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Cysteine sulfenic Acid as an Intermediate in Disulfide Bond Formation and Nonenzymatic Protein Folding[†]

Douglas S. Rehder and Chad R. Borges*

Molecular Biomarkers, The Biodesign Institute at Arizona State University, Tempe, Arizona 85287

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ABSTRACT: As a posttranslational protein modification, cysteine sulfenic acid (Cys-SOH) is well established as an oxidative stress-induced mediator of enzyme function and redox signaling. Data presented herein show that protein Cys-SOH forms spontaneously in air-exposed aqueous solutions of unfolded (disulfide-reduced) protein in the absence of added oxidizing reagents, mediating the oxidative disulfide bond formation process key to in vitro, nonenzymatic protein folding. Molecular oxygen (O_2) and trace metals [e.g., copper(II)] are shown to be important reagents in the oxidative refolding process. Cys-SOH is also shown to play a role in spontaneous disulfide-based dimerization of peptide molecules containing free cysteine residues. In total, the data presented expose a chemically ubiquitous role for Cys-SOH in solutions of free cysteine-containing protein exposed to air.

In the early 1960s, Anfinsen and colleagues conducted seminal studies in protein disulfide bond formation and folding, showing that unfolded (disulfide-reduced) proteins will spontaneously and completely refold, regenerating full protein activity, over a period of approximately a day in the absence of denaturants (*1*−*6*). For decades, it has been known that free thiol-containing molecules will, over time, spontaneously form intra- and/or intermolecular disulfide bonds when stored exposed to air in aqueous solution. Notably, however, thiols do not spontaneously oxidize one another to generate disulfide bonds in the absence of an oxidizing reagent (*7*). Moreover, an intermediate oxidized form of sulfur has not been clearly identified as a ubiquitous intermediate in the spontaneous formation of disulfide bonds in vitro. With the discovery that disulfide bond formation and protein folding in vivo constitute an enzyme-catalyzed process that takes place in a matter of seconds, interest in identifying an oxidized sulfur intermediate relevant to nonenzymatic protein folding seems to have waned. Even so, such an intermediate remains important in peptide and protein research/production systems that, intentionally or not, involve non-enzyme-mediated disulfide bond formation and protein folding. Knowledge of the identity of such an intermediate may allow for a better degree of control in manipulating in vitro protein systems.

Biologically, cysteine sulfenic acid (Cys-SOH)[1] formation within folded protein molecules can serve as a means of regulating protein activity, helping to absorb oxidative insults (*8*, *9*), informationally "register" such insults and/or alter protein activity (*10*−*20*), mediate redox signaling (*16*, *17*, *21*), and generally deflect (*14*−*16*, *22*−*26*) what otherwise might have been injurious oxidative damage. In most studies on protein Cys-SOH, exogenous oxidants such as hydrogen peroxide are required to build up significant quantities of Cys-SOH within fully folded proteins.

In general, Cys-SOH is a transient intermediate that until the past decade has been difficult to study. It has been understood that Cys-SOH likely renders the sulfur atom of cysteine electrophilic to the point of being susceptible to nucleophile attack (i.e., by a proximal thiol/thiolate anion) (*10*, *27*), yet though they now appear to play a key role in the biochemical regulation of many different proteins, Cys-SOH modifications have, historically, been difficult to study because of their inherent instability outside of their native "cocoon-like" protein environment (*9*, *10*, *28*−*31*). Recent developments in analytical technologies and molecular probes, particularly those based on the specific alkylation (or irreversible "covalent trapping") of Cys-SOH residues with 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (*10*, *32*−*39*), have produced a great deal of interest and a flurry of research activity centered on this unique protein modification.

The purpose of this study was to determine whether cysteine-sulfenic acid serves as an oxidized sulfur intermediate that mediates air-induced disulfide bond formation and nonenzymatic protein folding. Using dimedone as a well-established (*10*, *32*−*39*) mass-shifting molecular probe of Cys-SOH (Scheme 1) and bovine ribonuclease A (RNase A) as a model protein[2], we report here the observation that air- and trace metal-generated Cys-SOH is a non-site-specific intermediate in the disulfide bond formation process that occurs as part of the in vitro, nonenzymatic protein folding process.

EXPERIMENTAL PROCEDURES

Reagents. All chemicals, including ribonuclease A type III-A from bovine pancreas, were purchased from Sigma-Aldrich.

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*To whom correspondence should be addressed: Molecular Biomarkers, The Biodesign Institute at Arizona State University, P.O. Box 876601, Tempe, AZ 85287. Telephone: (480) 727-9928. Fax: (480) 727-9464. E-mail: chad.borges@asu.edu.

[1]Abbreviations: Cys-SOH, cysteine sulfenic acid; RNase A, bovine ribonuclease A.

[2]Bovine RNase A (UniProtKB/Swiss-Prot accession number P61823) is a 124-amino acid single-chain protein containing eight cysteine residues, all of which are involved in intramolecular disulfide bonds. It was employed as a model protein in the seminal protein folding studies conducted by Anfinsen and colleagues in the early 1960s (*1*−*6*). To the greatest degree possible, the folding conditions employed by Anfinsen have been replicated here.

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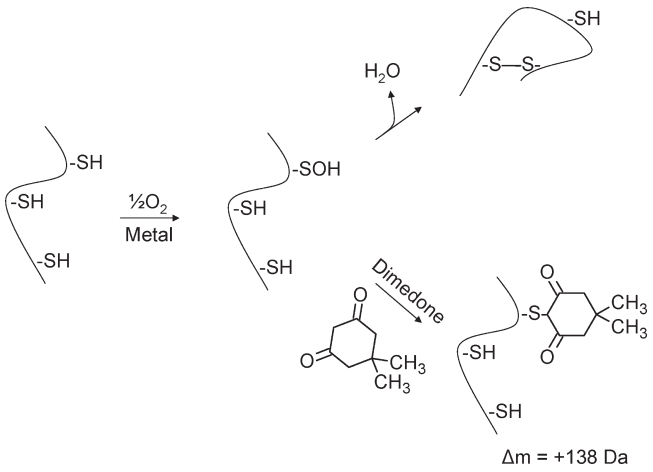
Scheme 1: Proposed Reaction Pathway for in Vitro Generation of Disulfide Bonds and Protein Folding[a]



[a]Dimedone reacts covalently and irreversibly with Cys-SOH, making it an effective probe for the existence of Cys-SOH (*10*, *32*−*39*). Detection of covalent dimedone-modified protein in the experiments described here indicates the production of protein Cys-SOH. By logical extension of previous studies of the reactivity of intra- and intermolecular sulfhydryl groups with Cys-SOH (*7*, *40*), such detection suggests that Cys-SOH is an oxidized sulfur intermediate that mediates in vitro disulfide bond formation and protein folding. Early experiments by Anfinsen and colleagues (*2*−*4*, *6*) showed that in vitro protein folding goes to completion and full restoration of protein activity.

Synthetic peptide "IGF 57−70" (H_2N-ALLETYCATPAKSE-CO_2H) was purchased from American Peptide. Amicon centrifugal concentration units were from Millipore. Endoproteinase Lys-C from *Lysobacter enzymogenes* was obtained from Roche. Spilfyter Hands-in-Bag atmospheric chambers were purchased from VWR.

Protein Reduction and Refolding. RNase A was dissolved at a concentration of 10 mg/mL in freshly prepared 8 M urea (pH 8.6) [adjusted with 5% (w/v) methylamine (*2*)] containing 50 mM dithiothreitol and incubated in a rotary shaker at 800 rpm and 37 °C for 1 h. One hundred microliters of the reduced protein solution was adjusted to pH 3.5 with glacial acetic acid and then loaded into an Amicon Ultra-4 5 kDa MWCO centrifugal concentration unit containing 4 mL of 0.1 M acetic acid. The sample was then centrifuged in a swing-bucket rotor for 20 min at 4000*g*. The retentate was then rediluted with 4 mL of 0.1 M acetic acid, and the cycle of concentration and redilution was conducted a total of five times, resulting in a greater than 1 million-fold dilution of urea and dithiothreitol.

The protein concentration of the final ~250 μL retentate was generally found to be ~120 μM by absorption spectrophotometry at 276 nm [ε = 9390 M^{-1} cm^{-1} for reduced RNase A (*4*)]. To verify complete reduction, a 5 μL aliquot was removed and alkylated with 5 μL of 50 mM maleimide dissolved in 50 mM ammonium acetate (pH 5) and incubated at 50 °C for 15 min. The alkylated RNase A was then analyzed intact by mass spectrometry to verify complete reduction by means of an anticipated 8 × 97 Da mass increase (Figure S1 of the Supporting Information).

For each set of refolding conditions examined, two aliquots of purified, unfolded RNase A were diluted to 0.075 mg/mL (5.5 μM), adjusted to pH 8 with a saturated solution of Tris base, and left to spontaneously refold at room temperature in the presence or absence of 50 mM dimedone. An additional negative control of native (folded) RNase A was also incubated in the presence of 50 mM dimedone. When employed, additives such as metals [50 μM $CuSO_4$ or $Fe_2(SO_4)_3$] and metal chelators (1 mM EDTA and 0.1 mM DTPA) were added from 100−200× stock solutions (in 0.1 M acetic acid or Tris acetate buffer) just prior to the adjustment of solution pH and addition of protein. Final volumes were typically 1 mL. For incubations under controlled atmospheric conditions, samples were placed into a Spilfyter Hands-in-Bag atmospheric chamber that was filled and continually pressurized with high-purity nitrogen or oxygen. Samples were withdrawn with a gel loading pipet tip through a resealable pinhole in the top of the chamber.

Assessment of Refolding Progress. We monitored refolding progress (in the absence of dimedone) by taking 5 μL aliquots of the sample and mixing them with 5 μL of 50 mM maleimide in 0.2 M acetic acid (giving a final pH of ≤5). Samples were incubated at 50 °C for 15 min and then diluted to a final concentration of 1 μM in starting LC solvent before the immediate injection of 2 μL on trap. (Dimedone-containing samples were not alkylated, but simply diluted to a final concentration of 1 μM in starting LC solvent before 2 μL was injected on trap.)

Refolding progress was monitored by maleimide alkylation of partially refolded protein and analysis of samples by ESI-MS. As demonstrated by the raw mass spectra (Figures S1 and S3 of the Supporting Information), the procedure employed resulted in sulfhydryl-specific protein alkylation with essentially no alkylation of protein amino groups. To determine the precise degree to which proteins were refolded, deconvoluted ESI mass spectra were integrated. Peak areas were then summed, and the fraction of protein in each folding state [i.e., zero, one, two, three, or four disulfides (see Figure S3 of the Supporting Information)] was determined by dividing the area of the appropriate representative peak by the total area. These fractions were then weighted by folding state according to the following equation:

$$R_p = (FSS_4 \times 1 + FSS_3 \times 0.75 + FSS_2 \times 0.5 + FSS_1 \times 0.25 + FSS_0 \times 0) \times 100$$

where R_p represents the percentage of protein refolded and FSS_n represents the fraction of protein containing n disulfide bonds. Thus, for example, if two equal-area peaks were observed in a deconvoluted ESI mass spectrum representing protein molecules with three disulfides and protein molecules with four disulfides, the percentage of protein refolded would be reported as 87.5%. Notably, this summary method of reporting protein refolding progress as a single numerical value is useful for comparison with similar non-mass spectrometric techniques, but it mutes data on folding-state molecular statistics (which are available in the raw data) that might be considered informative with respect to certain kinetic models of folding (which were not under consideration here). In unmuted form, i.e., without applying the equation given above, the raw data that are acquired using this technique contain more detailed information about protein disulfide status than is generally available with other non-mass spectrometric techniques.

Incubation of the Cysteine-Containing Peptide with Dimedone. Three microliters of a 670 μM solution of a single-free-cysteine-containing peptide (H_2N-ALLETYCATPAKSE-CO_2H) (also known as IGF 57−70) was added to 96 μL of freshly prepared 0.15 M ammonium bicarbonate (pH 7.1) and 1 μL of 1 M dimedone in ethanol, giving final peptide and

dimedone concentrations of 20 μM and 10 mM, respectively. A control without dimedone was prepared in parallel. The samples were then allowed to sit exposed to air for 20 h in the dark at room temperature, at which point they were diluted 30-fold in MALDI matrix solution and analyzed by MALDI-MS and MALDI-MS/MS (TOF/TOF) as described below.

ESI-LC/MS. A trap-and-elute form of sample concentration/solvent exchange rather than traditional LC was employed. Samples were injected with a Spark Holland Endurance autosampler in microliter pick-up mode and loaded with an Eksigent nanoLC*1D at a rate of 10 μL/min [90/10 water/acetonitrile mixture containing 0.1% (v/v) formic acid, solvent A] onto a protein cap trap (Michrom Bioresources, Auburn, CA) configured for unidirectional flow on a six-port divert valve. After 2 min, the divert valve position was automatically toggled and the rate of flow over the cartridge changed to 1 μL of solvent A/min (running directly to the ESI inlet) which was immediately ramped over 5 min to a 10/90 water/acetonitrile mixture containing 0.1% (v/v) formic acid. By 7.2 min, the run was completed and the flow back to 100% solvent A.

The bulk of the RNase A eluted between 3.5 and 5 min into a Bruker MicrOTOF-Q (Q-TOF) mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the m/z range of 50−3000. ESI settings for the Agilent G1385A capillary nebulizer ion source were as follows: end plate offset, −500 V; capillary voltage, −4500 V; nebulizer gas, nitrogen at 2 bar; dry gas, nitrogen at 3.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 2 GHz. Rate control for the spectra was by summation at 1 Hz.

One to two minutes of recorded spectra was averaged across the chromatographic peak apex of RNase A elution. The ESI charge-state envelope was deconvoluted with Bruker DataAnalysis version 3.4 to a mass range of 1000 Da on either side of any identified peak.

Proteolytic Digestion of RNase A. Reduced RNase A that had been incubated in air for more than 20 h in the absence or presence of dimedone was digested overnight at 37 °C and pH 7.5 with Lys-C at a protein to protease mass ratio of 3/1. One microliter of the resulting digestion mixture was added to 4 μL of MALDI matrix solution (see below) and allowed to dry on the MALDI target.

MALDI-MS. Peptides were mixed with MALDI matrix solution [33% (v/v) acetonitrile containing 0.4% (v/v) TFA and saturated with α-cyano-4-hydroxycinnamic acid], spotted onto a gold-surfaced MALDI target, and allowed to dry. Single-stage and LIFT-TOF/TOF mass spectra were recorded on a Bruker Ultraflex MALDI-TOF/TOF instrument. Externally calibrated mass spectra were acquired in positive ion mode with the reflector engaged. For LIFT-TOF/TOF experiments, the precursor ion selection width was set on an individual peptide basis to ensure that no undesired parent ions would contaminate the MS/MS spectra. Instrument voltages and other parameters were optimized for peptide resolution and sensitivity; at least 7500 laser shots were acquired per sample to ensure excellent ion counting statistics.

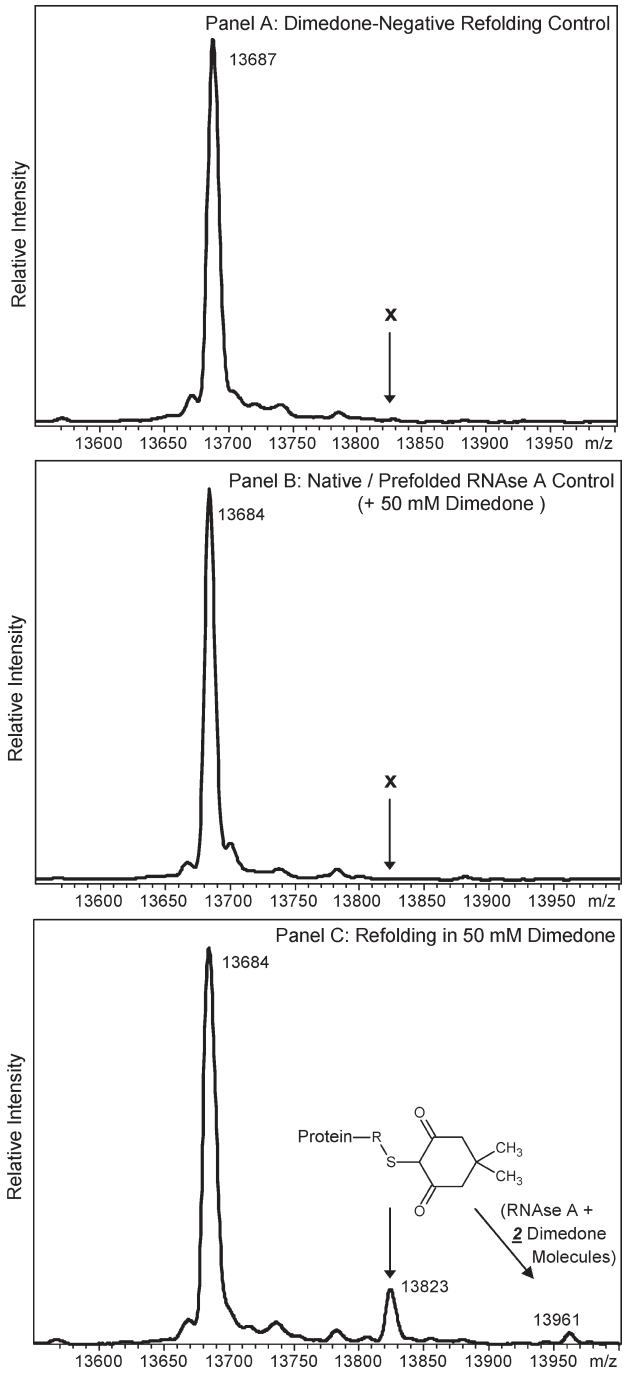


FIGURE 1: Charge-deconvoluted singly charged ESI mass spectra. (A) Negative control in which reduced RNase A was allowed to refold in the absence of dimedone at room temperature and pH 8 and exposed to air. X indicates the absence of a peak at the expected m/z value of dimedone-modified RNase A. (B) Negative control in which native (nonreduced/folded) RNase A was exposed to refolding conditions in the presence of 50 mM dimedone. (C) Reduced and purified RNase A refolded in the presence of 50 mM dimedone. The calculated MH^+ mass of fully reduced RNase A is 13691.3 Da and that of fully folded RNase A 13683.2 Da.

RESULTS

Strategy. Scheme 1 depicts the hypothesized ambient oxygen- and trace metal-induced formation of Cys-SOH in an unfolded protein molecule. Previous studies have demonstrated that in the absence of a trapping agent (which allows for detection of Cys-SOH) a facile reaction occurs between hydrogen peroxide-generated protein Cys-SOH and intramolecular thiols (*40*). [Intermolecular protein thiols are also reactive with peroxide-generated Cys-SOH (*7*, *41*).] We therefore reasoned that if Cys-SOH were to be detected within a refolding protein, then it must be serving as an oxidized sulfur intermediate in the in vitro formation of intramolecular disulfide bonds.

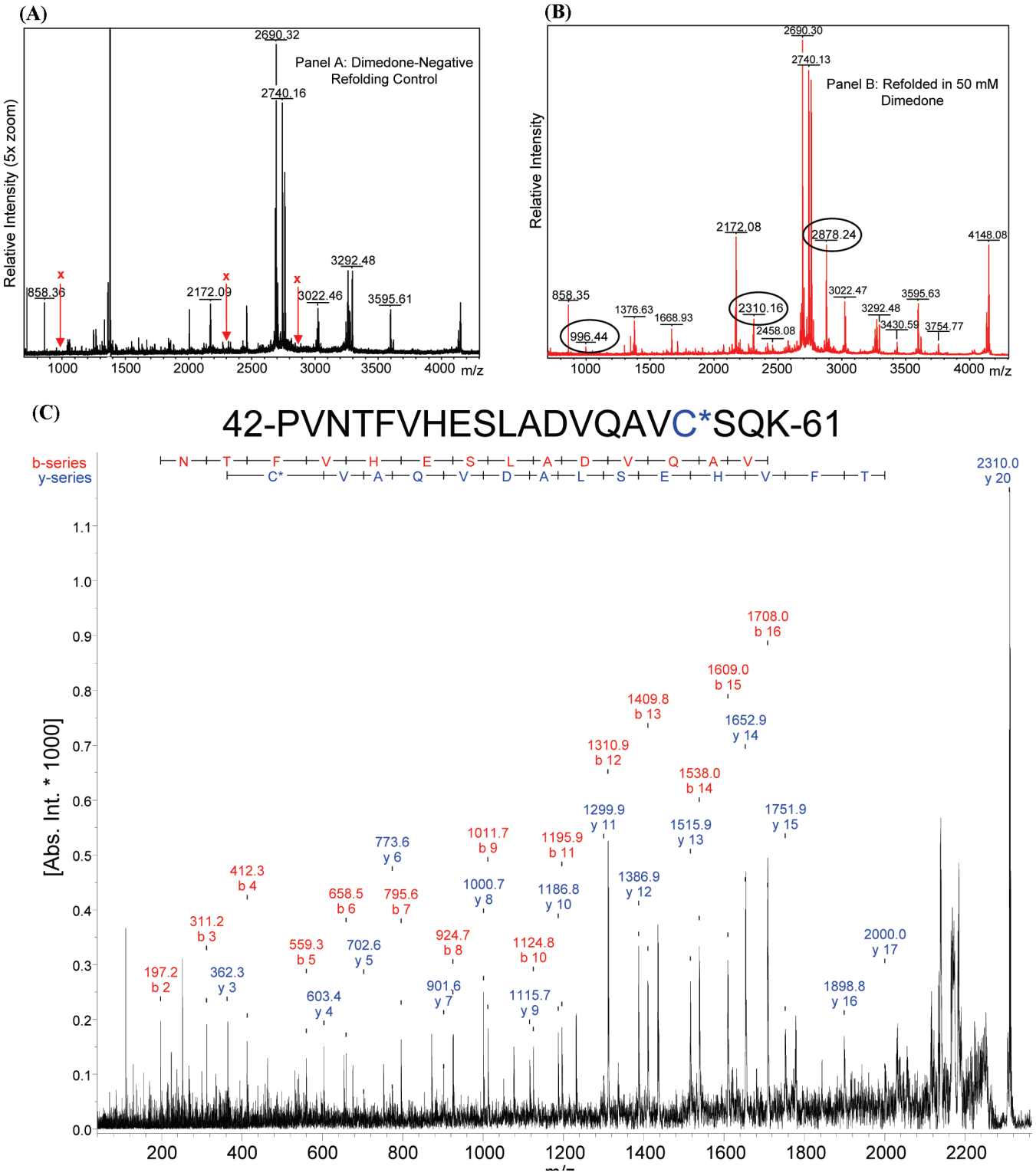


FIGURE 2: Reflector-mode MALDI mass spectra of Lys-C-digested RNase A. (A) RNase A that was refolded in the absence of dimedone. X indicates the absence of peaks that are found when RNase A is refolded in the presence of dimedone. (B) RNase A refolded in the presence of 50 mM dimedone. Circled m/z values indicate peaks representative of covalent dimedone-modified RNase A peptides. These peptides are shifted up in mass by that of covalently attached dimedone (138.07 Da). (Noncovalently attached dimedone would shift the mass by 140.08 Da.) Table 1 provides a list of pertinent calculated and observed masses. Though not visible, isotopic clusters are baseline-resolved; hence, the indicated m/z values are monoisotopic. (C) MALDI-MS/MS (TOF/TOF) spectrum of the peptide represented at m/z 2310 in panel B. Assigned peaks are accurate within 0.3 Da. The y-ion series indicates that the cysteine residue is shifted up in mass by 138 Da. MS/MS spectra for the dimedone-modified peptide ions represented by peaks at m/z 996 and 2878 in panel B are available as Supporting Information.

Trapping of Cys-SOH during Protein Refolding. To determine the potential role of air-generated Cys-SOH in disulfide bond formation and in vitro, nonenzymatic protein folding, disulfide-reduced RNase A was incubated at room temperature with exposure to air [analogous to the seminal experiments in protein folding by Anfinsen and co-workers (*1*−*6*)] in the presence of the Cys-SOH trapping reagent dimedone (*32*−*39*). Deconvoluted ESI mass spectra of intact RNase A incubated in

Table 1: Calculated and Observed Monoisotopic Masses of Cysteine-Containing Peptides and Their Corresponding Covalent Dimedone-Modified Peptides That Were Observed upon MALDI-TOF Analysis of Lys-C-Digested RNAse A That Was Refolded in the Presence of 50 mM Dimedone[a]

	calculated m/z	observed m/z
RNase A Y92−K98		
unmodified	858.38	858.35[b]
dimedone-modified	996.45	996.44
RNase A P42−K61		
unmodified	2172.08	2172.08
dimedone-modified	2310.14	2310.16
RNase A F8−K31		
unmodified	2740.12	2740.13
dimedone-modified	2878.19	2878.24
IGF 57−70		
unmodified	1496.76	1496.78
dimedone-modified	1634.83	1634.86
disulfide-linked dimer	2990.49	2990.54

[a]IGF 57−70 is the synthetic peptide (H_2N-ALLETYCATPAKSE-CO_2H) incubated for 20 h in the presence of dimedone (see Figure 4). Observed m/z values aligned within 20 ppm of the calculated m/z values (with the single exception indicated). [b]Mass accuracy outlier at 35 ppm.

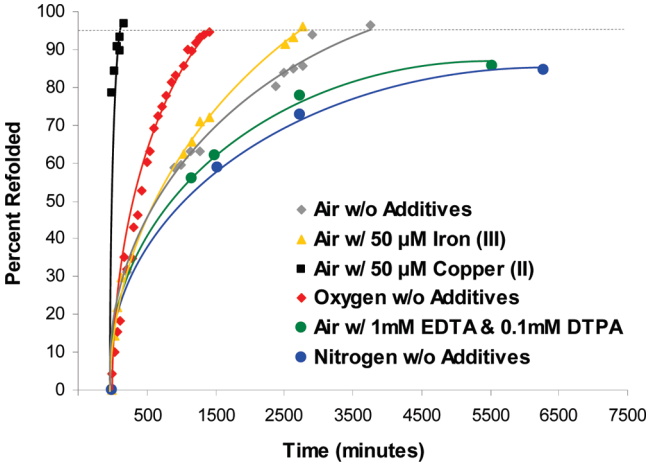


FIGURE 3: RNAase A refolding progress over time under different atmospheric and solution conditions. Gray diamonds represent data for RNase A refolding under air without special additives.

the presence of dimedone were acquired (Figure 1). Mass shifts caused by covalent dimedone reaction products were present. Control experiments conducted in the absence of dimedone and, separately, in the presence of dimedone but with native (fully folded) RNase A did not result in dimedone-modified RNase A as demonstrated (Figure 1).

Verification of Formation of a Covalent Dimedone−Protein Product. To verify that dimedone was indeed covalently bound to RNase A (because of its reaction with Cys-SOH), the samples described in Figure 1 (panels A and C) were digested with Lys-C and analyzed by MALDI-MS and MALDI-MS/MS (TOF/TOF). Mass spectra of the resulting peptide mixtures were acquired (Figure 2). With one exception, observed monoisotopic m/z values aligned within 20 ppm of the calculated m/z values (Table 1). The average mass accuracy was 13.7 ppm (including the noted outlier). MS/MS (TOF/TOF) spectra for three of these peptides containing nonredundant cysteine residues were acquired; one of these spectra is shown as Figure 2C (the others are available as Figure S2 of the Supporting Information). In agreement with the single-stage (peptide mapping) MALDI mass spectra, each of these MS/MS spectra confirms the covalent attachment of dimedone to the suspected cysteine residue.

Roles of Oxygen and Metals. Given its elemental composition, it is clear that formation of Cys-SOH requires oxygen. We hypothesized that diatomic oxygen from air contributes to Cys-SOH formation through the mediation of trace metals in solution. To elucidate the roles of ambient oxygen (O_2) and trace metals on the rate of protein refolding, additional experiments were conducted under an oxygen atmosphere, under a nitrogen atmosphere, or in the presence of added copper(II), iron(III), or metal chelators (Figure 3). Samples of RNase A were allowed to refold until either the refolding process was >95% complete or 4 days had passed, whichever came first. In the cases of refolding under a nitrogen atmosphere or refolding in the presence of metal chelators (in air), 4 days passed before 80% refolding was reached. It took just more than 2 days for RNase A to refold under air without added metals. The addition of 50 μM iron(III) had an only marginal effect on this rate. Refolding under a pure oxygen atmosphere was >95% complete within 24 h, and refolding in the presence of 50 μM copper(II) was >95% complete in <2 h. For each sample described in Figure 3, parallel samples were refolded in the presence of dimedone. In each case, covalent dimedone−RNase A reaction products were discovered as described above. (A time course showing the degree of covalent incorporation of dimedone into refolding RNase A over time is available as Figure S4 of the Supporting Information.) Notably, none of the experimental alterations to the refolding environment resulted in formation of cysteinesulfinic acid (-SO_2H).

Cys-SOH Mediates Spontaneous Formation of Peptide Dimers. The non-site-specific nature of Cys-SOH formation during RNase A refolding suggested a generalized chemical phenomenon. To verify the hypothesis that Cys-SOH forms spontaneously on free thiols that are dissolved in neutral-to-slightly alkaline aqueous solution and exposed to air, a 20 μM solution of the single-free-cysteine-containing peptide IGF 57−70 (H_2N-ALLETYCATPAKSE-CO_2H) was placed under such conditions in the presence of dimedone for approximately 20 h. MALDI-TOF mass spectra were recorded (Figure 4). Besides showing the expected formation of ample quantities of disulfide-linked homodimer, panels B and C confirm that dimedone was found covalently attached to the cysteine residue of the monomeric peptide, indicating that Cys-SOH had formed under the mild conditions of neutral-to-slightly alkaline pH and exposure to air.

DISCUSSION

The data shown here demonstrate air- and trace metal-induced formation of Cys-SOH as part of the reduced RNase A protein molecule. When allowed to proceed for a sufficiently long period of time, the refolding process completes and returns full activity to the RNase A molecule (*2*−*4*, *6*). Notably, arsenite is considered a reducing agent specific for Cys-SOH that does not affect protein disulfides (*13*, *42*). As such, if Cys-SOH is involved in the formation of protein disulfide bonds, arsenite should inhibit in vitro protein folding. Such inhibition was recently documented well by Ramadan et al. (*43*). Thus, considering (1) the detection of covalent dimedone−protein reaction products on cysteine residues within the unfolded protein in the absence of added

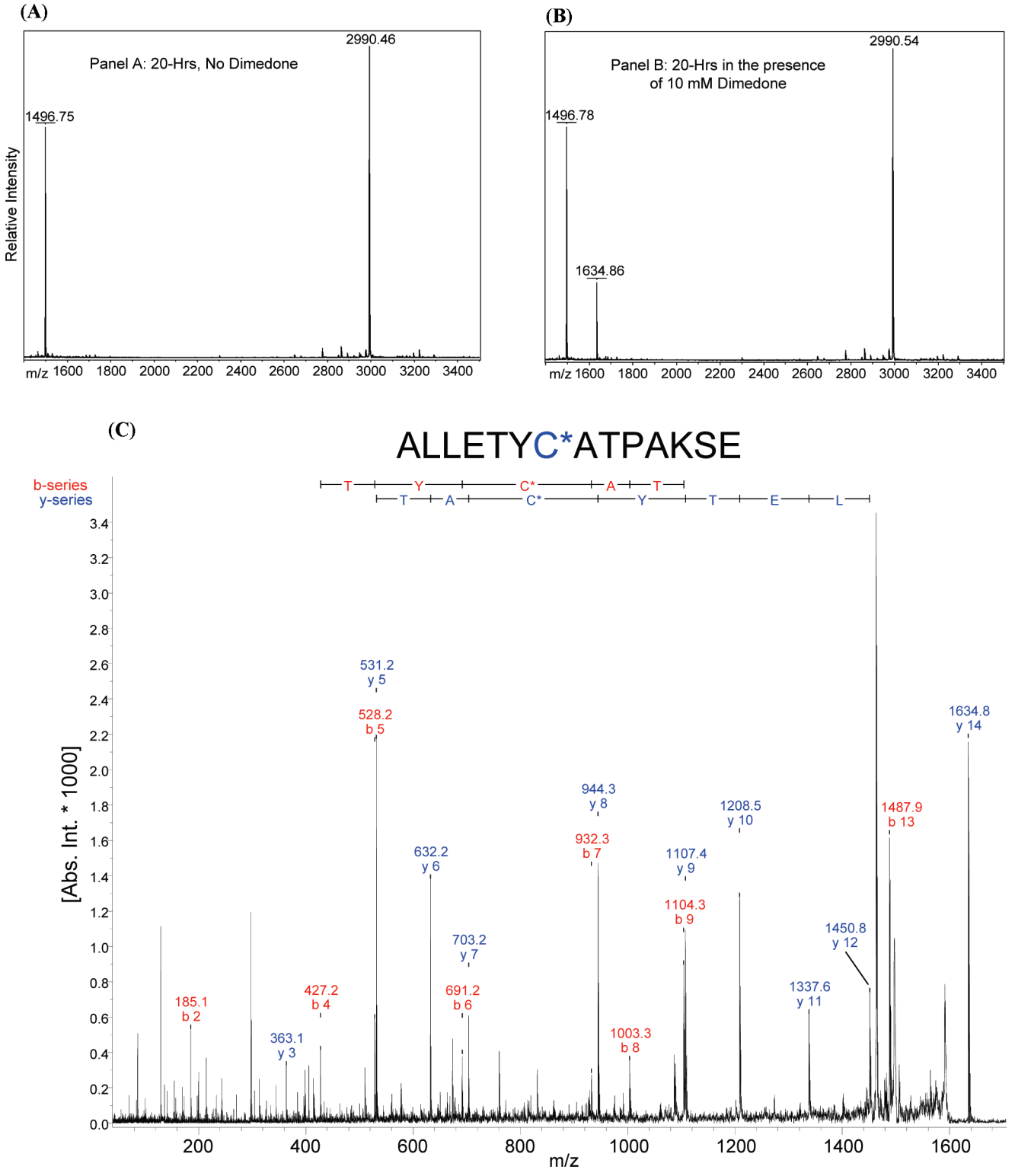


FIGURE 4: MALDI mass spectra of synthetic peptide IGF 57−70 (H_2N-ALLETYCATPAKSE-CO_2H) incubated for 20 h at room temperature and pH 7.1 in the (A) absence of dimedone and (B) presence of 10 mM dimedone. Isotopic clusters are baseline resolved, and indicated *m*/*z* values are monoisotopic. The final rows in Table 1 provide a list of pertinent calculated and observed masses. (C) MALDI-MS/MS (TOF/TOF) spectrum of the dimedone-modified peptide at *m*/*z* 1634. The b- and y-ion series indicate that the cysteine residue is shifted up in mass by 138 Da.

oxidants, (2) the fact that arsenite inhibits oxidative protein folding in vitro (*43*), (3) the fact that no detectable Cys-SOH forms within a folded RNase A molecule (Figure 1B), and (4) the known reactivity of free sulfhydryl groups with Cys-SOH (*7*, *8*, *10*, *21*, *40*, *41*, *44*−*46*), it is logical to conclude that ambient oxygen-induced Cys-SOH serves as an intermediate in the spontaneous disulfide bond formation process that takes place as part of in vitro, nonenzymatic protein folding. In 2007, Johansson and Lundberg (*45*) presented evidence of the spontaneous formation of protein−glutathione mixed disulfides. On the basis of inhibition of the process with dimedone and (separately) arsenite, they suggested that the mixed disulfides were formed via a Cys-SOH intermediate. Here we extend their studies by physically trapping and directly analyzing a spontaneously formed Cys-SOH

intermediate that mediates intramolecular disulfide bond formation and in vitro protein folding. Additionally, we show that such natural Cys-SOH species mediate formation of disulfide bonds between free cysteine-containing peptides, suggesting a ubiquitous role for Cys-SOH in the spontaneous, frequently undesirable formation of disulfide-linked intermolecular dimers.

The data presented in Figure 3 demonstrate that molecular oxygen and trace metals are important reagents in the spontaneous in vitro generation of Cys-SOH. Compared to a "natural" folding rate of ~2 days in air (gray diamonds), refolding under nitrogen or in the presence of metal chelators (EDTA and DTPA) in air takes several days; however, refolding under an oxygen atmosphere takes ~1 day, and refolding in the presence of 50 μM copper(II) takes just a couple of hours. Notably, Anfinsen observed complete refolding of RNase A in air in ~20−24 h (*3*, *5*). The differences between Anfinsen's results and those reported here may be due to the fact that we were able to utilize highly pure deionized water and virgin polypropylene test tubes that are likely to minimize trace metal contamination compared to reagents and reaction vessels available several decades ago.

The apparent plateau phases reached by samples folding under nitrogen and in the presence of metal chelators (Figure 3) may indicate that the extremely small quantities of available oxygen and trace metals, respectively, were effectively depleted during the refolding process, and that because of this the refolding process may never be fully complete under these conditions. The mechanisms and rate laws governing formation of Cys-SOH are the subjects of future investigation.

Notably, the means by which the folding process was monitored in these studies using maleimide alkylation and monitoring by mass spectrometry provides a unique viewpoint into the protein folding process by allowing for direct determination of the relative number of protein molecules in each state of disulfide bond formation at any given point in time. Though it is not obvious by the way the data are plotted in Figure 3, as explained in Experimental Procedures, each data point was gleaned via a mass spectrum in which information about the relative abundance of protein molecules with zero, one, two, three, or four disulfide bonds was unambiguously provided (see Figure S3 of the Supporting Information for a series of illustrative mass spectra). Via examination of these relative populations of protein folding states over time, it is clear that formation of the last disulfide bond is the slowest step in the refolding of RNase A. For example, the sample containing copper(II) was nearly 80% folded within 1 min but took almost 2 h to reach >95%. These observations support the RNase A disulfide folding mechanism asserted by Creighton more than 30 years ago (*47*, *48*).

CONCLUSIONS

The data presented here demonstrate that oxygen- and trace metal-generated Cys-SOH is a ubiquitous modification of cysteine residues in solution and an intermediate that mediates in vitro, nonenzymatic disulfide bond formation and protein folding.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Supporting mass spectra and graph. This material is available free of charge via the Internet at http://pubs.acs.org.

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