT 11.4*) to use for protein cleavage is not straightforward and in part depends on the susceptibility of the protein to the cleaving reagents and the position of cysteine residues in the protein sequence. Ideally, the cleavage agent selected should produce peptides with only one disulfide bond and should be active at a pH <7. In practice, a combination of cleavage agents is often required to produce a series of peptides suitable for disulfide bond assignment (Chong and Speicher, 2001; Roszmusz et al., 2001; Chong et al., 2002). Specific cleavage agents such as trypsin, endoproteinase Glu-C, and CNBr have the advantage of producing easily identified fragments because the cleavage boundaries are well defined; however, the specificity of these reagents may not permit sufficient numbers and locations of cleavage sites for production of peptides linked by a single disulfide bond. To circumvent this problem, broad specificity cleavage methods (e.g., partial acid hydrolysis, pepsin, subtilisin) can be employed. The major disadvantage of using broad specificity cleavage agents is that the complexity of the peptides generated is greatly increased, making the isolation and identification of disulfide-linked peptides much more difficult. Also, such cleavages are more likely to produce very short cysteine containing peptides that may occur multiple times in the sequence, particularly for very large proteins or proteins with multiple highly homologous domains.

Cleavage agents such as CNBr (see Alternate Protocol 1), 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP), endoproteinase Glu-C, and pepsin are favored because of their capacity to work effectively at low pH. The repertoire of proteases with low pH optima is limited, but often sufficient cleavage can be achieved at suboptimum pH conditions. To compensate, proteases with alkaline pH optima, such as trypsin, are typically used at higher concentrations with longer incubation times. In this respect, immobilized proteases are preferred to avoid contamination of the sample with large amounts of protease autolytic products (see Basic Protocol). A number of potentially useful immobilized proteases (e.g., endoproteinase Glu-C, trypsin, pepsin, α -chymotrypsin) are available from MoBiTec and other suppliers. A list of cleavage agents with typical reaction conditions for disulfide bond mapping is shown in Table 1. In situations where proteolytic and chemical cleavages are not able to produce peptides with single disulfide-linkages, partial reduction and alkylation methods can be employed (see Alternate Protocol 2). This strategy depends on tandem MS (*UNIT 16.10*) to identify the cysteine residues and will work well with cleavage agents, such as trypsin, that produce peptide fragments that are typically less than ~30 residues.

Separation and Identification of Disulfide-Containing Peptides

Off-line separation, usually with UV detection, needs to be used for most workflows that use MALDI MS for peptide analysis. Off-line separation can also be useful for situations where large or complex fragments need to be further fragmented and/or analyzed by multiple approaches. However, as noted above, off-line methods require much more starting protein sample compared with on-line LC-MS or LC-MS/MS analysis. For off-line separation of cleavage products, the method chosen depends mainly on the fragment sizes. For large fragments such as those generated by CNBr cleavage, gel-filtration chromatography is often useful (see Alternate Protocol 1). For separation of smaller fragments, such as those

produced by protease digestion, RP-HPLC with 0.1% TFA in a water/acetonitrile binary system is the separation method of choice. This system is capable of detecting minor changes in the hydrophobic character of the peptides, such as those caused by reduction of intrachain disulfide bonds, making it a highly sensitive method for detecting disulfide-containing peptides (see below). In addition, the RP-HPLC solvents have a pH of ~2, which is optimal for preventing disulfide scrambling.

The general strategy to identify disulfide-containing peptides is to detect alterations to peptide mobility as a consequence of disulfide bond reduction. An early yet elegant approach was the use of diagonal paper electrophoresis of peptides with performic acid cleavage of disulfide bonds between the first and second dimension separation (Brown and Hartley, 1966). Similar approaches are still used, except that paper electrophoresis is replaced by higher sensitivity methods, such as comparison of peptide mobilities by RP-HPLC in the nonreduced and reduced states (see Basic Protocol). Changes in the hydrophobicity of dissociated peptides after reduction can easily be detected by this method (UNIT 11.6).

LC-MS can also be used to directly analyze the unfractionated nonreduced and reduced states to rapidly provide information on disulfide linkages (Lu et al., 2015). The major difference compared with off-line reverse phase with UV detection is that the 0.1% TFA in the water/acetonitrile HPLC solvent system is replaced with 0.1% formic acid. Interchain disulfides are identified by the appearance of two signals in the reduced digest that sum to the mass of a single signal plus nominally 2 Da (mass of two H) in the nonreduced digest, while intrachain disulfides are identified by the mass increase of 2 Da after reduction. The mass spectrometric approach works best with simpler proteins, and high performance mass spectrometers with high mass accuracy and resolution are required for analyzing relatively high molecular masses, especially peptides with intrachain disulfide bonds. While it is quite feasible to perform direct mass spectrometric analysis of cleavage fragments with and without reduction, it can sometimes be desirable to isolate the peptide fragments by off-line RP-HPLC prior to mass analysis as this allows repeated and additional analyses to be performed on the isolated peptides.

Sequence Determination of Disulfide-Containing Peptides

On-line LC-MS/MS with collision-induced dissociation (CID) or high energy, requires the least amount of sample and does not require well-separated peptides (Pitt et al., 2000; Schnaible et al., 2002b). By coupling liquid chromatography with tandem MS (LC-MS/MS), disulfide linkages can be determined from the unfractionated tryptic digest (Yen et al., 2000). The mass spectrometric approach is comparatively faster and requires small quantities of samples, making it the method of choice when sample is limiting. Another major advantage of LC-MS/MS is the ability to determine the disulfide linkages of simple multiple disulfide-containing complexes, such as three peptide chains linked by two disulfide bonds or two peptide chains with one intra- and one interchain disulfide bond. However, the fragmentation data from a disulfide-containing complex is complicated and can be challenging to interpret. However, the availability of software capable of

automatically identifying disulfide-linked peptides from MS/MS spectra has improved the data analysis (Xu et al., 2008; Lu et al., 2015).

BASIC PROTOCOL

TRYPSIN CLEAVAGE AND DISULFIDE-BOND MAPPING OF PROTEINS

In this method, the protein is denatured and digested with immobilized trypsin under nonreducing conditions in the presence of intermediate concentrations of a denaturant such as urea. The proteolytic fragments generated are separated by RP-HPLC and disulfide-containing complexes are identified by comparing the reduced and nonreduced chromatograms. The peaks of interest are further characterized by MALDI-TOF MS (*UNIT 16.2*) and/or tandem MS (*UNIT 16.10*).

Materials

Urea buffer (see recipe)

Protein sample, lyophilized or aqueous (i.e., in mildly acidic buffer without serine protease inhibitors and with known concentration)

Argon

Urea, solid (for aqueous proteins only)

1M HCl

1M NaOH

50 mM sodium phosphate, pH 6.5 (*APPENDIX 2E*)

Reaction buffer: 3 M urea/50 mM sodium phosphate, pH 6.5

Trifluoroacetic acid (TFA)

Reducing solution, pH 8.0 (see recipe)

Solvent A: 0.1% TFA in H₂O

Solvent B: 0.1% TFA in 95% acetonitrile

50% acetonitrile/0.1% TFA

Immobilized tosylphenylalanine chloromethylketone (TPCK)-treated trypsin F7m column (MoBiTec)

10-ml syringe

HPLC system, with 1.0 mm C18 reversed-phase column (e.g., ZORBAX 300SB-C18), UV detector, fraction collector, peak detector, and chart recorder

C18 MicroSpin columns (The Nest Group Inc.)

Additional reagents and materials for RP-HPLC (*UNIT 11.6*) and MALDI MS analysis of peptides (*UNIT 16.2*), or tandem MS (*UNIT 16.10*)

Denature protein

- 1a. *For lyophilized proteins:* Add 100 μ l of urea buffer to 10–100 pmol lyophilized protein in a microcentrifuge tube. Blanket with argon, seal, and vortex lightly to dissolve.

Use freshly prepared buffer and high purity urea to minimize accumulation of cyanate from urea.

- 1b. *For aqueous proteins:* Dissolve solid urea in 100–1,000 pmol of protein solution to a final concentration of 9 M and readjust pH to 6.5 with 1 M HCl or NaOH as appropriate.

The protein should be prepared in a mild acidic buffer without phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, aprotinin, or other serine protease inhibitors to prevent inactivation of trypsin (UNIT 11.1). As in step 1a, use freshly prepared buffer and high purity urea to minimize accumulation of cyanate from urea. The final volume should be 100 μ l.

2. Incubate sample 30 min at 37°C to ensure protein denaturation.

Avoid temperature higher than 37°C and prolonged incubation (>1 hr) to minimize carbamylation of cysteine and lysine residues. The rate of carbamylation is greatly reduced at pH 6.5 compared with higher pH. As denatured protein is more susceptible to disulfide scrambling (see Strategic Planning and Critical Parameters and Troubleshooting), proceed to cleavage immediately. Do not store denatured protein at this stage.

3. Dilute denatured protein solution with 200 μ l of 50 mM sodium phosphate, pH 6.5, to lower the urea concentration to 3 M.

Other proteases have different tolerance for urea (UNIT 11.1). Also, deglycosylate if necessary prior to digestion (UNIT 12.4).

Equilibrate immobilized-trypsin F7m column

4. Equilibrate a TPCK-treated trypsin F7m column with 10 ml reaction buffer at room temperature. Connect the top of the column to a 10-ml syringe filled with reaction buffer and apply pressure to assist the flow.

The column contains 200 μ l F7m matrix, which has large pores and is suitable for proteins up to 10^7 Da. Immobilized trypsin is preferred because a larger amount of immobilized protease can be used without appreciable contamination of reaction solutions with protease autolytic products.

5. Microcentrifuge the column 5 sec at 2000 rpm to remove residual buffer.

Digest protein

6. Load the protein solution onto the column as follows.
 - a. Apply protein solution to the column and allow it to flow through by gravity over a period of ~1 hr.

- b. During this time, continue to reapply the sample onto the column as it elutes from the column.
- c. After passing the entire sample through the column approximately six times, cap the column outlet and incubate overnight at room temperature.

Repeated application of the sample onto the column and overnight incubation ensure more efficient protease cleavage.

7. Microcentrifuge the column 5 sec at 2000 rpm to collect the digested sample.
8. Remove residual peptides with two successive washes of 200 μ l reaction buffer, followed by microcentrifuging 5 sec at 2000 rpm.

Column can be reused after re-equilibration and should be stored in buffer without urea.

9. Combine digest and washes. Remove half the sample for reduction with Tris(2-carboxyethyl)phosphine (TCEP; see steps 10 to 12). Add TFA to the remainder of the sample to a final concentration of 2% (pH ~2) and proceed to step 13 with this aliquot.

Samples should be analyzed by RP-HPLC within ~24 hr (store at 0° to 4°C until analyzed). Otherwise, store samples at –80°C. At pH 2, disulfide scrambling is largely inhibited.

Reduce disulfide bonds of digest with TCEP

10. Add an equal volume of reducing solution, pH 8.0, to the saved aliquot. Mix and blanket reaction mixture with argon.

Although TCEP can function at acidic pH, reduction of disulfide bonds is most effective at pH 7 to 8. An inert atmosphere prevents reoxidation of cysteines to disulfides.

11. Incubate the mixture 1 hr at 37°C.
12. Adjust the pH of the reduced tryptic digest by adding TFA to a final concentration of 2%.

This reduces the pH to ~2 for RP-HPLC analysis.

Separate reduced and nonreduced peptides by RP-HPLC

13. Equilibrate an RP-HPLC column at 50 μ l/min in 98% solvent A/2% solvent B (UNIT 11.6).

A 1.0 mm inner diameter $\times$ 150 mm C18 column, 300-Å pore size, 5- μ m particle size) or comparable column is suitable for this purpose. See UNIT 11.6 for details on RP-HPLC separation of peptides.

14. If necessary, determine the optimal separation gradient for the protein or set of digestion conditions. Once this has been determined, inject the reduced (step 12) and nonreduced (step 9) samples separately onto the column.

The separation gradient will need to be optimized for different proteins/digests to obtain the best separation for all the components. Refer to UNIT 11.6 for examples. For optimization, inject 5 pmol aliquots of each sample. Once a satisfactory gradient has been established, inject 80% of the remaining samples in 500-pmol aliquots to avoid overloading the column.

15. Elute peptides using the optimized acetonitrile gradient and collect all fractions with the help of a peak detector and fraction collector. Store eluted peptides at 4°C (up to a month) or –80°C (indefinitely).

Identify disulfide-linked peptides by MALDI-MS

16. Analyze all peaks in the nonreduced and reduced RP-HPLC chromatogram by MALDI-MS (UNIT 16.2).

Large peptides are mixed 1:1 with a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 33% CH₃CN and 0.1% TFA. Peptides <5 kDa are applied to a MALDI sample plate precoated with a saturated solution of nitrocellulose and α-cyano-4-hydroxycinnamic acid (1:4 w/w) in 2-propanol and acetone (1:1 v/v). Since each chromatographic peak could have a mixture of peptides with differing ionization efficiency, it is advisable to perform the MALDI-MS with more than one matrix (see UNIT 16.2). MALDI-MS is usually sufficient to identify peptides generated by high specificity proteolytic or chemical cleavages, and is faster and consumes less sample than ESI-MS or LC-MS/MS. Ambiguous peptides can be confirmed by tandem MS (UNIT 16.10).

17. Compare RP-HPLC chromatograms of reduced and nonreduced tryptic digests. Confirm the presence of disulfide complexes by reducing the appropriate fractions.

Peaks in the nonreduced chromatogram that disappear or have a decreased peak area after reduction probably contain disulfide-linked peptides. New peaks corresponding to constituent peptides of these disulfide-linked complexes are usually present in earlier eluting fractions of the reduced chromatogram. Figure 3 shows an example of the reduced and nonreduced chromatograms of a tryptic digest.

Reduce RP-HPLC fractions to confirm presence of disulfide complexes

18. Add 8 µl reducing solution to 2 µl RP-HPLC fractions from the nonreduced digest that contains the suspected disulfide-linked peptides. Mix and blanket reaction mixture with argon.
19. Incubate 1 hr at 37°C to reduce disulfide bonds.
20. Desalt the reaction solution using a C18 clean up Tip or equivalent following manufacturer's instructions.

TCEP can interfere with efficient ionization of peptides and should be removed prior to MS analysis. Alternatively, if the sample is concentrated enough, dilute the reduced sample at least 2- to 4-fold with 0.1% TFA for MALDI-MS analysis.

21. Elute peptides with 2 μ l of 50% acetonitrile/0.1% TFA and analyze by MALDI-MS (*UNIT 16.2*).

Appearance of two new peptides having a combined mass (MH⁺) that is 3 Da higher than the nonreduced peptide complex indicates a single interpeptide disulfide bond. An internal peptide disulfide is identified by a mass increase of 2 Da compared with the nonreduced peptide. The disulfide assignment is complete if the complex contains a total of two cysteine residues (e.g., T3 complex in Figure 2). Cysteine residues not involved in disulfide linkages are identified from RP-HPLC fractions containing cysteinyl peptides that do not display retention time shifts or mass changes after reduction.

Confirm peptide sequence and (if necessary) choose an alternate cleavage strategy

22. Confirm sequence of disulfide-containing peptides by tandem MS (*unit 16.10*) as needed.
23. If reduction of the disulfide complex resulted in more than two peptides or at least one of two peptides contains multiple cysteines perform one of the following steps:
 - a. Dry the RP-HPLC fractions completely (~1 hr) in a Speedvac. Choose a new cleavage agent (Table 1), resuspend in appropriate denaturing buffer, and digest. Repeat steps 9 to 22.
 - b. Alternatively, analyze the fractions by partial reduction and alkylation (see Alternate Protocol 2).

ALTERNATE PROTOCOL 1

CYANOGEN BROMIDE CLEAVAGE OF PROTEIN

Proteins or large peptide complexes are cleaved at the C-terminal side of methionine residues in 70% formic acid using CNBr. The low pH of the cleavage reaction prevents disulfide scrambling from occurring. The fragmentation is usually monitored by SDS-PAGE and the cleavage products are subsequently separated by preparative HPLC gel-filtration for disulfide mapping. Due to the low occurrence of methionine residues in most proteins, CNBr cleavage usually generates relatively large fragments. Therefore, this procedure is often used as the first cleavage step for disulfide mapping of large proteins (>50 kDa) or proteins with large domains containing multiple disulfide bonds, which are usually resistant to denaturation and proteolysis.

Additional Materials (also see Basic Protocol)

Glacial and 5% acetic acid

Solution of purified protein with known sequence

88% and 70% formic acid

CNBr

Acetonitrile

Desalting column (i.e., Bio-Rad Econo-Pac 10DG or equivalent)

Fraction collector

Glass vial with Teflon-lined cap

Aluminum foil

Additional reagents and materials for desalting protein samples (*unit 8.3*) and SDS-PAGE (*unit 10.1*)

Prepare sample for CNBr cleavage

1. Add glacial acetic acid to ~10 nmol purified protein solution to a final concentration of 5%. Vortex to mix.

CNBr cleavage, like most chemical cleavages, tend to be incomplete; therefore, a higher amount of starting material is required. The protein quantity given (10 nmol) is a good starting amount, especially when CNBr is used as the first cleavage step in multistep disulfide mapping experiments.

2. Desalt the acidified protein sample on a desalting column (*UNIT 8.3*). Use 5% acetic acid to elute the protein.

The acidic pH inhibits disulfide bond scrambling.

3. Collect 0.5-ml fractions for a total of 20 fractions. Determine the absorbance at 280 nm for each fraction and analyze by 1-D SDS-PAGE (*UNIT 10.1*) to verify elution of the protein. Pool all protein-containing fractions.
4. Lyophilize samples and reconstitute with 400 μ l of 88% formic acid. Vortex to dissolve sample. Keep a 20- μ l aliquot for SDS-PAGE.

Cleave protein with CNBr

5. Prepare a CNBr stock solution (0.5 mg/ μ l) immediately before use in a chemical fume hood using the following procedure:
 - a. Weigh an empty glass vial and Teflon-lined cap, then move to the hood and transfer a small amount of CNBr into the vial.
 - b. Tightly cap the vial, and weigh it again.
 - c. Determine the amount of CNBr in the vial (in grams) and dissolve in the hood using 5 ml acetonitrile per 0.5 g CNBr.
 - d. Wrap the vial in aluminum foil and keep it in the hood.

CAUTION: CNBr is extremely toxic and must be neutralized with NaOH. See UNIT 11.4 for details.

This procedure is necessary as balances generally do not function properly in fume hoods.

6. Determine the Met content of the protein and calculate the amount of CNBr required for 100-fold molar excess.

For example, 10 nmols of a protein with 10 Met residues will require $10 \text{ nmol} \times 10 \times 100 = 10 \text{ } \mu\text{mol}$ CNBr. This is equivalent to $10 \text{ } \mu\text{mol} \times 0.106 \text{ mg/} \mu\text{mol} = 1.06 \text{ mg}$ CNBr.

7. Add appropriate amount of CNBr stock solution (0.1 mg/ μl) to the sample from step 4 and add water to a final volume of 500 μl . Vortex to mix. Blanket reaction mixture with argon.

The final concentration of formic acid is ~70%.

8. Wrap the tube with aluminum foil. Incubate 24 hr at room temperature in the hood.

The reaction must be carried out in complete darkness to avoid unwanted side-reactions. CNBr cleavage will result in peptide bond cleavage on the C-terminal side of methionines with conversion of the methionine to a mixture of homoserine and homoserine lactone. The predominant form is homoserine lactone which has a residue mass of 83.04 Da. The change in mass should be taken into consideration when identifying CNBr peptides using MS.

Determine cleavage success or failure

5. Dry the reaction mixture with a Speedvac (~1 hr) and resolubilize the sample in 400 μl of 70% formic acid. Repeat the lyophilization and reconstitute in 500 μl urea buffer.

CAUTION: The CNBr will be in the trap of the Speedvac. Transfer to the chemical hood to discard CNBr and drain trap.

6. Analyze the cleavage reactions with reducing and nonreducing SDS-PAGE (*unit 10.1*).

By loading proportional amounts of the total sample at each step, the success of the cleavage reaction can be easily determined from the intensities of protein staining and cleavage failure or excessive losses can be easily traced to a particular step.

Separate and analyze cleavage products

1. Separate the cleavage products by HPLC gel-filtration chromatography (*unit 8.3*).

The authors typically use two TSK columns, G3000 SW_{XL} and G2000 SW_{XL}, connected in series with a running buffer of 10 mM sodium phosphate (pH

6.5)/150 mM NaCl/7 M urea, pH 6.5, at a flow rate of 0.6 ml/min at 4°C. (The running buffer is prepared fresh daily.) If the sample is separated at 4°C, the urea in the sample must be decreased from 9 to 7 M using buffer without urea.

2. Analyze separated fragments with reducing and nonreducing SDS-PAGE (*unit 10.1*) and MALDI-MS (*UNIT 16.2*).

Fragments containing disulfide linkages can be identified easily after reduction by changes in migration.

3. Analyze disulfide-containing fragments as described (see Basic Protocol, steps 3 to 23).

ALTERNATE PROTOCOL 2

PARTIAL REDUCTION AND ALKYLATION OF DISULFIDE BONDS

Protein fragments with multiple disulfide linkages are partially reduced with Tris(2-carboxyethyl)phosphine (TCEP) to generate a series of isoforms with only one disulfide bond reduced. The nascent free thiols are immediately alkylated with excess iodoacetamide. The partially reduced and alkylated fragments are separated by RP-HPLC. Each isoform is characterized by MALDI-MS (*UNIT 16.2*) and/or tandem MS (*UNIT 16.10*) to reveal the position of the alkylated cysteine residues. This procedure is useful for dissecting protein fragments with multiple disulfide bonds when cleavage with proteases/chemicals fails to generate appropriate peptides with a single disulfide bond.

Additional Materials (also see Basic Protocol)

Reducing solution, pH 3.5 (see recipe)

RP-HPLC-purified peptide complex (see Basic Protocol)

1 M alkylation solution (see recipe)

0.3% and 10% TFA

1. Add an equal volume of reducing solution, pH 3.5 to ~100 pmol (~80 µl) of a RP-HPLC purified peptide complex containing multiple disulfides. Incubate reaction mixture 3 min at room temperature.

The reduction rate can be controlled by altering the amount of TCEP in the reaction mixture and the incubation time. It is necessary to optimize the reaction to generate a good yield of different disulfide forms. The yield of the different partially reduced isoforms can be conveniently determined by RP-HPLC.

2. Remove an aliquot (15% of reaction volume) and add to two volumes of 0.3% TFA. Store the reaction mixture on dry ice until ready for RP-HPLC analysis.

Dilution with 0.3% TFA reduces the pH to ~2 and decreases the acetonitrile content of the reaction mixture so that peptides can bind to the RP-HPLC column.

3. Vigorously pipet the remaining partially reduced peptide solution into a microcentrifuge tube containing an equal volume of 1 M alkylation solution. Mix immediately by pipetting up and down a few times.

To minimize disulfide scrambling, it is important to add the partially reduced peptides into the alkylation buffer and not vice versa. This will prevent a transient pH increase before a sufficient concentration of alkylation reagent is present.

4. Wrap reaction tube with aluminum foil and incubate 30 min at 37°C.

Protect the alkylation solution and reaction from light to minimize photolytic production of iodine, a strong oxidant for thiols.

5. Terminate the reaction by adding 10% TFA to a final concentration of 1.3% (pH ~2.0).

Acidify the reaction quickly to prevent disulfide scrambling. Analyze the reaction immediately by RP-HPLC to prevent artifactual alkylation of other amino acid residues by the high concentration of iodoacetamide.

6. Separate both reaction mixtures (with and without alkylation) immediately by RP-HPLC (*unit 11.6*).

The alkylated peptides have different retention times than the nonalkylated peptides. Ideally, the number of peptide peaks from the partially reduced sample with alkylation should be the same as the partially reduced sample without alkylation. In practice, more peaks are normally observed in the alkylated sample due to some disulfide scrambling during alkylation; however, these peaks are relatively minor and should not pose a problem in the disulfide bond assignments.

7. Analyze all the RP-HPLC peaks by MALDI-MS (*UNIT 16.2*) or LC-MS.

Disulfide-linked peptides with a single reduced disulfide have a mass increase of 116 Da due to alkylation. When subjected to MALDI-MS analysis, disulfide-linked peptides often undergo in-source fragmentation of the disulfide bond to yield the constituent peptides. Alkylated constituent peptides have a mass increase of 57 Da over free cysteine. If the MALDI in-source fragmentation is not sufficient, the masses of the constituent peptides can be determined after reduction as described (see Basic Protocol, steps 18 to 21).

8. Verify the sequence and locate the alkylated cysteines by tandem MS (*UNIT 16.10*).

Figure 4 shows an example of the partial reduction and alkylation of a multiple disulfide-containing complex.

Theoretically, one isoform is sufficient to define a two disulfide-linked complex, and two isoforms for a three disulfide-linked complex, etc. In practice, additional isoforms are often obtained that can provide confirmation to the disulfide assignments.

SUPPORT PROTOCOL

DETERMINATION OF TOTAL DISULFIDE BONDS

The total number of disulfide bonds can be determined by comparing the molecular masses of the protein of interest obtained after (1) reduction and alkylation of all SH groups and (2) alkylation of the original free SH groups without reduction of disulfide bonds. Reduction is carried out with TCEP, and alkylation is performed with iodoacetamide. An alternative method to determine the number of disulfide bonds is to determine the free sulfhydryl content of the protein with Ellman's reagent (*UNIT 15.1*). The colorimetric method is, however, less sensitive and requires nanomoles of protein.

Additional Materials (also see Basic Protocol)

Protein solution

Guanidine buffer (see recipe)

Reducing solution, pH 6.5 (see recipe)

80 mM alkylation solution (see recipe)

0.3% TFA

50% acetonitrile/0.1% TFA

1. Mix 2 μ l (~10 pmol) protein solution with 15 μ l guanidine buffer. Blanket with argon.
8 M urea can be used in place of guanidine-HCl.
2. Incubate sample 30 min at 37°C to denature protein.
3. Remove 5 μ l denatured protein (step 2) and reduce by adding an equal volume of reducing solution, pH 6.5. Blanket with argon and incubate reaction 1 hr at 37°C.
4. Remove 5 μ l reduced protein (step 3) and alkylate with an equal volume of 80 mM alkylation solution. Blanket with argon and incubate reaction 1 hr at 37°C.
To minimize disulfide scrambling and avoid incomplete modification, perform a series of alkylations with different incubation times to determine the minimal incubation period required for successful alkylation. The success of the reduction and alkylation procedure can be gauged from the experimental total number of cysteine residues (N_{Cys} , steps 9 to 10) compared to the expected number determined from the protein sequence.
5. In parallel, alkylate 5 μ l denatured but nonreduced protein (step 2) as described in step 4.
6. Add an equal volume of 0.3% TFA to the remaining denatured protein (step 2), reduced protein (step 3), reduced and alkylated protein (step 4) and nonreduced alkylated protein (step 5).
7. Remove reaction buffer components using C18 MicroSpin columns according to the instructions provided by the manufacturer.

8. Elute peptides from C18 MicroSpin columns with 2 μ l of 50% acetonitrile/0.1% TFA.
9. Determine the molecular masses of denatured protein (M_{step2}), reduced protein (M_{step3}), reduced and alkylated protein (M_{step4}), and nonreduced alkylated protein (M_{step5}) by MALDI-MS (*UNIT 16.2*).
Mix sample with 1:1 with a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 33% CH₃CN and 0.1% TFA, and apply to target plate.
10. Calculate the number of cysteine residues (N_{Cys}):

$$(N_{\text{Cys}}) = (M_{\text{step4}} - M_{\text{step3}})/57.$$

where M_{step4} is the mass of the denatured, reduced, alkylated protein and M_{step3} is the mass of the denatured reduced protein.

S-Carboxamidomethylation of a cysteine residue with iodoacetamide results in a mass increase of 57 Da.

11. Calculate the number of free sulfhydryl groups (N_{SH}):

$$(N_{\text{SH}}) = (M_{\text{step5}} - M_{\text{step2}})/57.$$

where M_{step5} is the mass of the denatured, nonreduced, alkylated protein and M_{step2} is the mass of the denatured nonreduced protein.

12. Use this information to calculate the number of disulfide bonds:

$$\text{No. disulfide bonds} = (N_{\text{Cys}} - N_{\text{SH}})/2.$$

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps.

Alkylation solution, 80 mM

Dissolve the following in 8 ml H₂O:

0.15 g iodoacetamide (SigmaUltra; 80 mM final)

5.73 g guanidine hydrochloride (Thermo Fisher Scientific; 6 M final)

0.06 g sodium phosphate monobasic, anhydrous (50 mM final)

Adjust pH to 7.5 with 10 M NaOH

H₂O to 10 ml

Protect solution from light

Prepare fresh daily

Alkylation solution, 1 M

1.85 g iodoacetamide (SigmaUltra; 1 M final)

2 ml 1 M HEPES, pH 7.5 (200 mM final)

40 μ l 0.5 M EDTA, pH 7.5 (2 mM final)

H₂O to 10 ml

Protect solution from light

Prepare fresh daily

Guanidine buffer

Dissolve the following in 8 ml H₂O:

5.73 g guanidine hydrochloride (Thermo Fisher Scientific; 6 M final)

0.06 g sodium phosphate monobasic, anhydrous (50 mM final)

Adjust pH to 6.5 with 10 M NaOH

H₂O to 10 ml

Store up to one week at 23°C

Reducing solution, pH 3.5

Dissolve the following in 9 ml H₂O:

0.143 g with Tris(2-carboxyethyl)phosphine (TCEP; Thermo Fisher Scientific; 50 mM final)

0.105 g citric acid monohydrate (50 mM final)

Adjust pH to 3.5 with 10 M NaOH

H₂O to 10 ml

Prepare fresh daily

Reducing solution, pH 6.5

Dissolve the following in 8 ml H₂O:

0.057 g TCEP (Thermo Fisher Scientific; 20 mM final)

5.73 g guanidine hydrochloride (Thermo Fisher Scientific; 6 M final)

0.06 g sodium phosphate monobasic, anhydrous (50 mM final)

Adjust pH to 6.5 with 10 M NaOH

Adjust volume to 10 ml with H₂O

Prepare fresh daily

Reducing solution, pH 8.0

Dissolve the following in 9 ml H₂O:

0.057 g TCEP (Thermo Fisher Scientific; 20 mM final)

0.158 g ammonium bicarbonate (200 mM final)

Adjust pH to 8.0 with 10 M NaOH

Adjust volume to 10 ml with H₂O

Prepare fresh daily

Urea buffer

Dissolve the following in about 10 ml H₂O:

0.12 g sodium phosphate monobasic, anhydrous (50 mM final)

10.8 g urea (9 M final)

Adjust pH to 6.5 with 10 M NaOH

Adjust volume to 20 ml with H₂O

Prepare immediately before use

COMMENTARY

Background Information

The thiol (SH) group of cysteine is one of the most reactive amino acid side chains. In proteins, cysteines are usually present in the reduced form (with a free SH group) or in the oxidized form as cystines (two cysteines linked by a disulfide bond). Free SH groups are often found in the active site of proteins where they are typically involved in substrate binding and catalysis. Disulfide bonds are common post-translational modifications that are critical for stabilizing the native structures of extracellular domains of membrane bound proteins. Disulfide-bond linkages can occur between two cysteines in the same polypeptide chain (intrachain disulfide) or between two different polypeptides (interchain disulfide). Disulfide bond linkages cannot be predicted with any certainty and must be determined experimentally. Determination of disulfide bond linkages in proteins will provide insights into their three-dimensional structures, and because disulfide linkages are strongly conserved evolutionarily, conserved disulfide pairing patterns can be used to confirm common origins of domains with very low sequence homology.

Cleavage-based strategies—Due to the importance of disulfide bonds in the structure and function of proteins, a number of strategies have been devised to determine disulfide linkages. In a common strategy (see Basic Protocol) that has been applied successfully to numerous proteins, the protein of interest is subjected to extensive chemical and/or proteolytic cleavage in its nonreduced state to generate disulfide-linked peptides which are identified by comparing the RP-HPLC separation of peptides produced in the nonreduced versus the reduced state. The HPLC separation can be performed either off-line with UV

detection or on-line with an ESI mass spectrometer as the detector. In the off-line approach, the disulfide-linked peptides are subsequently isolated and analyzed by MS (see Strategic Planning and below).

Partial reduction and alkylation strategies—In cases where protein domains are extensively cross-linked by multiple disulfide bonds that cannot be separated into distinct fragments containing single disulfide bonds, the partial reduction and alkylation strategy (see Alternate Protocol 2) introduced by Gary (1993) is often used to selectively reduce one or two disulfide bonds at low pH with TCEP while leaving the remaining disulfides unmodified. The partially reduced disulfides are rapidly alkylated with iodoacetamide and separated for characterization by MS (*UNIT 16.2*). Two major advantages of this approach are that accessible cleavage sites between disulfides are not required and the low pH reduction reaction with TCEP minimizes disulfide scrambling.

In a variation of the partial reduction and alkylation method, nascent thiols of partially reduced proteins are cyanylated with the reagent 2-nitro-5-thiocyanobenzoic acid (NTCB) at pH 8, or with 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP) at pH 3 (Wu et al., 1996; Wu and Watson, 1997). Both cyanylating reagents are able to react with TCEP quantitatively, which is useful to terminate the partial reduction reaction (Schnaible et al., 2002a). Cyanylation with CDAP is preferred as the reaction can be carried out at low pH where disulfide scrambling is minimized. The cyanylated cysteines are susceptible to cleavage at the N-terminal side of the residues at alkaline pH (Jacobson et al., 1973). Following cleavage of the cyanylated cysteines and complete reduction of the remaining disulfide linkages, MS analysis allows the identification of the initially reduced disulfide bonds (Wu and Watson, 1997). The advantages of this method are that the reactions are performed at low pH and the ability to cleave at the cyanylated cysteines avoids the need for separate proteolytic/chemical cleavage steps. After cleavage, the cyanylated cysteine is converted to a cyclic derivative (Jacobson et al., 1973), resulting in peptides with blocked N termini that are refractory to Edman sequencing but can be determined using MS approaches.

MALDI MS—In addition to cleavage by reducing agents, disulfide bonds can undergo spontaneous dissociation when analyzed by MALDI MS (Patterson and Katta, 1994) or in source reduction in ESI instruments (Cramer et al., 2017; Li et al., 2018). This observation has led to MS being used to directly analyze HPLC fractions of digested proteins for disulfide-containing peptides without the need for chemical reduction (Qin and Chait, 1997; Schnaible et al., 2002a,b). In addition to producing the constituent peptides, fragmentation of the disulfide bonds by MALDI or ESI in-source decay also produces various combinations of disulfide-linked peptides which can be used for successful determination of disulfide linkages. The identity of the peptides and location of the disulfide bonds can subsequently be determined by tandem MS. On instrument reduction is comparatively faster and requires relatively small quantities of samples compared with off-line HPLC peptide mapping, making it the method of choice, particularly when sample is limited and the disulfide linkages is not highly complex.

Recently, a similar approach, employing ETD fragmentation, has been used for identification of disulfide bonds. Unlike CID or HCD, ETD has been demonstrated to cleave preferentially at the disulfide bond instead of the peptide backbone. Disulfide bonds have been identified directly from non-reduced protein digests using ETD, followed by CID or HCD fragmentation for sequence information (Wang et al., 2011; Ni et al., 2013; Liu et al., 2014).

Critical Parameters and Troubleshooting

Disulfide scrambling

pH.: In disulfide mapping, the most critical concern is disulfide scrambling. Disulfide scrambling occurs readily at pH 8, when the protein conformation is perturbed by chaotropic reagents or other nonphysiological conditions. This pH dependency is due to conversion of thiol groups (pKa 8.3) to thiolate anions (S⁻). The nucleophilic thiolate anion can attack the sulfur of another disulfide bond, forming a new “scrambled” disulfide bond. The other sulfur is released as a new thiol/thiolate which can continue the exchange with other disulfide bonds. The exchange process occurs more readily when the protein contains free cysteines which can provide the catalytic source of thiolate anions, but it also occurs when proteins whose cysteines are all participating in disulfide bonds are exposed to denaturants and mild reducing conditions at alkaline pH. Since the exchange mechanism is caused by the thiolate anion, the process can be reduced by a factor of ten for each pH unit below the thiol pKa. For example, with trypsin digestion, a >30-fold reduction in disulfide exchange can be achieved by performing the digest at pH 6.5 instead of the optimum, pH 8. Therefore, acidic conditions are always preferred in disulfide mapping. See Strategic Planning for more information on the pH issue with regard to sample preparation and choice of cleavage agents.

Alkylation.: As mentioned above, proteins with both disulfide bonds and free cysteines are more susceptible to disulfide scrambling, especially under denaturing conditions. To overcome this problem, free cysteines can be alkylated prior to protein denaturation (Yen et al., 2000; Zhang et al., 2002). For this purpose, *N*-ethylmaleimide (NEM) or other maleimide derivatives are useful alkylating agents that have been used successfully at acidic pH, from 3.0 to 6.5 (Bures et al., 1998; Yen et al., 2000; van den Hooven et al., 2001; Schnaible et al., 2002a; Zhang et al., 2002). Alternatively, the free cysteines can be cyanylated with CDAP, which works well at pH 3.0 where disulfide scrambling is minimized (Wu and Watson, 1998); however, cyanylated cysteines can undergo undesired cleavage at basic pH. Peptides with cyanylated cysteines are best analyzed by mass spectrometric approaches (Wu and Watson, 1998; Schnaible et al., 2002a). Following alkylation, the alkylated protein can be purified by gel filtration with mobile phase containing denaturing agent. In addition to minimizing disulfide scrambling, alkylation will also prevent the highly reactive free SH group from undergoing oxidation to form disulfide or carbamylation when urea is used as a protein denaturant (Lippincott and Apostol, 1999).

Partial reduction and alkylation.: Among the protocols presented in this unit, disulfide scrambling is most likely to occur in the partial reduction and alkylation protocol during the alkylation step with iodoacetamide (Gary, 1993). In the authors experience, disulfide

scrambling is usually minor when the protocol conditions are followed, and the disulfide-exchanged peptides can be easily identified by comparing the RP-HPLC chromatograms of the alkylated and nonalkylated partial reduction products (see Alternate Protocol 2). In addition, multiple isoforms are often obtained that can be related to the same disulfide linkage and this redundancy increases the confidence of disulfide assignments; however, if disulfide scrambling is significant, the alkylation step can be performed with NEM instead of iodoacetamide (Bures et al., 1998; van den Hooven et al., 2001; Schnaible et al., 2002a). As mentioned above, NEM, although a bulkier reagent, has been reported to work at the low pH of 3, where disulfide scrambling is minimal.

Cleavage agents—Very often, due to a high density of disulfide bonds in some proteins, the protein of interest cannot be cleaved readily with specific proteases to yield satisfactory amounts of proteolytic fragments, even though denaturing agents are used. The problem can be aggravated in some cases with the use of proteolytic cleavages at suboptimum pH conditions. If this occurs, the authors suggest using chemical cleavages such as CNBr (see Alternate Protocol 1) to provide initial fragmentation (Chong et al., 2002). Alternatively, limited cleavage with broad specificity agents such as pepsin can be used (Roszmusz et al., 2001); however, as mentioned above (see Strategic Planning), the use of broad specificity cleavage agents can complicate the separation and identification of the fragments. An alternative to chemical or broad specificity cleavages is the partial reduction and alkylation procedure (Bures et al., 1998; Schnaible et al., 2002b). If partial reduction and alkylation is performed prior to cleavage, the protein of interest is first denatured to ensure that the reducing agent will ideally have similar access to all disulfide bonds. This will result in the generation of a maximum number of isoforms that can be used to locate and confirm disulfide bonds.

Because some chemical reagents used for cleavage and modification of proteins are highly reactive, it is important to keep in mind that prolonged incubations can often result in undesirable artifactual modifications or cleavages. Therefore, do not incubate reactions longer than required and remove or inactivate the reagent promptly after the reaction has been completed. Commonly observed modifications are oxidation of methionine and tryptophan residues, carbamylation of lysines and cysteine residues in the presence of urea, and conversion of N-terminal glutamine to pyroglutamate. With iodoacetamide, extended reaction can lead to undesirable alkylation of lysine, histidine, and methionine residues. These modifications increase the heterogeneity of the cleavage products which will complicate peptide separation and data analysis as well as reduce the yields of desired products.

Anticipated Results

The protocols detailed in this unit have been successfully used to determine the disulfide linkages of a number of proteins. Cleavage of proteins to generate fragments suitable for disulfide assignment is the most critical step. With a starting amount of about 1 nmol or less, the whole procedure can usually be successfully performed using off-line peptide mapping. More protein may be required if it contains a large number of disulfide bonds, or it is very large and requires additional chemical or enzymatic cleavage. Because determination of

disulfide linkages is usually performed using recombinant proteins, protein quantity is typically not a limitation.

Digestion with specific proteases, such as trypsin, will normally generate disulfide-linked peptides that are <50 residues and unlinked peptides that are <20 residues. With a protein of ~25 kDa, most of the peptides produced can easily be resolved by RP-HPLC with good recovery. A starting amount of 1nmol should yield time point analyses of disulfide-linked peptides that are at least 20 pmol, which is usually sufficient for downstream analysis strategies including additional cleavages or partial reduction and alkylation. Peptides in the range of 10–100 fmol/μl are usually sufficient for MALDI-MS analysis (*UNIT 16.2*), whereas tandem MS (*UNIT 16.10*) can identify peptides in the low fmol to high attomole on-column range.

Time Considerations

The time needed to complete the disulfide mapping depends largely on the complexity and number of disulfide bonds. In general, chemical and proteolytic cleavage usually requires an overnight incubation. An RP-HPLC gradient of ~1 hr is normally sufficient. With control and blanks included, several hours may be required. MALDI-MS or tandem MS analysis coupled with data analysis will require one or two days. In general, the entire Basic Protocol will require 1 to 2 weeks, while the Alternate Protocols 1 and 2 require ~3 days. The Support Protocol can be completed within a day. Overall, assignment of all disulfide linkages in a 30-kDa protein containing six disulfides is likely to be a several month project due to the multiple step strategies required and the fact that some methods need to be optimized and a few methods may not work satisfactory even after optimization, requiring alternative strategies.

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Key Reference

Lakbub, J.C., Shipman, J.T., and Desaire H. 2018. Recent mass spectrometry-based techniques and considerations for disulfide bond characterization in proteins. *Anal. Bioanal. Chem.* 410:2467–2484.

A recent comprehensive review of alternative experimental approaches.

Figure 1.
Overall strategy for location of disulfide bond in proteins.

Figure 2.

Disulfide bond determination of the GA733-2 antigen. The protein contains six disulfide bonds (gray lines) located within the first 115 residues of the protein. The locations of the cysteine residues involved in disulfide linkages and some of the tryptic cleavage sites are shown. Digestion of the protein with trypsin generated three major disulfide-containing complexes: T1, T2, and T3. The T1 complex was deglycosylated and further cleaved chemically with BNPS-skatole to generate single disulfide-linked peptides. The partial reduction and alkylation strategy was used to determine the three disulfide linkages of the T2 complex. The alkylated cysteine residues are indicated by the italicized lowercase "c." MALDI-MS (*UNIT 16.2*) and Edman sequencing (*UNIT 11.10*) of the T2-R3 and T2-R5 complexes revealed disulfide-bond formation between Cys6-Cys36, and between Cys15-Cys25, respectively. Based on the two disulfide bond assignments, the third disulfide bond is deduced to form between Cys4-Cys23.

Figure 3.

RP-HPLC separation and identification of the disulfide-linked tryptic peptides. Tryptic digest of the recombinant GA733–2 protein was separated on a ZORBAX 300SB-C18 column before (bottom panel) and after (top panel) reduction with TCEP. Major peaks, which disappeared following reduction are indicated by T1 to T3. The peptide constituents of the complexes are shown in Figure 2. T2* and T3* indicate incomplete tryptic cleavages of T2 and T3, respectively. Single peptides that appear as a result of reduction are indicated in the top panel by their residue numbers. Peptides were identified by MALDI-MS and, in some cases, by automated Edman sequencing (*UNIT 11.10*). Adapted with permission of ASBMB from Chong and Speicher (2001).

Figure 4.

RP-HPLC analysis of partial reduced and alkylated T2 disulfide-containing complex. Peptides were separated on a ZORBAX 300SB-C18 column. **(A)** T2 control before partial reduction and alkylation. **(B)** T2 after partial reduction with TCEP and alkylation with iodoacetamide. The structure of the labeled peptides is shown in Figure 2. Peptides were identified by MALDI-MS (*UNIT 16.2*) and the position of the alkylated cysteines was determined by Edman sequencing (*UNIT 11.10*). The small peak marked with an asterisk showed a molecular mass indicative of T2 with two alkylated cysteines, although the amount of this species was negligible compared with the major species (T2-R3). Adapted with permission of ASBMB from Chong and Speicher (2001).

Table 1

Proteases and Chemical Cleavage Agents Suitable for Use in Disulfide Bond Mapping

Cleavage agent	Cleavage residue	Buffer ^a	Temp	Time	Reference
<i>Chemical</i>					
BNPS-skatole	Trp	0.1% TFA	37°C	1 hr	Chong and Speicher (2001)
CDAP	Cys	0.1 M citrate, pH 3.0	23°C	15 min	Wu (2008)
Cyanogen bromide	Met	70% formic acid; 0.1–0.5 M HCl	23°C	16 hr	Chong et al. (2002); Andreev et al. (2010)
Partial acid hydrolysis	Broad	0.25 M oxalic acid	100°C	8 hr	Zhou and Smith (1990)
<i>Protease</i>					
Chymotrypsin ^b	Trp, Tyr, Phe	50 mM sodium phosphate, pH 5.5	37°C	16 hr	Schnaible et al. (2002a)
Endoproteinase Glu-C (V8 proteinase)	Glu, Asp	100 mM sodium acetate, pH 4.0	24°C	16 hr	Lippincott and Apostol (2002)
Endoproteinase Lys-C	Lys	25 mM Tris-Cl, pH 6.8	37°C	24 hr	Leal et al. (1999)
Pepsin	Broad	0.02 N HCl, pH 2.0	37°C	16 hr	Bures et al. (1998)
Subtilisin	Broad	50 mM sodium phosphate, pH 6.5	37°C	16 hr	Chong et al. (2002)
Thermolysin	Broad	0.1M triethylamine·HCl, pH 6.0	37°C	16 hr	Bures et al. (1998)
Trypsin	Lys, Arg	50 mM sodium phosphate, pH 6.0	37°C	16 hr	Schnaible et al. (2002a)
Trypsin (immobilized)	Lys, Arg	50 mM sodium phosphate, pH 6.5	23°C	16 hr	Chong and Speicher (2001)

^aUrea or guanidine-HCl is commonly included in the buffer to denature the protein. See Appendix 2E for sodium phosphate and Tris-Cl buffer recipes.

^bChymotrypsin also cleaves a number of other peptide bond with widely varying efficiency.