A Tris (2-Carboxyethyl) Phosphine (TCEP) Related Cleavage on Cysteine-Containing Proteins

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Introduced in the late 1980s as a reducing reagent, Tris (2-carboxyethyl) phosphine (TCEP) has now become one of the most widely used protein reductants. To date, only a few studies on its side reactions have been published. We report the observation of a side reaction that cleaves protein backbones under mild conditions by fracturing the cysteine residues, thus generating heterogeneous peptides containing different moieties from the fractured cysteine. The peptide products were analyzed by high performance liquid chromatography and tandem mass spectrometry (LC/MS/MS). Peptides with a primary amine and a carboxylic acid as termini were observed, and others were found to contain amidated or formamidated carboxy termini, or formylated or glyoxylic amino termini. Formamidation of the carboxy terminus and the formation of glyoxylic amino terminus were unexpected reactions since both involve breaking of carbon–carbon bonds in cysteine. (J Am Soc Mass Spectrom 2010, 21, 837–844) © 2010 American Society for Mass Spectrometry

The cleavage of peptide bonds at cystine residues by cyanide has been reported previously [1]. Its mechanism involves scission of the disulfide bond to yield a sulfhydryl and a thiocyanate group. The latter cyclizes to form an unstable acyliminothiazolidine moiety that hydrolyzes spontaneously, resulting in cleavage of the peptide bond at the N-terminal side of cysteine residues [2]. Selective cleavage of cysteine residues is most commonly performed with 2-nitro-5-thiocyanatobenzoic acid (NTCB) in two distinct steps, cyanylation and cleavage [3–9]. Since only reduced cysteines are cyanylated by NTCB, this method allows differentiation of free sulfhydryl groups from those forming disulfide bonds. This specific reaction of cysteine residues has been exploited to cleave proteins into appropriate sizes for structural studies and for the characterization of free sulfhydryl groups and disulfide linkages in proteins [10–15].

In the last two decades, Tris (2-carboxyethyl) phosphine (TCEP) has been extensively used in a wide range of applications to reduce disulfide bonds, to maintain free sulfhydryl groups, and for subsequent structural analysis in proteins [16–22]. Among all reducing reagents, TCEP is often preferred due to its strong reducing power, high stability, wide pH range, and odorless nature. To date, only a few reports have been published discussing its side reactions. Recently, a side reaction was observed, in which a cysteine residue was found to be converted to alanine by heating in the presence of TCEP [23]. Another side reaction of TCEP, albeit not TCEP specific, was also observed. In a reduction and cleavage reaction, photoactive yellow protein (PYP) with azido homoalanine substituted for its methionine residues was found to be cleaved by TCEP, dithiothreitol (DTT), or 2-mercaptoethanol [24]. In the last reaction, the protein was cleaved at azido homoalanine residues, which were incorporated in vivo. All other protein cleavages mentioned above occurred at cysteine residues.

In this work, we report the observation of cysteine-containing proteins being cleaved slowly when they are treated with TCEP under mild conditions. Cysteine residues are cleaved via multiple pathways, such that peptides with heterogeneous termini are formed, similar to protein cleavages resulting from cysteine cyanylation. A unique feature of this TCEP side reaction is

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that the formation of some peptides requires cleavage of strong carbon–carbon bonds. This is unexpected under mild conditions. The cleavage was observed in six unrelated proteins and peptides that were tested in our laboratory. It does not appear to be sequence-specific; however, the reaction rate was found to vary with pH and amino acid sequence.

The cleavage of cysteine-containing proteins was first observed in medium-sized proteins that had been stored at 4 °C in the presence of TCEP. It was later found that the observed cleavage happens to both proteins and peptides as long as the sequence contains cysteine residues. To simplify identification of the reaction products, a synthetic peptide containing a single cysteine was used in this work.

Experimental

Chemicals and Reagents

The model molecule is a synthetic peptide with 16 residues: SGTASVVCLLDNFYPR (monoisotopic mass 1741.85 Da, from human Ig κ chain C region). It was synthesized by AnaSpec (Fremont, CA). TCEP solution (catalog no. 646547; Sigma, St. Louis, MO, USA) and powder (catalog no. T2556; Invitrogen, Carlsbad, CA, USA) were used in the study. The cleavage reaction was performed by incubating the synthetic peptide at 0.04 mg/mL (~23 μM) with 10 mM TCEP for 24 h at 37 °C in the following buffers: (1) 50 mM glycine-HCl (Sigma, catalog no. G8898), pH 2.2; (2) 50 mM citrate (citric acid, Sigma, catalog no. 251275; sodium citrate dihydrate, Sigma, catalog no. C8532), pH 4.0; (3) 50 mM MES (CalBiochem, catalog no. 475893; Darmstadt, Germany), pH 6.0; (4) 50 mM Tris (Sigma, catalog no. T8443), pH 8.0; (5) 50 mM borate (CalBiochem, catalog no. 203667), pH 10.0. The pH of all buffers was adjusted using 0.1 M NaOH (J. T. Baker, catalog no. 5635-02, Phillipsburg, NJ, USA) or 0.1 M HCl (J. T. Baker, catalog no. 5620-02). Cleavage in the presence of TCEP was found to happen at both 4 °C and 37 °C. In this study, 37 °C was used to accelerate the reaction.

HPLC and MS Conditions

In the LC/MS and LC/MS/MS experiments, the reaction products were separated using gradient elution from a 2.1 × 250 mm, C18 column (5 μm, 300 Å, 218TP52; Grace, Deerfield, IL, USA) on a Waters Alliance 2695 HPLC system (Waters, Milford, MA, USA). The column temperature was 35 °C. The mobile phases were 0.1% formic acid (Fluka, catalog no. 56,302, Seelze, Germany) in water (A) and 0.1% formic acid in acetonitrile (B) (J. T. Baker, catalog no. 9017-33) at a flow rate of 0.2 mL/min. With respect to mobile phase A, the gradient was 95%–50%, 50%–20%, 20%–95%, and 95% constant in 30, 8, 2, and 15 min, respectively. Analysis of the chromatographic eluate was carried out using a QTOF SYNAPT mass spectrometer (Waters) operating in positive electrospray ionization mode. Data-dependent analysis (DDA) and product ion modes were used to acquire product ion spectra for the resultant peptides. The electrospray voltage and the cone voltage were set at 3000 and 30 V, respectively.

For accurate mass measurements, the reaction products were analyzed by LC/MS on an LTQ-FT mass spectrometer (Thermo Scientific, San Jose, CA, USA) interfaced to a Waters Acquity ultra performance liquid chromatography system. Chromatographic separations were achieved employing a 2.0 × 100 mm, 1.7 μm, Acquity BEH-C18 column (Waters) with gradient elution at 0.6 mL/min. The column temperature was 50 °C. Mobile phase A was water with 0.1% formic acid and mobile phase B was 98:2 acetonitrile:water with 0.1% formic acid. A linear gradient was formed from 10% to 80% mobile phase B over 11.6 min. The final composition was held for 1.5 min before returning to the initial conditions. Positive ESI Fourier transform mass spectrometry (FT-MS) data were acquired from *m/z* 200 to 2000. The instrument was operated at 25,000 resolution. Instrumental settings follow: capillary temperature 320 °C, capillary voltage 10 V; tube lens voltage 40 V; ESI metal needle option; ESI needle voltage 5 kV; sheath gas 80 arbitrary units (arbs); auxiliary gas 20 arbs; sweep gas 15 arbs.

Results and Discussion

Heterogeneous Reaction Products

At least eight reaction products were detected by LC/MS after the model peptide was incubated with TCEP overnight at 37 °C. Among them, six peptides have been identified by LC/MS and LC/MS/MS analysis. Their monoisotopic masses were determined to be 618.34, 619.40, 646.32, 1036.56, 1064.57, and 1108.55 Da. The masses of the two unidentified reaction products were determined to be 1103.6 and 1106.6 Da, respectively. In the following discussion, these reaction products and their parent 16-mer peptide are denoted as F618, F619, F646, F1036, F1064, F1108, and P16, respectively. Among the six, F619 and F1036 were identified by MS/MS as peptides containing the first seven and the last eight residues of P16, respectively (data not shown). They would be formed when the peptide bonds flanking the cysteine residue in P16 were hydrolyzed. Although the protein cleavage observed in the presence of TCEP is unusual, the existence of products generated by peptide bond hydrolysis is not unexpected. Peptide bonds are the weakest within the protein backbone with slightly positive free energies [25]; therefore, they are the least stable. The observation of F619 and F1036 illustrates one aspect of this TCEP side reaction in which the cysteine residues are in effect "extracted".

Reaction Products from the Amino Side of P16

The product ion spectra of doubly charged F618 and F646 (Figure 1a and b) are similar. The spectra in the region below *m/z* 602 are virtually the same. Since the b-series ions [26] from b3 through b5 were observed, these two peptides originated from the amino side of P16. In addition, all identified b-series ions (b2 through b6) as well as the doubly protonated molecular ions show a strong tendency to lose H_2O. This is consistent with the amino acid sequence of P16, which contains Ser residues at positions 1 and 5, and a Thr residue at position 3. The product ion spectra indicate that the first six residues in both peptides were unmodified and that the C-terminal residue was modified. Since the terminal Val contains a relatively nonreactive side chain, the modification was most likely on the backbone of the C-terminus. Further studies using accurate mass measurement indicated that the elemental compositions of F618 and F646 were [C25 H46 O10 N8] and [C26 H46 O11 N8], respectively.

Determination of the elemental composition by FTMS was done by matching all potential molecules to a measured mass, and using constraints to eliminate false hits. For example, the protonated F618 ion was observed at *m/z* 619.34,184, indicating that its molecular weight was 618 Da. Based on the nitrogen rule, this peptide should contain an even number of nitrogen atoms. Since F618 is from the N-terminal side containing at least seven residues (Figure 1), and each amino acid contains one nitrogen atom, the constraint is that the protonated F618 should contain a minimum of eight nitrogen atoms. As a result, only the elemental composition [C25 H47 O10 N8] with a theoretical mass of 619.34,097 satisfies the constraint. Therefore, the elemental composition of F618 was determined to be [C25 H46 O10 N8], after removing a proton from the formula. A summary of the elemental compositions for the peptide products is shown in Table 1.

The elemental composition of peptide F619, SGTASVV, is [C25 H45 O11 N7]; therefore, the elemental gain/loss of F618 and F646 relative to F619 are +H+N−O and +H+C+N, respectively. To identify the source of the additional nitrogen atom in F618 and F646, and to rule out the possibility of an unknown contaminant insert-

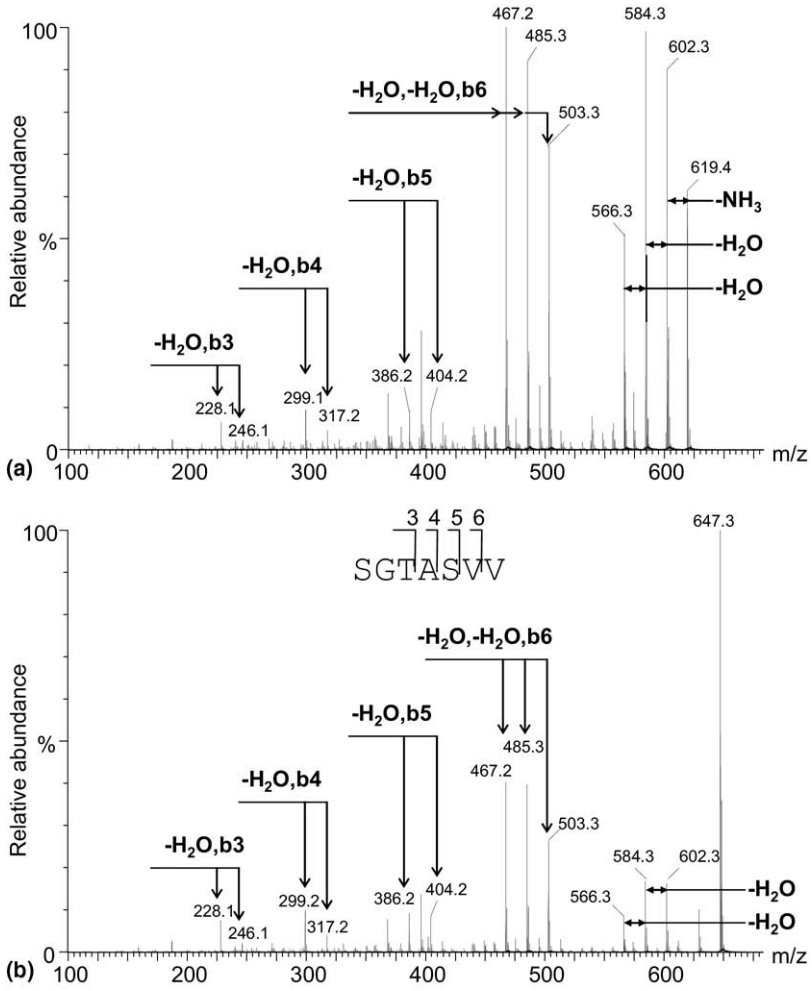


Figure 1. Product ion spectra of doubly charged F618 (**a**) and F646 (**b**), indicating the two peptides contain the N-terminal side of the model peptide, P16, after the cysteine residue was cleaved. The first six residues appeared to be intact and the seventh was modified.

Table 1. Summary of accurate mass measurements for peptide products

Measured $(M + H)^+$	Theoretical $(M + H)^+$	Relative mass difference (ppm)	Elemental composition
619.34184	619.34097	1.41	C_{25} H_{47} O_{10} N_8
647.33670	647.33588	1.26	C_{26} H_{47} O_{11} N_8
1109.52756	1109.52620	1.22	C_{51} H_{73} O_{16} N_{12}
1065.53877	1065.53637	2.25	C_{50} H_{73} O_{14} N_{12}

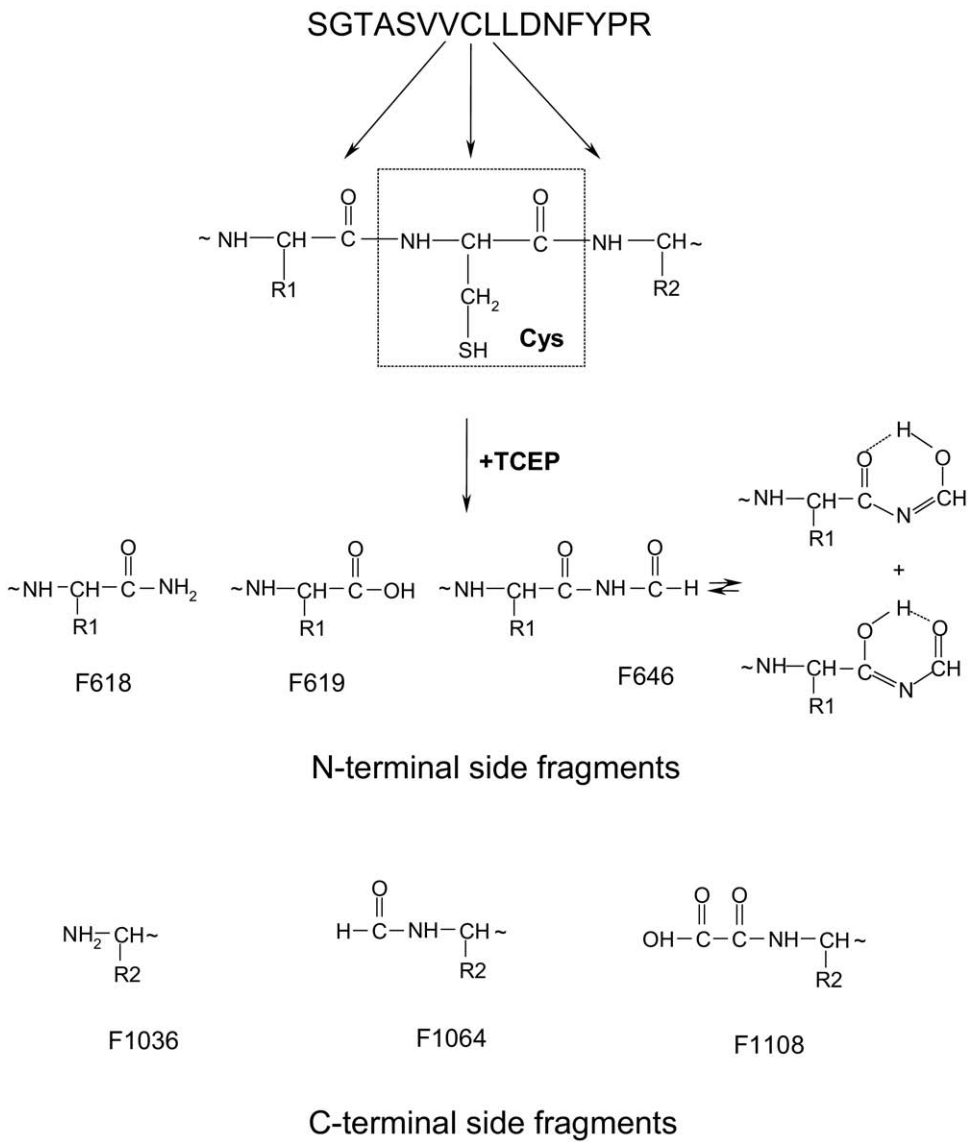
ing a nitrogen atom into the peptides during cleavage of P16, a surrogate P16′ was prepared in which its cysteine residue was ^{15}N labeled. The labeled peptide was treated with TCEP using the same reaction conditions. The masses of the corresponding peptides F618′ and F646′ were determined to be 619.39 Da and 647.40 Da, respectively, which were 1 Da larger than F618 and F646. This indicated that the additional nitrogen atom in both F618 and F646 came from the fractured cysteine. Before P16 is cleaved, the local structure near cysteine is ~CHR-CO-NH-CHR_1~. After cleavage, the C-terminal structure of F619 is ~CHR-CO-OH. With an elemental gain/loss of +H+N−O relative to F619, the only reasonable structure for F618 is SGTASVV-NH2. In other words, F618 contains the seven N-terminal residues of P16 and is amidated at the C-terminus, as shown in Scheme **1**. Further support for an amidated C-terminus is the observation of an intense ion at *m/z* 602.3 in the product ion spectrum of F618 (Figure 1a). This loss of 17 Da is consistent with a neutral loss of NH_3 from protonated F618. A similar amidated C-terminus has been reported for an antibody after being stored for 2 wk at 45 °C, and was believed to result from β-elimination at the cysteine residue [27]. This reaction product has also been observed in free radical-mediated oxidation and in the amidation pathway of proteins [28–30]. The C-terminus of F646 also contains the nitrogen atom from the fractured cysteine, with an additional carbon and hydrogen, supporting its assignment as a formamidated C-terminus, ~CO-NH-CO-H. The presence of a formamidated C-terminus could be indicative of formation of a ring structure consisting of the same six atoms as shown in Scheme **1**. In our experiment, a mixed population of F646 was observed in two mass spectrometrically identical species eluting at different times, with a peak ratio of 5:1 (data not shown); therefore, in aqueous solution, F646 appears to be an equilibrium mixture of the formamide structure and a proposed six-member ring structure (Scheme **1**). This observation provides further evidence to support the identification of F646 as a peptide containing a formamidated C-terminus. Other isomers of the ring structure may exist and contribute to the mass spectrometrically identical species of F646.

Even though an amine could potentially react with formic acid to form formamide, the generation of F646 does not proceed via this mechanism. This peptide was still observed after the HPLC system had been thoroughly flushed and the formic acid in the HPLC mobile phases was replaced by acetic acid.

Reaction Products from the Carboxy Side of P16

The product ion spectra of doubly charged F1064 and F1108 (Figure 2a and b) are also similar, as both contain the y-series ions [26] from y1 through y6. This indicates F1064 and F1108 were formed from the C-terminal side of P16, and the last six residues of these two peptides were unmodified. In the product ion spectra of F1064 and F1108, the additional loss of 17 Da accompanying most y-ions was consistent with the neutral loss of NH_3 from the Arg at the C-terminus or from the Asn. From the product ion spectra alone, we were unable to determine whether the side chains of the Leu residues were modified; however, it is unlikely that either side chain was involved in any modifications due to their relatively inert nature. The observed modifications on F1064 and F1108 were most likely at the amino termini. By accurate mass measurement, the elemental compositions of F1064 and F1108 were determined to be [C50 H72 O14 N12] and [C51 H72 O16 N12], respectively. Since the elemental composition of F1036, the C-terminal 8-mer LLDNFYPR, is [C49 H72 O13 N12], the elemental difference between F1064 and F1036 is +C+O. Based on this small difference in elemental composition as well as the structures of the N-terminal peptide products (F619, F646) that were previously identified, the CO on F1064 most likely originate from the cysteine in P16. Therefore, we propose that F1064 is formylated at the N-terminus, as shown in Scheme **1**.

F1108 appears to have strong structural similarities with F1036 and F1064 (Figure 2), with structural differences based on stable leaving groups as shown in Figure 3. The product ion spectrum of protonated F1108 contains intense ions at *m/z* 1065.2, 1047.2, and 1037.2, which correspond to neutral losses of 44 Da, 62 (= 44+18) Da, and 72 (= 44+28) Da, respectively. Since the elemental difference between F1108 and F1064 is +C+2O, and between F1108 and F1036 is +2C+3O, the ions at *m/z* 1065.2, 1047.2, and 1037.2 are consistent with losses of CO_2, CO_2 + H_2O, and CO_2 + CO, respectively. The loss of CO_2 has been shown to be a typical feature in the product ion spectra of pyruvic acid derived compounds [31, 32]. Based on this and the limited number of structural possibilities for addition of two carbon and three oxygen atoms to F1036, we propose that F1108 has a glyoxylic N-terminus: HO-CO-CO-Leu-, as shown in Scheme **1**. Similar to this N-terminal structure, a pyruvylated N-terminus (CH3-CO-CO~) was observed on a cleaved cystine in an antibody following degradation at an elevated-temperature [27]. Cleavage products with N-termini containing adjacent carbonyls have also been observed at other amino acid residues during oxidative cleavage of proteins [28–30]. This indicates that the formation of adjacent carbonyls is not cysteine specific; rather, it probably is a common degradation pathway for protein backbone cleavage.



Scheme 1. Local bond structure of the cysteine residue in the model peptide, P16, and the proposed amino and carboxy terminal structures of the six cleavage products.

pH Dependence and Yield

The yields of the six peptide products and P16 are pH-dependent as shown in Figure 4, which illustrates the ion counts for each species at different pH conditions. The error bars depicting standard deviations were calculated from three replicate injections from the same incubation conditions. As shown in Figure 4, P16 has a decreasing stability as pH increases from pH 2-10, with a minimum at pH 8. All six N-terminal and C-terminal peptide products show an increasing yield in this pH range with a maximum at pH 8. The decrease in the amount of P16 observed and the corresponding increase in the yields of all identified peptide products at pH 8 indicate: (1) the initial cleavage reaches its maximum at pH 8; (2) all peptides are direct products of the cleavage reaction, or subsequent reactions for their formation is not sensitive to pH changes in the range from pH 6 to 10, such that their yields are determined mainly by the initial cleavage step. Based on the complexity of the reaction products, it is unlikely that a single cleavage reaction generates the multiple peptides observed. It should also be noted that the dependence of peptide product yields on pH can be significantly different for other proteins and peptides (data not shown).

For most proteins and peptides, a few percent of the total population are cleaved if they are treated with 1–10 mM TCEP at 37 °C overnight (data not shown). The reaction also happens at lower temperatures with a slower reaction rate. It was observed that in the presence of 2 mM TCEP, about 40% of a protein was cleaved after being stored at 4 °C for a period of 2 wk (data not shown). Based on our preliminary data, the major peptide products from

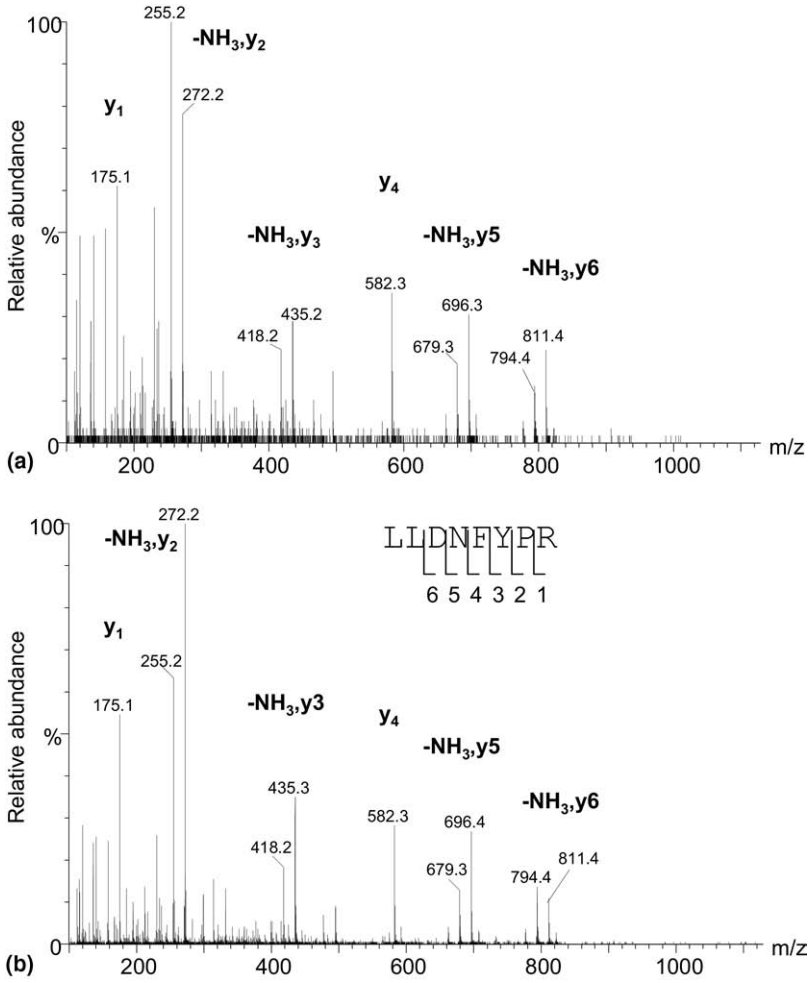


Figure 2. Product ion spectra of doubly charged F1064 (**a**) and F1108 (**b**) indicating the two peptides originate from the C-terminal side of P16 after the cysteine residue was cleaved. The last six residues appeared to be intact.

the amino side of P16 contain an amidated or a formamidated C-terminus. The major carboxy side peptide products contain a primary amine or a glyoxylic N-terminus.

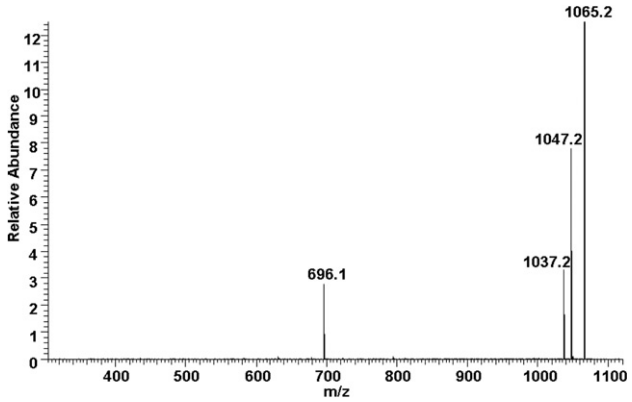


Figure 3. Product ion spectrum of protonated F1108 indicating structural similarities with F1064 (*m/z* 1065.2), and F1036 (*m/z* 1037.2). The neutral leaving groups of 44 Da and 28 Da are consistent with CO_2 and CO, respectively.

Reaction Mechanism

The pathways for this TCEP side reaction are not completely elucidated at the moment. Based on the complexity of the products, several steps may have been involved in the reactions that resulted in the cysteine residue being fractured in multiple ways giving rise to peptide products with various termini. The N- and C-terminal peptides, F619 and F1036, with unmodified residues result from the peptide bond hydrolysis step in which these two peptides are separated from the neighboring cysteine or its fractured moieties. Since TCEP is not known as a proteolytic agent, it is unlikely that it can cleave the two peptide bonds directly. The cleavage most likely occurs after the cysteine residue has been attacked and fractured in the presence of TCEP. Since amidated peptides have also been observed in oxidized proteins [28, 29], and on antibodies degraded at elevated temperatures [27], it appears to be a product from one of the major degradation pathways of the cysteine residue in an activated state. For this side reaction, the amidated peptide could result from β-elimination, after the sulfur atom in

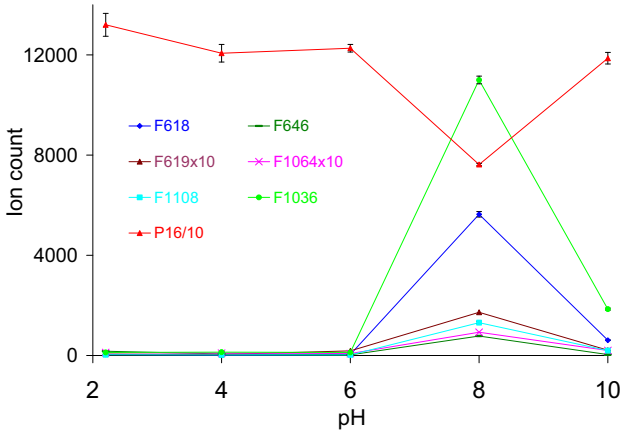


Figure 4. pH dependence of the model peptide, P16, and the yields of six identified cleavage products based on ion count. To facilitate comparison, the ion count of P16 is reduced by a factor of 10, the ion counts of F619 and F1064 are increased by a factor of 10, in the figure. The error bars depict standard deviations calculated from three injections from the same incubation conditions.

cysteine is extracted from the side-chain. Assuming that the extra carbon atom at the C-terminus is from the fractured cysteine, the formation of F646 involves breaking two C–C bonds, which must overcome a very large energy barrier. Another large energy barrier is involved in the formation of F1108 when the C–C bond between the α carbon and its side-chain carbon is broken. In general, C–C bond breaking appears to be a unique feature in this TCEP side reaction. This cleavage is unusual, since carbon–carbon bonds are relatively strong with bond energies of about 350 kJ/mol. Under mild conditions, it has been known that carbon–carbon bonds can be cleaved by the molecular oxygen [33], a radical formation [34], the rhodium metal ion [35], and some enzymes [36–42]. This unique feature of C–C bond cleavage is not observed in other cysteine related protein fragmentation pathways. Although this TCEP side reaction shares some similarities with cleavages caused by oxidation and elevated-temperatures, most reaction products of this side reaction are different from other reaction pathways. This indicates that TCEP does not function as a catalyst for protein cleavage; rather, it reacts with and fractures the cysteine residue differently from other known pathways.

The current study was performed on a synthetic peptide containing a cysteine residue. Conceptually, the same TCEP-related cleavage reaction could occur with proteins/peptides containing disulfide bonds. The pH dependence of the reaction rate may be different for different proteins and peptides, but our data indicate that the major N-terminal products contain formamidated and amidated carboxy termini, with a mass difference of 28 Da. When this mass difference is observed for protein cleavage products in the presence of TCEP, there is a strong indication that TCEP related cleavage may have taken place. Since the purity of TCEP is not 100%, the possibility of the side reaction being caused by some unknown contaminants in TCEP can not be completely ruled out until its mechanism is well determined. However, since TCEP from different vendors and from different forms (crystal versus solubilized solution) did not show an obvious difference in reactivity, that possibility is quite low.

Conclusion

The data presented in this study show that cysteine-containing proteins are cleaved in the presence of TCEP under mild conditions. Several reaction steps or pathways may exist when cysteine residues are cleaved, generating peptides with various termini. These results suggest that care should be taken when proteins are treated with TCEP, especially for an extended period of time, to prevent extensive protein degradation. The structures of two peptide products indicate that some carbon–carbon bonds are broken when cysteine residues are cleaved in the presence of TCEP. This is unexpected under mild conditions and is not observed in other cysteine related cleavages. More studies are needed before the mechanism for this TCEP side reaction on cysteine-containing proteins can be determined.

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References

1. Wood, J. L.; Catsimpoolas, N. Cleavage of the Peptide Bond at the Cystine Amino Group by the Action of Cyanide. *J. Biol. Chem.* **1963,** *238,* 2887–2888.
2. Catsimpoolas, N.; Wood, J. L. Specific Cleavage of Cystine Peptides by Cyanide. *J. Biol. Chem.* **1966,** *241,* 1790–1796.
3. Jacobson, G. R.; Scheffer, M. H.; Stark, G. R.; Vanaman, T. C. Specific Chemical Cleavage in High Yield at the Amino Peptide Bonds of Cysteine and Cystine Residues. *J. Biol. Chem.* **1973,** *248,* 6583–6591.
4. Degani, Y.; Patchornik, A. Cyanylation of Sulfhydryl Groups by 2-Nitro-5-Thiocyanobenzoic Acid. High-Yield Modification and Cleavage of Peptides at Cysteine Residues. *Biochemistry* **1974,** *13,* 1–11.
5. Stark, G. R. Cleavage at Cysteine after Cyanylation. *Methods Enzymol.* **1977,** *47,* 129–132.
6. Lu, H. S.; Gracy, R. W. Specific Cleavage of Glucosephosphate Isomerases at Cysteinyl Residues Using 2-Nitro-5-Thiocyanobenzoic Acid: Analyses of Peptides Eluted from Polyacrylamide Gels and Localization of Active Site Histidyl and Lysyl Residues. *Arch. Biochem. Biophys.* **1981,** *212,* 347–359.
7. Papayannopoulos, I. A.; Biemann, K. Amino Acid Sequence of a Protease Inhibitor Isolated from *Sarcophaga bullata* Determined by Mass Spectrometry. *Protein Sci.* **1992,** *1,* 278–288.
8. Wu, J.; Gage, D. A.; Watson, J. T. A Strategy to Locate Cysteine Residues in Proteins by Specific Chemical Cleavage Followed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Anal. Biochem.* **1996,** *235,* 161–174.
9. Wu, J.; Watson, J. T. A Novel Methodology for Assignment of Disulfide Bond Pairings in Proteins. *Protein Sci.* **1997,** *6,* 391–398.
10. Sutherland, C.; Walsh, M. P. Phosphorylation of Caldesmon Prevents its Interaction with Smooth Muscle Myosin. *J. Biol. Chem.* **1989,** *264,* 578–583.
11. Pedroni, S.; Lecomte, M. C.; Gautero, H.; Dhermy, D. Heterogeneous Phosphorylation of Erythrocyte Spectrin β Chain in Intact Cells. *Biochem. J.* **1993,** *294,* 841–846.
12. Bahler, M.; Benfenati, F.; Valtorta, F.; Czernik, A. J.; Greengard, P. Characterization of Synapsin I Fragments Produced by Cysteine-Specific Cleavage: A Study of Their Interactions with F-actin. *J. Cell. Biol.* **1989,** *108,* 1841–1849.
13. San Agustin, J. T.; Leszyk, J. D.; Nuwaysir, L. M.; Witman, G. B. The Catalytic Subunit of the cAMP-dependent Protein Kinase of Ovine

Sperm Flagella Has a Unique Amino-Terminal Sequence. *J. Biol. Chem.* **1998,** *273,* 24874–24883.
14. Daniel, R.; Caminade, E.; Martel, A.; GoYc, F. L.; Canosa, D.; Carrascal, M.; Abian, J. Mass Spectrometric Determination of the Cleavage Sites in *Escherichia coli* Dihydroorotase Induced by a Cysteine-specific Reagent. *J. Biol. Chem.* **1997,** *272,* 26934–26939.
15. Shin, H. J.; Chong, C. K.; Chang, S. I.; Choi, J. D. Structural and Functional Role of Cysteinyl Residues in Tobacco Acetolactate Synthase. *Biochem. Biophys. Res. Commun.* **2000,** *271,* 801–806.
16. Ruegg, U. T.; Rudinger, J. Reductive Cleavage of Cystine Disulfides with Tributylphosphine. *Methods Enzymol.* **1977,** *47,* 111–126.
17. Kirley, T. L. Reduction and Fluorescent Labeling of Cyst(e)ine-Containing Proteins for Subsequent Structural Analysis. *Anal. Biochem.* **1989,** *180,* 231–236.
18. Burns, J. A.; Butler, J. C.; Moran, J.; Whitesides, G. M. Selective Reduction of Disulfides by Tris-(2-Carboxyethyl)-Phosphine. *J. Org. Chem.* **1991,** *56,* 2648–2650.
19. Han, J. C.; Han, G. Y. A Procedure for Quantitative Determination of Tris-(2-Carboxyethyl)-Phosphine, an Odorless Reducing Agent More Stable and Effective Than Dithiothreitol. *Anal. Biochem.* **1994,** *220,* 5–10.
20. Gray, W. R. Disulfide Structures of Highly Bridged Peptides: A New Strategy for Analysis. *Protein Sci.* **1993,** *2,* 1732–1748.
21. Fischer, W. H.; Rivier, J. E.; Craig, A. G. In Situ Reduction Suitable for Matrix Assisted Laser Desorption/Ionization and Liquid Secondary Ionization Mass Spectrometry Using Tris-(2-Carboxyethyl)-Phosphine. *Rapid Commun. Mass Spectrom.* **1993,** *7,* 225–228.
22. Tam, J. P.; Lu, Y. A.; Liu, C. F.; Shao, J. Peptide Synthesis Using Unprotected Peptides through Orthogonal Coupling Methods. *Proc. Natl. Acad. Sci. U.S.A.* **1995,** *92,* 12485–12489.
23. Wang, Z.; Rejtar, T.; Zhou, Z. S.; Karger, B. L. Desulfurization of Cysteine-Containing Peptides Resulting from Sample Preparation for Protein Characterization by Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2010,** *24,* 267–275.
24. Back, J. W.; David, O.; Kramer, G.; Masson, G.; Kasper, P. T.; de Koning, L. J.; de Jong, L.; van Maarseveen, J. H.; de Koster, C. G. Mild and Chemoselective Peptide-Bond Cleavage of Peptides and Proteins at Azido Homoalanine. *Angew. Chem. Int. Ed.* **2005,** *44,* 7946–7950.
25. Martin, R. B. Free Energies and Equilibria of Peptide Bond Hydrolysis and Formation. *Biopolymers* **1998,** *45,* 351–353.
26. Roepstorff, P.; Fohlman, J. Proposal for a Common Nomenclature for Sequence Ions in Mass Spectra of Peptides. *Biomed. Mass Spectrom.* **1984,** *11,* 601.
27. Cohen, S. L.; Price, C.; Vlasak, J. β-Elimination and Peptide Bond Hydrolysis: Two Distinct Mechanisms of Human IgG1 Hinge Fragmentation Upon Storage. *J. Am. Chem. Soc.* **2007,** *129,* 6976–6977.
28. Garrison, W. M. Reaction Mechanisms in the Radiolysis of Peptides, Polypeptides, and Proteins. *Chem. Rev.* **1987,** *87,* 381–398.
29. Stadtman, E. R.; Levine, R. L. Free Radical-Mediated Oxidation of Free Amino Acids and Amino Acid Residues in Proteins. *Amino Acids* **2003,** *25,* 207–218.
30. Dean, R. T.; Fu, S.; Stocker, R.; Davies, M. J. Biochemistry and Pathology of Radical-Mediated Protein Oxidation. *Biochem. J.* **1997,** *324,* 1–18.
31. Chen, G.; Liu, Y. H.; Pramanik, B. N. LC/MS Analysis of Proteins and Peptides in Drug Discovery. In *HPLC for Pharmaceutical Scientists*; Kazakevich, Y. Ed.; John Wiley and Sons, Inc.: Hoboken, NJ, 2007; pp 837–899.
32. Prome, D.; Blouquit, Y.; Ponthus, C.; Prome, J. C.; Rosa, J. Structure of the Human Adult Hemoglobin Minor Fraction A_1b by Electrospray and Secondary Ion Mass Spectrometry. Pyruvic Acid as Amino-Terminal Blocking Group. *J. Biol. Chem.* **1991,** *266,* 13050–13054.
33. Norris, T.; Santafianos, D.; Bordner, J. An Unusual Carbon–Carbon Bond Scission Reaction with Molecular Oxygen under Mild Conditions: Formation of Piperidines from 1-Azabicyclo[2.2.2]Octanes. *J. Chem. Soc. Perkin Trans.* **1997,** *1,* 3679–3684.
34. Barondeau, D. P.; Kassmann, C. J.; Tainer, J. A.; Getzoff, E. D. The Case of the Missing Ring: Radical Cleavage of a Carbon–Carbon Bond and Implications for GFP Chromophore Biosynthesis. *J. Am. Chem. Soc.* **2007,** *129,* 3118–3126.
35. Gandelman, M.; Vigalok, A.; Konstantinovski, L.; Milstein, D. Discovery of the First Metallaquinone. *J. Am. Chem. Soc.* **2000,** *122,* 8797–8798.
36. Myers, R. W.; Wray, J. W.; Fish, S.; Abeles, R. H. Purification and Characterization of an Enzyme Involved in Oxidative Carbon–Carbon Bond Cleavage Reactions in the Methionine Salvage Pathway of Klebsiella Pneumoniae. *J. Biol. Chem.* **1993,** *268,* 24785–24791.
37. Shyadehi, A. Z.; Lamb, D. C.; Kelly, S. L.; Kelly, D. E.; Schunck, W. H.; Wright, J. N.; Corina, D.; Akhtar, M. The Mechanism of the Acyl–Carbon Bond Cleavage Reaction Catalyzed by Recombinant Sterol 14α-Demethylase of *Candida albicans* (Other Names are: Lanosterol 14α-Demethylase, P-450_{14DM}, and CYP51). *J. Biol. Chem.* **1996,** *271,* 12445–12450.
38. Foulon, V.; Antonenkov, V. D.; Croes, K.; Waelkens, E.; Mannaerts, G. P.; Van Veldhoven, P. P.; Casteels, M. Purification, Molecular Cloning, and Expression of 2-Hydroxyphytanoyl-CoA Lyase, a Peroxisomal Thiamine Pyrophosphate-Dependent Enzyme that Catalyzes the Carbon–Carbon Bond Cleavage During α-Oxidation of 3-Methyl-Branched Fatty Acids. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** *96,* 10039–10044.
39. Tuynman, A.; Spelberg, J. L.; Kooter, I. M.; Schoemaker, H. E.; Wever, R. Enantioselective Epoxidation and Carbon–Carbon Bond Cleavage Catalyzed by *Coprinus cinereus* Peroxidase and Myeloperoxidase. *J. Biol. Chem.* **2000,** *275,* 3025–3030.
40. Cryle, M. J.; De Voss, J. J. Carbon–Carbon Bond Cleavage by Cytochrome P450BioI (CYP107H1). *Chem. Commun.* **2004, (**1), 86–87.
41. Müller, M.; Gocke, D.; Pohl, M. Thiamin Diphosphate in Biological Chemistry: Exploitation of Diverse Thiamin Diphosphate-Dependent Enzymes for Asymmetric Chemoenzymatic Synthesis. *FEBS J.* **2009,** *276,* 2894–2904.
42. Cicchillo, R. M.; Zhang, H.; Blodgett, J. A. V.; Whitteck, J. T.; Li, G.; Nair, S. K.; van der Donk, W. A.; Metcalf, W. W. An Unusual Carbon–Carbon Bond Cleavage Reaction during Phosphinothricin Biosynthesis. *Nature* **2009,** *459,* 871–874.