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Partial Reduction and Two-Step Modification of Proteins for Identification of Disulfide Bonds

Volker Schnaible,* Stephan Wefing, Anne Bücker, Sybille Wolf-Kümmeth, and Daniel Hoffmann

Center of Advanced European Studies and Research, Stiftung caesar, Friedensplatz 16, 53111 Bonn, Germany

An experimental protocol was established to combine partial reduction, cyanylation, and a second modification step for the assignment of disulfide bonds in proteins that are resistant to proteolysis under native conditions. After proteolysis, disulfide bonds were assigned via MALDI mass spectrometry with subsequent semiautomatic interpretation using the program SearchXLinks, which enumerates all possible combinations of proteolytic fragments for all observed monoisotopic masses. The putative assignment of disulfide bonds was confirmed by ISD and PSD fragmentation of the corresponding protonated molecules.

Disulfide bond formation is a posttranslational modification that strongly stabilizes the 3D structure of proteins.[1] Therefore, identification of disulfide bonds or free cysteine residues can give valuable information on a protein's structure and function. Modern mass spectrometric methods such as MALDI-MS[2] and ESI-MS[3] offer the opportunity to detect and analyze biomolecules rapidly even if they are only present in small quantities. To identify disulfide bonds, the protein under investigation is enzymatical or chemically cleaved into peptides. The digestion mixture is separated chromatographically, and the components are analyzed by mass spectrometry. Putatively linked peptides are identified by mass mapping. The identity of disulfide-linked peptides is then confirmed either by reduction of the disulfide bond to liberate the peptides involved in the disulfide bond or by MS/MS techniques.[4−7] However, this approach depends on the ability to cleave the protein of interest into peptides. Proteins containing many disulfide bonds are often difficult to digest with specific proteases.[8] Thermal denaturation cannot be used to enhance proteolysis because disulfide bonds are opened by β-elimination at higher temperatures,[9,10] which leads to disulfide reshuffling.

Partial reduction and alkylation was used by several groups to facilitate proteolytic degradation.[11−16] However, when iodoacetamide is used as the alkylating agent, disulfide reshuffling occurs at pH 8 even with reaction times as short as 30 s and iodoacetamide concentrations up to 2.2 M.[12,13] Partial reduction using TCEP under acidic conditions and alkylation with NEM derivatives can be performed at pH values as low as 4.6 to prevent disulfide reshuffling during alkylation.[14,15] The incubation of proteins containing free thiol groups with high concentrations of alkylating agents, such as 0.1 M NEM, prior to denaturation was successfully applied to prevent disulfide scrambling.[16] As the NEM modification of partially reduced proteins is not quantitative, disulfide reshuffling during proteolysis remains an issue. Proteolysis at pH values lower than 8 is an effective method to avoid disulfide reshuffling at this stage.[8, 16]

Partial reduction and cyanylation at pH 3 was successfully applied for the assignment of disulfide bonds.[17,18] However, this approach is only successful if all protein derivatives generated by partial reduction and cyanylation can be separated chromatographically. Information about disulfide bonds is then gathered by chemical cleavage at the N-terminus of cyanylated cysteinyl residues from those protein species with only one disulfide bond opened. Thus, in this procedure, unmodified cysteinyl residues do not prevent the correct assignment of disulfides.

Cyanylating agents such as NTCB and CDAP react quantitatively with TCEP,[19] which allows quenching of the reduction without removal of TCEP. In this study, we combined partial reduction, cyanylation, and a second modification step with NEM or IAM to enhance modification of cysteinyl residues prior to proteolysis. Interpretation of mass spectra was performed semiautomatically using the SearchXLinks[20] program. SearchXLinks

* Corresponding author: (e-mail) schnaible@caesar.de.

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was developed to identify all sorts of cross-linked peptides, including peptides linked by disulfide bonds. To this end, SearchXLinks generates all possible combinations of optionally modified linear or cross-linked peptide fragments for each singly charged monoisotopic peak in the mass spectrum. Assigned disulfide bonds were verified via MALDI-ISD/PSD. We used hen egg white lysozyme[21−23] and bovine ribonuclease A as two well-studied model systems[24−27] to establish an experimental protocol that minimizes disulfide reshuffling during analysis. In the following, data are presented which describe assignment of all native disulfide bonds of RNAseA after a single HPLC run.

EXPERIMENTAL SECTION

Materials. α-Cyano-4-hydroxycinnamic acid, bombesin, bovine RNAseA, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, chymotrypsin (TLCK-treated), hen egg white lysozyme, human adrenocorticotropic hormone fragment (18−39), human angiotensin II, *N*-ethylmaleimide, tris(2-carboxyethyl)phosphine, and trypsin (TPCK-treated) were purchased from Sigma-Aldrich (Deissenhofen, Germany). Ultrapure water (specific conductivity, 18.2 MΩ/cm) was produced by a MilliQ device (Millipore, Eschborn, Germany). Endoproteinase GluC (sequencing grade), was purchased from Roche Diagnostics (Mannheim, Germany). Acetonitrile (gradient grade), guanidinium chloride for biochemistry, Lichrosorb C_8, 10-μm particle size, methanol (HPLC-grade), Na_2HPO_4, and trifluoroacetic acid (Uvasol) were bought from Merck (Darmstadt, Germany). Somatostatin-28 was from Bachem (Basel, Switzerland).

Chemical Modifications. For comparison of proteolysis conditions, partial reduction of RNAseA (300 μg) was carried out in a semipreparative scale with a final protein concentration of 4 μg/μL in 50 mM phosphate buffer, pH 4, containing 6 M GuaCl and 5 mM EDTA. A 7.5-μL aliquot of a freshly prepared TCEP stock solution (20 μg/μL, 70 mM) in 300 mM phosphate buffer, adjusted to pH 4, was added (molar ratio of TCEP/RNAseA, 24). Reduction proceeded for 10 min at 25 °C in a thermomixer. A 10-μg aliquot of RNAseA was taken for HPLC analysis as reduction control. Cyanylation was performed by addition of 5 μL of CDAP stock solution (96 μg/μL, 408 mM) in ACN (molar ratio CDAP/RNAseA, 96). Cyanylation was carried out for 10 min at 25 °C in a thermomixer. Another 10-μg aliquot of RNAseA was taken for HPLC analysis as cyanylation control. To 48 μL of the reaction mixture, 6.4 μL of a NEM stock solution (1 M freshly prepared in ACN) was added to a final NEM concentration of 0.1 M. pH was raised to 5 by addition of 9 μL of 300 mM phosphate buffer, pH 6.3. NEM modification was carried out for 20 min at 25 °C in a thermomixer. For preparation of partially reduced and NEM-modified RNAseA, the reagent concentrations and reaction times were identical; only the cyanylation step was omitted. The modification has also been carried out with 60-μg aliquots of RNAseA in a final volume of 17 μL, which was still manageable with standard pipets. Protein and reagent concentrations and reactions times were identical to the semipreparative conditions.

Removal of Reagents. Reagents were removed using C_8 microcolumns. C_8 microcolumns were prepared in 10-μL Eppendorf filtertips by applying 30 μL of a C_8-Lichrosorb slurry (75 mg/mL in methanol). After sedimentation of RP material, excess methanol was removed. The column bed was washed with two 20-μL aliquots of 0.1% TFA in water. Aliquots of the reaction mixture containing 60 μg of RNAseA were applied on one microcolumn. Reagents were removed by washing with five 30-μL aliquots of 0.1% TFA in water. Protein derivatives were eluted in two steps using 7.5-μL aliquots of 80% ACN/0.1% TFA. All liquid handling on the column was performed by air pressure produced with a 2-mL disposable plastic syringe. In all steps except the last elution step, care was taken to prevent dry running of the microcolumns. The eluate was diluted with 0.1% TFA to reduce acetonitrile content to less than 10%, and an aliquot was analyzed by RP-HPLC to determine protein concentration. The main part of the eluate was stored at −20 °C until proteolysis.

Proteolytic Digest. RNAseA microcolumn eluates were diluted with the appropriate buffer for proteolysis. Typically 5−15-μg aliquots were used. Chymotryptic digest of RNAseA derivatives was performed overnight (e/s = 1/50) in phosphate buffer solution (pH 5.5, 6, and 8, respectively) containing 2 M GuaCl. Chymotrypsin was added from a stock solution (0.1 μg/μL in 1 mM HCl). Trypsin and GluC digests were also performed overnight in phosphate buffer solution (pH 6 and 8). Trypsin and GluC (e/s = 1/50 for each protease) were added simultaneously from stock solutions (trypsin 0.1 μg/μL in 1 mM HCl, GluC 0.1 μg/μL in MilliQ water).

HPLC Separation and MALDI-MS. Protein and peptide separation was carried out on a HP 1100 chromatographic system (Agilent Technologies, Waldbronn, Germany) using a Zorbax RP18 (5 μm, 300 Å, 2.1 × 150 mm) column. A binary gradient with 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) was applied at a flow rate of 0.3 mL/min. RNAseA derivatives were analyzed using a 45-min gradient. After 5 min of equilibration at 5% solvent B, the acetonitrile content was raised to 27% in 7 min. Separation of RNAseA derivatives was accomplished using a shallow slope from 27 to 30% solvent B in 10 min. Proteolytic digest mixtures were separated using a linear gradient from 5 to 45% solvent B in 25 min after 5-min equilibration at 5% B. Total gradient time was 45 min. A total of 24 fractions (0.7 min each) were collected in 96-well microtiter plates using a MAPII robot (Bruker Daltonik, Bremen, Germany) under control of the MAP-Control 2.0.2 software package. Sample collection was started 10 min after injection. Dried droplet sample preparation of HPLC fractions for MALDI-MS with HCCA as matrix (saturated solution in 33% ACN/0.1% TFA) was performed by the MAPII robot. MALDI and MALDI-PSD spectra were automatically recorded in positive mode on a Bruker Reflex III Scout 384 instrument under control of TOF-Software release 5.0 (XACQ 4.0.4). External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of

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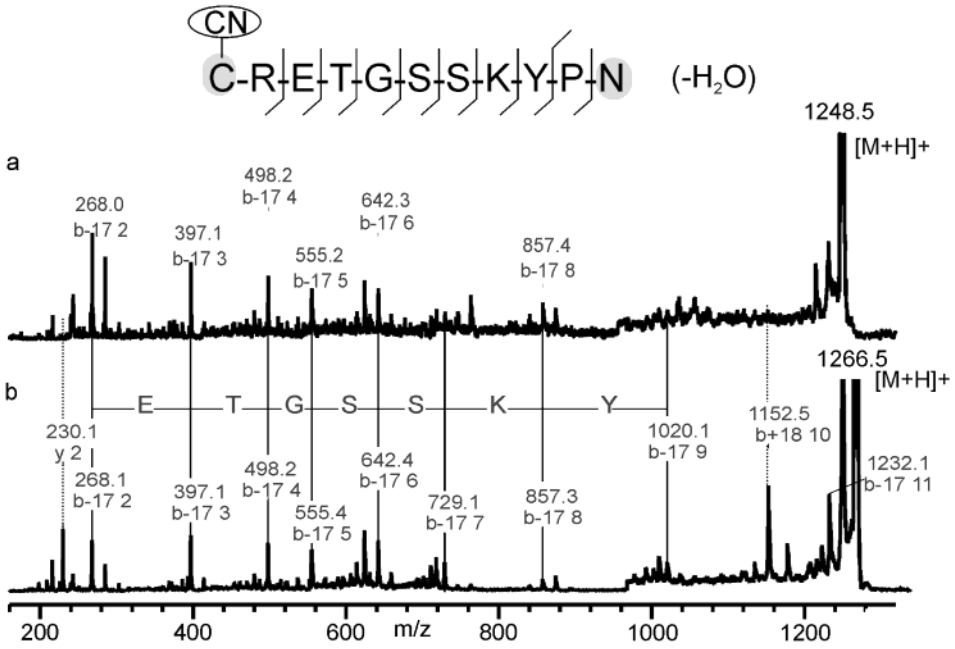

Figure 1. MALDI-PSD spectra of precursor ions at (a) *m*/*z* 1248.5 and (b) 1266.5.

human angiotensinII (*m*/*z* 1046.542), bombesin (*m*/*z* 1619.823), ACTH (18−39) (*m*/*z* 2465.199), and somatostatin-28 (*m*/*z* 3147.472). In parallel to the measurements, calibration was validated on three different 3 × 3 spot squares. The mixture of calibration peptides was measured using external calibration, which was carried out on the central spot of a 3 × 3 square. We observed a maximum mass error of 123 ppm. Therefore, we allowed for a maximum mass error of 150 ppm for interpretation of the peptide mass spectra. Visual inspection of MALDI spectra was performed using XMASS 5.1 NT. MALDI-PSD spectra were annotated with the BioTools 2.0 software package.

Semiautomatic Interpretation of Mass Spectra by SearchXLinks. MS peak lists were semiautomatically interpreted using the SearchXLinks[20] program (a detailed discussion of SearchXLinks will be published elsewhere). Cleavage, modification, and link rules were specified for data interpretation. All peptide combinations matching *m*/*z* values within a mass error of 150 ppm were generated, taking disulfide bonds into account. Optionally, all possibilities of different connectivities and modification sites for each set of connected peptides were also generated, which will be referred to as configurations. For the interpretation of tryptic digests, chymotryptic activity was also considered. Cleavage at the N-terminus of cyanylated cysteine residues was used as an additional cleavage rule if proteolysis had been carried out at pH 8. Methionine and tryptophane oxidation, cyanylation, NEM modification, and β-elimination of cyanylated cysteine residues[17] were chosen as modification rules. Furthermore, hydrolysis of the NEM moiety, formation of pyroglutamate,[28,29] and cyclization of asparagine residues during proteolysis were also taken into account. The current version of SearchXLinks does not permit one to define modifications that only occur at cleavage sites. Therefore, the latter two modification rules were allowed to occur at those amino acid residues that are theoretically located at cleavage sites of the applied protocol for proteolysis. This strategy causes false positive assignments, if the respective amino acid residues are modified and if they are present within a larger proteolytic fragment generated by SearchXLinks. These false positive assignments had to be discarded manually. No information about the known disulfide structure of RNAseA was used for data interpretation. Examples for a parameter and result file of SearchXLinks are included as Supporting Information.

RESULTS AND DISCUSSION

Identification of disulfide bonds by mass spectrometric methods is ideally performed with peptide fragments containing only one disulfide bond. Analysis of the amino acid sequence of RNAseA suggested the combination of trypsin and endoproteinase GluC as suitable for generating singly disulfide-linked peptide fragments. However, proteolysis of RNAseA using trypsin and GluC under native conditions (phosphate buffer, pH 8) did not generate satisfactory amounts of proteolytic fragments. Treatment with urea was successful, if the sample was boiled, which resulted in scrambling of disulfides. After partial reduction and cyanylation at pH 4, RNAseA derivatives appeared that were accessible to proteolysis under standard conditions (data not shown).

To interpret mass spectra with the help of the program SearchXLinks, knowledge of all potential amino acid modifications that may occur during the reduction/modification and proteolysis procedure have to be taken into account. In addition to the known modifications for the cyanylation reaction,[17] we identified another modification reaction: Two signals at *m*/*z* 1266.5 and 1248.5 were observed in mass spectra of HPLC fractions independent of the protease used for digestion. The signal at *m*/*z* 1266.5 was assigned to peptide (84−94), which is cyanylated at cysteine 84. Peptide (84−94) was generated by N-terminal chemical cleavage at Cys-84 and Cys-95. The PSD spectrum of the precursor ion at *m*/*z* 1266.5 (Figure 1b) contained a b-17 ion series of the cyanylated peptide (84−94). The PSD spectrum of the precursor ion at *m*/*z*

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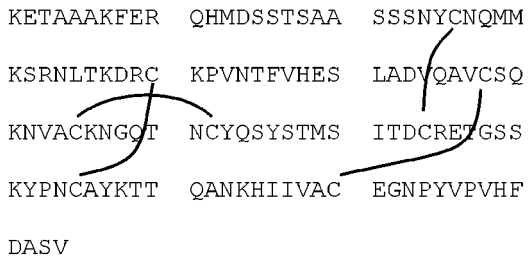

Figure 2. Sequence and native disulfide bonds of bovine ribonuclease A.

1248.5 (Figure 1a) also contained a b-17 ion series of the cyanylated peptide (84−94). In contrast to the PSD spectrum of the peak m/z 1266.5, the strong b+18 10 signal at m/z 1152 was missing in the PSD spectrum of the peak at m/z 1248.5. The occurrence of b+18 ($n - 1$) ions is frequently observed for peptides containing arginine[30] or that were cationized by alkali salts.[31] During fragmentation, the C-terminal hydroxy group shifts toward the penultimate residue. This fragmentation pathway is not available for the precursor ion at m/z 1248.5. Thus, the loss of 18 Da was attributed to cyclization of the C-terminal asparagine residue by loss of water.

Disulfide scrambling was observed upon analyzing HPLC fractions of proteolytic digests of reduced and cyanylated RNAseA. For the combined trypsin and GluC digest, the oxidized peptide (40−61) was detected at m/z 2401.1, which indicated that cysteine residues 40 and 58 were connected. Using chymotrypsin as protease, an intense signal at m/z 1992.8 (peptide 80−97, cysteine residues 84 and 95 connected) was observed in the MALDI mass spectrum (Figure 3a) of an HPLC fraction eluting at 25% solvent B. Throughout this study, the $[M + H]^+$ ion at m/z 1992.8 was used as one prominent indicator for disulfide scrambling when different modification and proteolysis conditions were compared. The sequence and the location of the native disulfide bonds in RNAseA are shown in Figure 2.

To avoid disulfide scrambling, a second modification step was performed by adding NEM to the reaction mixture after reduction and cyanylation. The pH was raised to 5 and NEM modification was carried out for 20 min at 25 °C. The reaction mixture was desalted on C_8 microcolumns and subjected to proteolysis. MALDI-MS analyses of chymotryptic digest fractions still indicated disulfide scrambling. As a result of the second modification step, the intensity of the peak at m/z 1992.8 for the doubly modified RNAseA was reduced in comparison to that of the coeluting cyanylated peptide (36−46) detected at m/z 1333.7 (Figure 3b). Using iodoacetamide instead of NEM had a similar effect. However, the reaction mixture had to be desalted after cyanylation, because TCEP and CDAP interfere with the iodoacetamide modification (data not shown). Another disadvantage of iodoacetamide is that the modification reaction has to be carried out at pH values above 7, which favors disulfide scrambling. A major reduction of the amount of scrambled disulfides was achieved by performing proteolysis at pH 6 (trypsin and GluC) and pH 5.5 (chymotrypsin), respectively. The spectrum shown in Figure 3c is dominated by a signal at m/z 1333.7. The intensity of the signal at m/z 1992.8

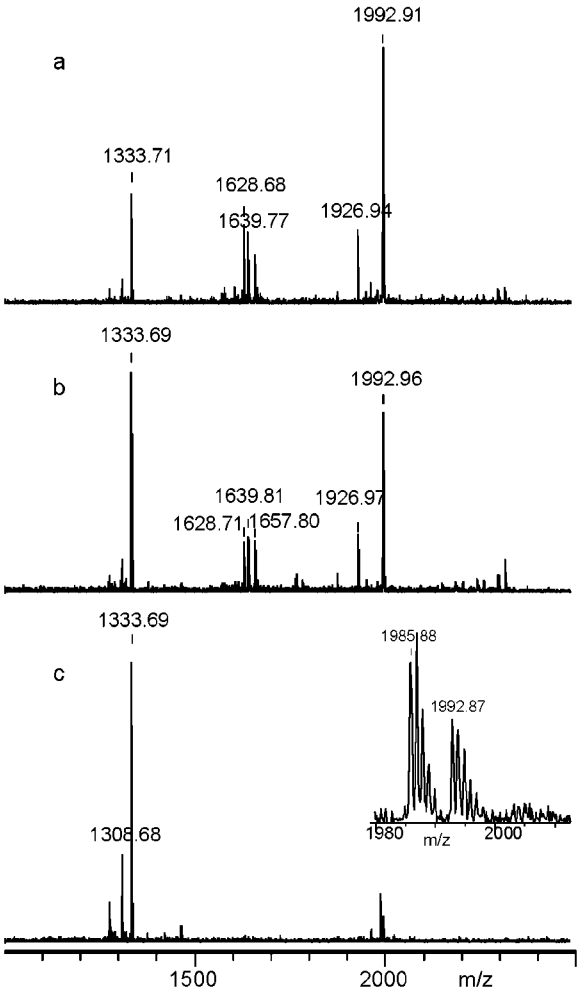

Figure 3. MALDI mass spectra of HPLC fractions of chymotryptic digest of RNAseA (a) partially reduced and cyanylated, pH 8, (b) partially reduced, cyanylated, and NEM-modified, pH 8, and (c) partially reduced, cyanylated, and NEM-modified, pH 5.5.

is significantly reduced. In HPLC fractions of the trypsin and GluC digest carried out at pH 6, no more scrambling products were identified (data not shown).

Addition of NEM prior to denaturation and TCEP addition prevented partial reduction of RNAseA. MALDI spectra of reaction mixtures of TCEP and NEM contained an intense signal at m/z 376, which indicates that TCEP and NEM react with each other. Thus, reversing the modification sequence to minimize disulfide scrambling is not possible (data not shown). However, if the modification of partially reduced RNAseA was carried out with NEM only, the reduction process was not terminated even at NEM concentrations of 0.1 M. This indicates that the addition of the cyanylating agent CDAP is necessary to stop the reduction in a controlled manner. Figure 4a shows a chromatogram of partially reduced, cyanylated, and NEM-modified RNAseA. Unmodified RNAseA accounted for about one-third of the total peak area in that chromatogram. The analysis of NEM-modified partially reduced RNAseA is shown in Figure 4c. A large amount of RNAseA derivatives were observed. Unmodified RNAseA accounted for 10% of the total peak area. Figure 4b shows the chromatogram of a proteolytic mixture of partially reduced, cyanylated, and NEM-modified RNAseA generated by combined trypsin and GluC digestion. About one-third of the total peak area

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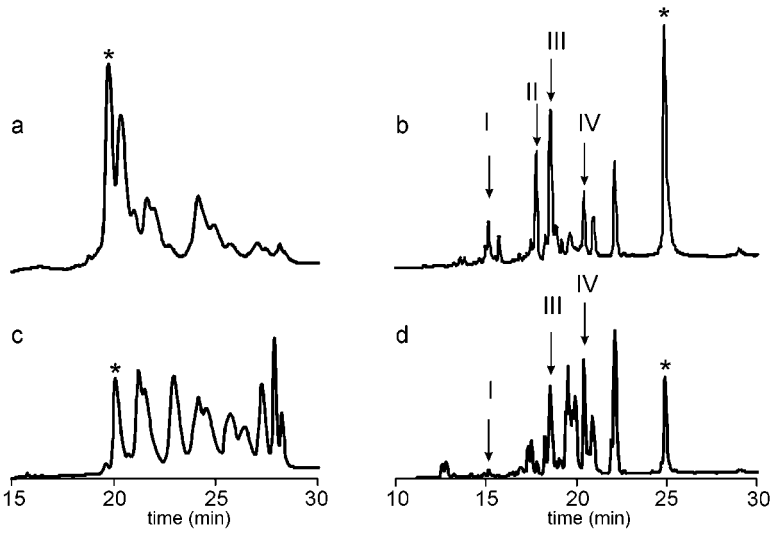

Figure 4. HPLC chromatograms of partially reduced and modified RNAseA derivatives: (a) cyanylated and NEM-modified; (c) NEM-modified; (b) and (d) represent (a) and (c) subjected to trypsin and GluC digestion at pH 6. Unmodified/noncleaved RNAseA is indicated by an asterisk. Arrows in (b) and (d) indicate representative fractions for identification of the native disulfide bonds.

Table 1. Number of Possible Assignments and Their Configurations for $[M + H]^+$ Ions Detected in HPLC Fractions of a Tryptic and GluC[a] Digest Performed at pH 6 Representing Disulfide-Bonded Peptides

	150 ppm[b]		20 ppm[b]	
m/*z*	no. assgn	no. conform	no. assgn	no. conform
1708.73	1	1	1	1
2028.95	3	4	1	1
2030.02	5	7	2	2
3301.23	19	50	3	5

[a] The cleavage rule allowed also for chymotryptic cleavage. [b] All linear, linked, and optionally modified peptides were generated by SearchXLinks for mass errors of 150 and 20 ppm, respectively.

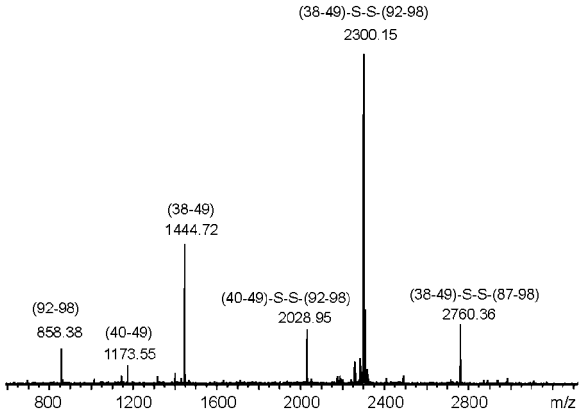

Figure 5. MALDI mass spectrum of an HPLC fraction (t_R = 18.4 min, labeled as III in Figure 4c) of tryptic and GluC digest of partially reduced, cyanylated, and NEM-modified RNAseA.

in the chromatogram represented noncleaved RNAseA eluting at 25 min. (Note that two different gradients were used for separation of RNAseA derivatives and proteolytic mixtures, respectively; see the Experimental Section). Highlighted fractions I−IV accounted for ~40% of the total peak area. These fractions contain peptides that were used for assignment of the four native disulfide bonds of RNAseA (see Table 2). The chromatogram of the separation of the trypsin and GluC digestion mixture of partially reduced and NEM-modified RNAseA is shown in Figure 4d. No fraction corresponding to fraction II in Figure 4b was present, and the peak area of fraction I was reduced 10-fold from 5.0 to 0.5% of the total peak area in the chromatogram. Thus, using exclusively NEM as modification reagent after partial reduction, two of the four native disulfide bonds of RNAseA are almost inaccessible. The termination of the reduction by addition of CDAP is a necessary step to generate a variety of RNAseA derivatives that are accessible to proteolysis but still contain a sufficient proportion of native disulfide bonds.

With respect to data analysis, it turned out that it was in general impossible to assign the observed peaks in a unique way. To limit the number of ambiguities, we started data interpretation with strict boundary conditions enabled, i.e., excluding unspecific cleavage or more than one disulfide bond per fragment. All peaks that were not assigned or not correctly assigned are then interpreted in a second run employing relaxed boundary conditions, e.g., including chymotryptic activity for trypsin. For interpretation of $[M + H]^+$ ions of partially reduced, cyanylated, and NEM modified RNAseA, it was necessary to include chymotryptic activity as an additional cleavage rule. The occurrence of a chymotryptic cleavage after Tyr-76 or Met-79 leads to singly disulfide-linked peptides for all disulfide bonds, which facilitates verification of the assignment by MS/MS. If proteolytic mixtures were generated at pH 6, chemical cleavage before cyanylated cysteine residues was not applied as a cleavage rule, since that cleavage occurs only with alkaline conditions. Because of the ambiguities, disulfide bonds had to be confirmed via MS/MS experiments. Table 1 summarizes the number of possible assignments and configurations for disulfide-linked peptides of the trypsin and GluC digest of RNAseA.

A simple MS/MS experiment is ISD fragmentation of the disulfide bond. This fragmentation is very useful if homogeneous HPLC fractions have been obtained.[32] If signals of the intact disulfide-linked peptide and the peptides released by ISD frag-

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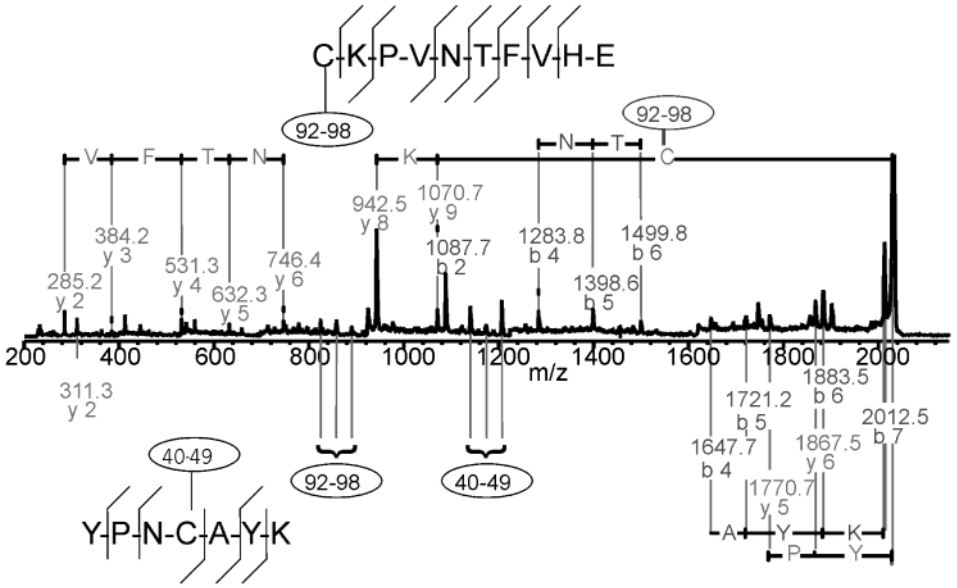

Figure 6. MALDI-PSD spectrum of the precursor ion at *m*/*z* 2028.9. Upper part: fragment ions corresponding to fragmentation of peptide (40−49). Lower part: fragment ions corresponding to fragmentation of peptide (92−98).

mentation of the disulfide bond are present in a mass spectrum, the *m*/*z* value of the disulfide-bonded peptide can be assigned by simply adding *m*/*z* values of the involved singly protonated peptides and by subtracting the mass of three hydrogens. The MALDI mass spectrum in Figure 5 shows a number of ions that represent overlapping disulfide-linked peptides (detected at *m*/*z* 2028.9, 2300.1, and 2760.4) and their ISD fragments (detected at *m*/*z* 858.4 (92−98), 1173.6 (40−49), and 1444.72 (38−49)). The correct assignment of the disulfide bond between cysteine residues 40 and 95 was confirmed by MALDI-PSD of the precursor ion at *m*/*z* 2028.9 shown in Figure 6. The spectrum contained a y-ion series of the peptide (40−49) and b- and y-ions of the peptide (92−98) with (40−49) connected to Cys-95. Additionally, two triplets with 32 and 34 Da mass difference were centered at *m*/*z* 858 and 1173 resulting from symmetric and asymmetric fragmentation of the disulfide bond by PSD. This PSD fragmentation behavior agrees with data published previously.[4−7] The identity of the ions at *m*/*z* 2300.1 and 2760.4 was also confirmed by PSD (data not shown).

If SearchXLinks generates a large number of possible assignments for one $[M + H]^+$ ion, ISD fragmentation is very useful to reduce the number of plausible assignments. As the MALDI mass spectra were calibrated externally, a maximum mass error of 150 ppm was accepted. For the $[M + H]^+$ ion at *m*/*z* 3301.2 (see Table 1), SearchXLinks generated 19 possible assignments within a mass error of 150 ppm. Disulfide connectivity and the location of modification sites within one assignment are often ambiguous; here, these 19 assignments represented 50 different configurations. For comparison, Table 1 also contains the number of assignments and configurations if a mass error of only 20 ppm would have been accepted. For the $[M + H]^+$ ion at *m*/*z* 3301.2, there were still three assignments generated, which makes MS/MS experiments essential for identification. In the MALDI mass spectrum presented in Figure 7, two pairs of $[M + H]^+$ ions separated by 129 Da were detected at *m*/*z* 1013.5/1142.5 and at *m*/*z* 3301.2/3430.3, respectively. The two smaller fragments were assigned by their *m*/*z* to the peptides (77−85) and (77−86),

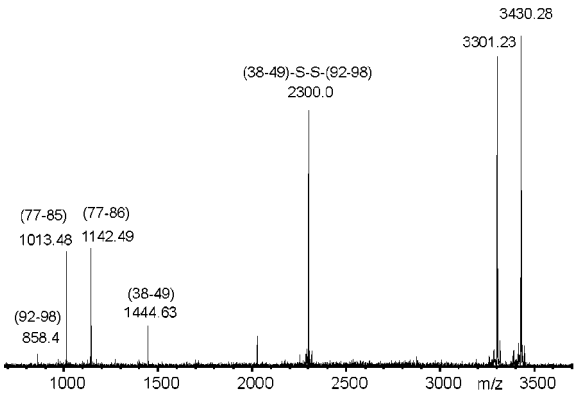

Figure 7. MALDI mass spectrum of an HPLC fraction (t_R = 19.1 min) of tryptic and GluC digest of partially reduced, cyanylated, and NEM-modified RNAseA.

containing free Cys-84. The cleavage after Tyr-76 indicated chymotryptic activity of the trypsin used. Thus, for data interpretation with SearchXLinks, cleavage after W, F, Y, L, and M was additionally taken into account. The mass difference of 129 Da between *m*/*z* 3301.2 and 3430.3 implied that these $[M + H]^+$ ions were precursor ions for *m*/*z* 1013.5 and 1142.5, respectively. Inspection of the result list generated for the peak at *m*/*z* 3301.2 for molecules containing peptide (77−85) reduced the number of possible assignments to two ((77−85)-S−S−(52−66)−S−S−(92−97) and (pyro11−31)−S−S−(77−85)). As formation of pyroglutamate upon proteolysis is well known in the literature,[28,29] the latter assignment is more reasonable than the first one, which involves three additional chymotryptic cleavage sites. PSD analysis confirmed the identity of the peak at *m*/*z* 3301.2 as (77−85) linked to pyro (11−31) (Supporting Information). Thus, ISD fragmentation is very helpful for reducing the complexity of the result list and for speeding up the data interpretation process.

The disulfide bond between Cys-65 and Cys-72 was assigned by PSD analysis of the precursor ion at *m*/*z* 1708.8, which represents (62−66) connected to (67−76) (data not shown). The

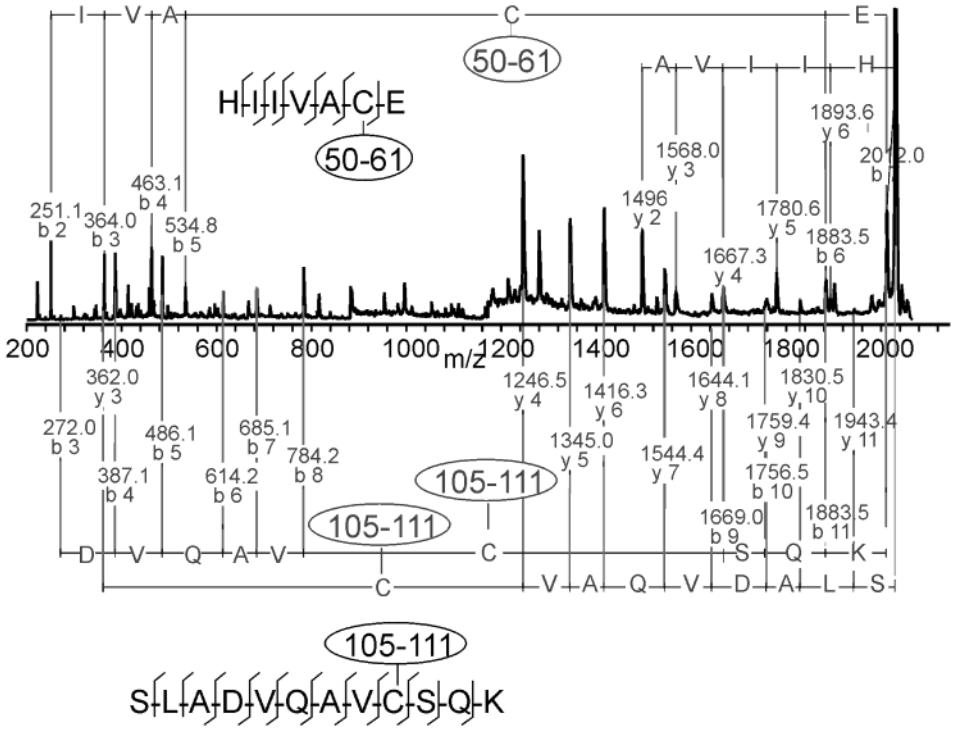

Figure 8. MALDI-PSD spectrum of the precursor ion at *m*/*z* 2030.0. Upper part: fragment ions corresponding to fragmentation of peptide (105−111). Lower part: fragment ions corresponding to fragmentation of peptide (50−61).

Table 2. List of All $[M + H]^+$ Ions Representing Disulfide-Linked Peptides after Tryptic and GluC Digestion Performed at pH 6 and HPLC Separation

m/*z*, exp	*m*/*z*, calc	error, ppm	peptide1	peptide2	modification	fraction label[b]
2982.12	2982.18	−21	(11−31)	(80−85)[a]	pyroQ−11	II
3111.17	3111.22	−17	(11−31)	(80−86)[a]	pyroQ−11	II
3301.24	3301.30	−19	(11−31)	(77−85)[a]	pyroQ−11	
3430.28	3430.34	−18	(11−31)	(77−86)[a]	pyroQ−11	
2488.05	2488.02	13	(15−31)	(80−85)[a]		
2617.09	2617.06	11	(15−31)	(80−86)[a]		
2300.02	2300.06	−17	(38−49)	(92−98)		III
2760.36	2760.29	27	(38−49)	(87−98)		III
2028.95	2028.93	11	(40−49)	(92−98)		III
2030.04	2030.01	16	(50−61)	(105−111)		IV
1708.73	1708.71	13	(62−66)	(67−76)[a]		I
2027.80	2027.83	−11	(62−66)	(67−79)[a]		
2857.20	2857.17	13	(62−66)	(67−86)	Cys-84, cyanylated	

[a] Fragment is generated by chymotryptic cleavage at Tyr-76 or Met-79, respectively. [b] Labels from Figure 4c.

PSD spectrum contained a b-ion series for the peptide (67−76) with (62−66) connected via a disulfide bond to Cys-72. For the peptide (62−66), a y-ion series was found. Furthermore, the characteristic triplet for the PSD fragmentation of the disulfide bond for the peptide (62−66) was centered at *m*/*z* 534.

The disulfide bond between Cys-58 and Cys-110 connecting the peptides (50−61) and (105−111) was confirmed by PSD analysis of the precursor ion at *m*/*z* 2030.0. The PSD spectrum (Figure 8) showed nearly complete b- and y-ion series for both connected peptides. Ions corresponding to symmetric disulfide bond fragmentation (*m*/*z* 784.4 and 1248.6) were concealed by the b8 and y4 ions of the disulfide-linked peptide (50−61) at *m*/*z* 784.2 and 1246.5, respectively.

The presented data demonstrate that the two-step modification procedure combined with proteolysis at pH 6 or lower enables a reliable assignment of disulfide bonds in a protein that is not amenable to proteolysis under native conditions. Table 2 contains a summary of all disulfide-linked peptide ions detected in the tryptic and GluC digest of partially reduced, cyanylated, and NEM modified RNAseA performed at pH 6.

CONCLUSIONS AND PERSPECTIVES

Partial reduction, cyanylation, and NEM modification is a useful method to identify disulfide bonds in proteins that are resistant to proteolysis with specific proteases. As the partial reduction step has to be carried out at high protein concentrations, minimizing sample volumes is essential if biologically relevant proteins shall be analyzed, which are only available in limited amounts. Therefore, the partial reduction and modification steps could be performed in a microfluidic device[33] or on prestructured sample supports[34] using piezospotting techniques. For identification of disulfide bonds or any other cross-link, it is important to first

generate a set of possibly connected fragments out of the MS data. As assignments are usually ambiguous, it is essential to verify putative cross-links by MS/MS methods. To avoid manual inspection of MS/MS spectra and to speed up the data interpretation process, we plan to extend SearchXLinks such that its output can be scored with the help of MS/MS data.

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GLOSSARY

ACN	acetonitrile
ACTH (18−39)	adrenocorticotropic hormone human fragment 18−39 (CLIP)
CDAP	1-cyano-4-dimethylaminopyridinium tetrafluoroborate
ESI-MS	electrospray ionization mass spectrometry
e/s	enzyme/substrate ratio
TFA	trifluororacetic acid
GuaCl	guanidinium chloride
HCCA	α-cyano-4-hydroxycinnamic acid
IAM	iodoacetamide
ISD	in source decay
NEM	*N*-ethylmaleimid
NTCB	2-nitro-5-thiocyanatobenzoic acid
MALDI-MS	matrix-assisted laser desorption /ionization mass spectrometry
PSD	postsource decay
RNAseA	ribonuclease A
TCEP	tris(2-carboxyethyl)phosphine
TLCK	*N*α-tosyl-L-lysine chloromethyl ketone
TPCK	tosyl-L-phenylalanine chloromethyl ketone.

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SUPPORTING INFORMATION AVAILABLE

Example parameter file for SearchXLinks: schnaible_et_al_si-_paramter_file_sxl.txt. Result file generated by SearchXLinks: schnaible_et_al_si_result_file_sxl.txt. PSD spectrum of the precursor ion at *m*/*z* 3301.2: schnaible_et_al_si_PSD_m_z_3301.tif. This material is available free of charge via the Internet at http://pubs.acs.org.

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