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FORUM REVIEW ARTICLE

Trisulfides in Proteins

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Abstract

Trisulfides and other oligosulfides are widely distributed in the biological world. In plants, for example, garlic, trisulfides are associated with potentially beneficial properties. However, an extra neutral sulfur atom covalently bound between the two sulfur atoms of a pair of cysteines is not a common post-translational modification, and the number of proteins in which a trisulfide has been unambiguously identified is small. Nevertheless, we believe that its prevalence may be underestimated, particularly with the increasing evidence for significant pools of sulfides in living tissues and their possible roles in cellular metabolism. This review focuses on examples of proteins that are known to contain a trisulfide bridge, and gives an overview of the chemistry of trisulfide formation, and the methods by which it is detected in proteins. *Antioxid. Redox Signal.* 15, 67–75.

Introduction

In proteins, trisulfides are variants of disulfides that contain an extra sulfur atom, creating an S-S-S bridge between two cysteine residues. This is a fairly rare post-translational modification that has been characterized in fewer than 10 proteins to date. Chemically, however, the formation of such a structure is easily rationalized. Polysulfide H_2S_n molecules are generated from hydrogen sulfide (H_2S or HS^-, depending on pH), for example in the presence of molecular oxygen (Fig. 1) (33, 39). Polysulfides of the general nature $R_1S_nR_2$, with varying numbers of covalently linked sulfur atoms, are found in numerous natural compounds. The sulfur atoms that are not bonded to heteroatoms are in the 0 (null) state, such as elemental sulfur. Naturally occuring polysulfides have been known for years, for example in garlic (24, 42), where diallyl trisulfide (Fig. 2A) has been intensively studied as a possible treatment for cancer and cardiovascular disease (4, 24, 57). Dimethyl trisulfide (Fig. 2B) is found with other sulfide-containing species in the breath of people with oral malodor (62), and in fungally-infected cancer wounds (55). Shiitake mushrooms, bitter beans, and the red algae *Condria californica* contain the antifungal and antibacterial cyclic pentasulfide, lenthionine (Fig. 2C) (26, 68).

Peptide-like compounds have also been shown to contain polysulfide forms [*e.g.,* glutathione trisulfide (GSSSG)], which was identified as an impurity in commercial glutathione disulfide (GSSG) (37). Trisulfide cystine was found as a degradation product in acid hydrolysates of wool (18).

Protein trisulfides

Trisulfide in proteins, as illustrated in Figure 3, were first reported in 1959 by Kun and Fanshier. They proposed that the enzymatic trans-sulfuration of *β*-mercaptopyruvate involved an enzyme intermediate with an extra sulfur atom in its disulfide bond, which was thought to be produced together with pyruvate (34). This interesting reaction was not studied further, and the proposed trisulfide enzyme was not isolated. Also in 1959, Hylin and Wood observed a polysulfide enzyme in the mercaptopyruvate trans-sulfuration reaction (23), but did not characterize the enzyme product. By denaturing spinach ferredoxin in presence of urea and oxygen, Petering *et al.* discovered a dimer containing a trisulfide bond (48).

In 1975, Calabrese *et al.* showed that the unusual absorption of superoxide dismutase (SOD) (Fig. 4A) at 320 nm was due to an extra sulfur molecule, which was concluded to originate from perthiol in the enzyme (9). Perthiols are thiols (RSH) with an extra sulfur atom (RSSH), which are intermediates in trisulfide formation. Perthiol formation in SOD was later proposed to occur during purification of SOD (8) in the laboratory. But it could not be ruled out that the proposed extra sulfur actually was in the form of a mixed tri- and polysulfide between the enzyme and an other thiol (8).

After these early observations, no reports of trisulfide protein were published until 1994, when Jespersen *et al.* reported a derivative of human growth hormone (hGH) with an extra sulfur atom covalently bound in a disulfide-bond (30) (Fig. 4B). Shortly thereafter, Parmentier *et al.* reported formation of a trisulfide byproduct during chemical synthesis of a disulfide-containing peptide (46). Interestingly, these

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$$2nH_2S + ½(2n-1)O_2 \rightarrow H_2S_{2n} + (2n-1)H_2O$$

FIG. 1. Polysulfide formation. Oxidation of sulfide leads to polysulfide formation, as illustrated by the general expression for linear chains.

studies also identified tetra- and pentasulfides that were generated under basic conditions by reaction of the disulfide-bonded peptide with elemental sulfur. Andersson *et al.* also reported trisulfide in the same hGH disulfide bond (1) as Jespersen *et al.*, but in hGH from a different source. These reports both placed the extra sulfur in the disulfide bond Cys182 to Cys189. The observation by Canova–Davis *et al.* of a trisulfide between Cys53 and Cys165 in hGH from a third source (10) (Fig. 4B) is interesting, but remains unconfirmed.

In addition to trisulfide-hGH, a trisulfide was found in Δ22-IL-6, a mutant variant of interleukin-6 in which the N-terminal 22 amino acids are absent, and Cys23 and Cys29, which are nonessential for activity, are substituted with serine (7) (Fig. 4C); and in salmon calcitonin (sCT) (67) (Fig. 4D). A trisulfide-containing vasopressin derivative was created by synthetic chemistry for the purpose of investigating receptor affinity and structure (41). Except for further studies on disulfide-hGH (58, 60) and a patent on how to minimize trisulfide formation in biopharmaceutical production of hGH (56), nothing was reported on trisulfide proteins for the next 10 years.

The subject was reopened when De Beus *et al.* reported that the long-standing mystery of an anomalous absorbance at 320–325 nm in superoxide dismutase (SOD) (Fig. 4A) (8, 9, 11, 38) was due to modifications in the Cys111–Cys111 bond of dimeric SOD (14). This led to a publication on trisulfide-SOD containing further characterization, ways to avoid formation of the trisulfide, and its possible existence *in vivo* (45). The full potential of polysulfide formation in SOD seems to be more extreme than in other proteins, as a heptasulfide bond between the two Cys111 in the SOD dimer has been reported (69).

Recently, a trisulfide-variant of human immunoglobulin-2 monoclonal antibody (mAb) was reported (49) (Fig. 4E), and trisulfide bonds have been found in multiple mAb subgroups (20). All structures in Figure 4 are discussed in detail below.

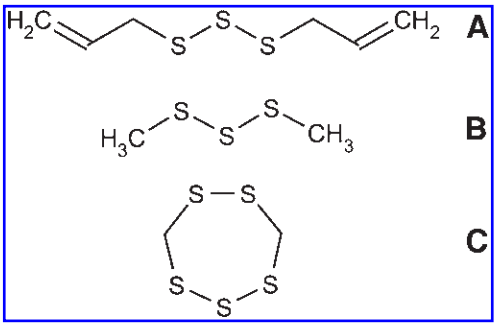

FIG. 2. Examples of natural polysulfides. (A) Diallyl trisulfide which has disulfide and tetrasulfide analogs, found in onions and plants of the *Allium* genus (28). **(B)** Dimethyl trisulfide found in oral malodor (62) and fungally infected cancer wounds (55). **(C)** Lenthionine, a cyclic trisulfide component of shiitake mushrooms, bitter beans, *Parkia speciosa* (26) and the red alga *Chondria californica* (68). Structures were drawn using the software Marvin 5.2, 2009, ChemAxon (http://www.chemaxon.com; last accessed Sept. 6, 2010).

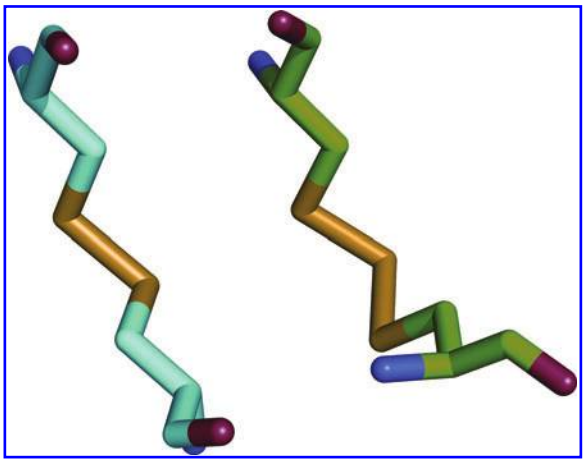

FIG. 3. Comparison of disulfide and trisulfide bonds. A sulfur atom is covalently bound between the two sulfides in a cystine bond to make a trisulfide bond. Bond angles and lengths have been generated using Marvin 5.2, ChemAxon (http://www.chemaxon.com). Figures 3 and 4 have been rendered using PyMOL v. 0.99; copyright by DeLano Scientific LLC. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Despite the growing number of reported trisulfide proteins, the mechanisms and general chemistry of trisulfide formation are not well characterized. In particular, not much attention has been paid to the kinetics and thermodynamics of trisulfide formation and within the area of protein redox chemistry, tri- and polysulfides have, until recently, been considered merely a curiosity. The trisulfide proteins characterized to date have been found *in vitro*, in purified proteins, so the trisulfides may have been introduced during purification. However, recent proteomics screens for characterization of a broad range of post-translational modifications, suggest that trisulfides may be present as genuine post-translational modifications *in vivo* (Michael L. Nielsen, personal communication).

This review will focus on possible biochemical reactions leading to trisulfide proteins, as well as provide an overview of reported trisulfide proteins, and their biochemical and physiological implications.

Trisulfide Chemistry

Chemical synthesis of trisulfide-containing peptides has been studied by several groups (13, 16, 35, 40) Cysteine-containing peptides are reacted with either elemental sulfur (S^0) (40) or organic compounds containing two or more sulfur atoms of the RS_nR form, where S is attacked by thiol of a cysteine and through different reaction mechanisms is incorporated in the peptide (13, 16, 35). Normally chemical synthesis of trisulfide peptides is carried out by the nucleophilic attack from cysteine sulfur, which means that the peptides have to be reduced prior to the synthesis, which makes the chemistry completely different from that described below.

Organic synthesis of trisulfide bonds in peptides have yielded the amount and trisulfide peptide form needed for nuclear magnetic resonance (NMR), or spectroscopy studies of trisulfide peptides versus native peptides. It has also allowed controlled chemical reactions in which reactants and products could easily be detected, providing valuable information about the reaction chemistry. Nonetheless, the chemical synthesis

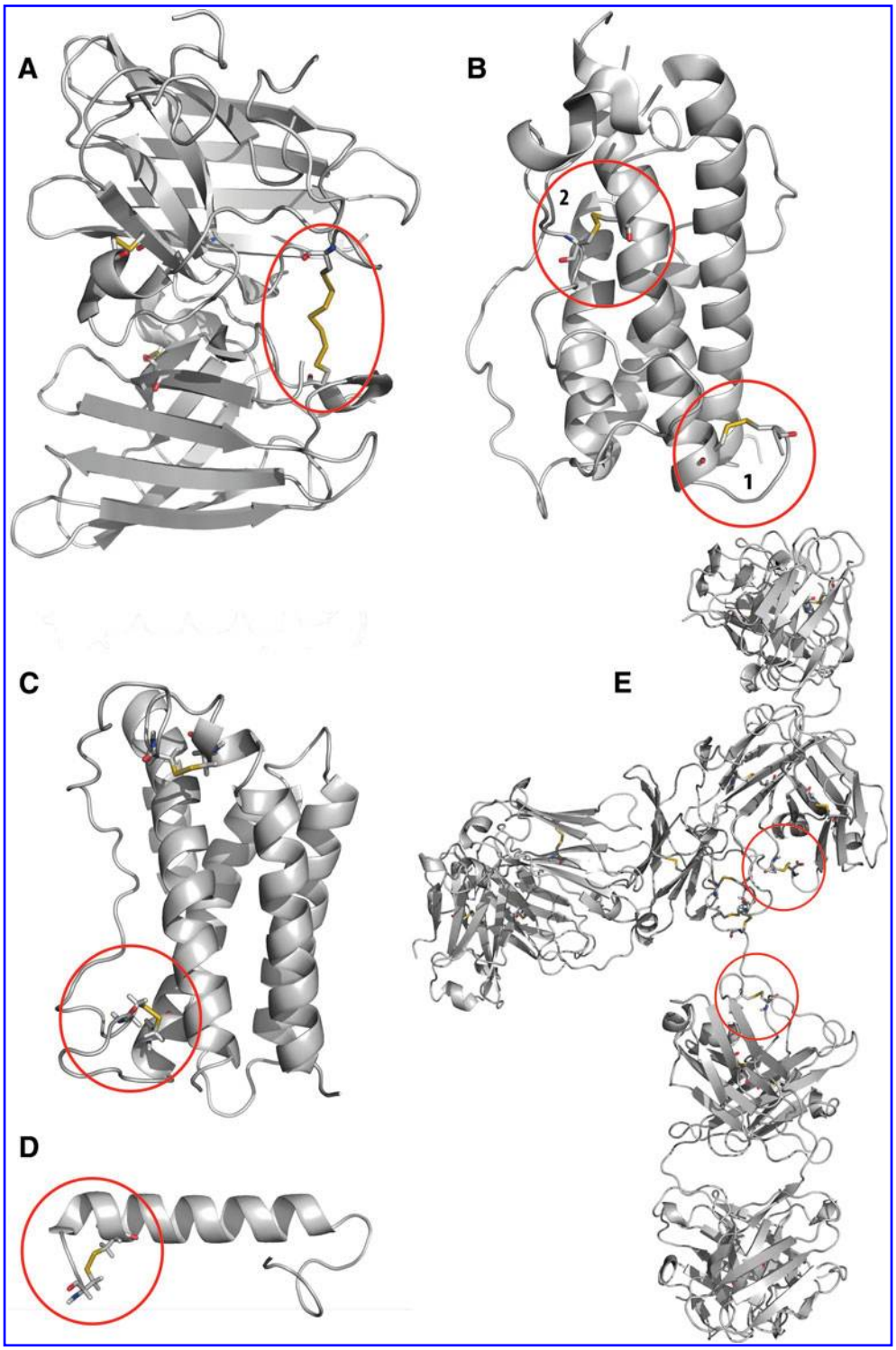

FIG. 4. Location of trisulfides in proteins. Structures of proteins with reported trisulfides or polysulfides. Note that only the structure of the heptasulfide in SOD has been solved. The other proteins that are illustrated here are in disulfide forms but the location of the putative trisulfide is marked with *red circles*. Reported location of trisulfides (*red ring*), **(A)** SOD1 (pdb id: 3K91); **(B)** hGH (pdb id: 1HGU); **(C)** wild-type IL-6 (pdb id: 1IL6); **(D)** sCT (pdb id: 1GLH); **(E)** IgG2 (pdb id: 1IGT). Numbers next to cystines on hGH refer to trisulfide locations reported by 1: Jespersen *et al*. (30) and Andersson *et al*. (1) or 2: Canova–Davis *et al*. (10). The IL-6 molecule shown might differ structurally from the Δ22-IL-6 in which the trisulfide was reported (7), but the structure of Δ22-IL-6 has not been solved. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

mechanism may not reflect the biochemistry that takes place *in vivo* or during biopharmaceutical production.

Putative reactions leading to protein trisulfide

No reaction mechanism for trisulfide formation in proteins, *in vivo* or *in vitro*, has been described. By adding sodium sulfide to different disulfide-containing peptides and proteins, Cavallini *et al.* (12) were able to detect perthiol formation in the peptides and proteins. The same method was used by Rao and Gorin, and by Purdie *et al.* to form trisulfide-cystine, trisulfide-homocystine, and trisulfide-cystamine (50, 51). Gu *et al.* showed that treating a disulfide-bonded protein with H_2S forms trisulfide proteins (20), and Jespersen *et al*. discuss the possibility that this led to the trisulfide they observed in hGH (30).

These reports all assume thiol–disulfide exchange reactions, in which sulfide is the nucleophilic component. Because thiol–disulfide exchange reactions already occur within the cell (29), we propose that trisulfide formation *in vivo* or in biopharmaceutical production *in vitro* may take place in similar ways (RWN and JRW, unpublished observations).

Trisulfide formation would require breaking a C-S-bond if the thiol–disulfide exchange reaction is carried out in the absence of sulfide. However, the breaking of thiol S-C bonds, which occurs in the endogenous production of sulfide (31), requires enzymatic catalysis by carbon–sulfur lyases. We propose the noncatalyzed reaction mechanism shown in Figure 5. A nucleophilic attack of the sulfide ion HS^- on a disulfide bond (Fig. 5, 1) creates a mixed perthiol and thiol intermediate (Fig. 5, 2). Since the perthiol of the protein (Protein-SSH) is likely to be significantly more reducing than the thiol group of the protein (Protein-SH) (27), the protein perthiol could carry out a nucleophilic attack on an oxidant (*e.g*. GSSG; Fig. 5, 3). The resulting intermediate links the protein to the thiol substituent through a trisulfide bond, with the other cysteine group forming a thiolate (Fig. 5, 4).

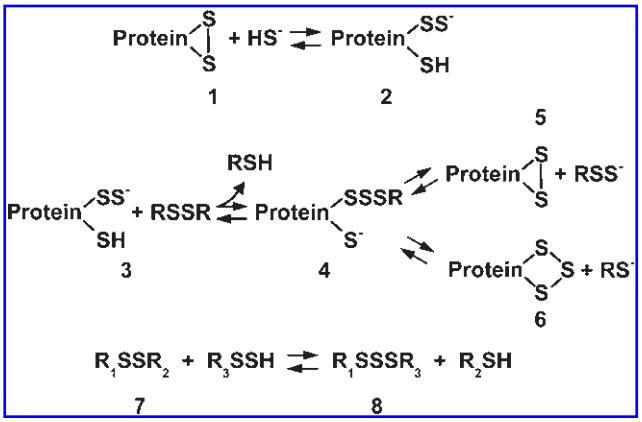

FIG. 5. Proposed trisulfide formation reaction. We suggest that trisulfide formation takes place in a reaction similar to a thiol–disulfide exchange, with the thiol exchanged for a sulfide (HS^-). The numbers from **1** to **8** refer to different major species. The numbers from **1** to **6** refer to the formation of protein trisulfides from H_2S. The numbers **7** and **8** refer to the reaction of small molecular perthiols on a disulfide, which actually is the reverse reaction going from **5** through **4** to **6**. Note that RSSH species, in analogy to peroxides, are termed persulfides.

Next, in an intermolecular reaction, the thiolate attacks the trisulfide bond (Fig. 5, 4). This will result in the reformation of the protein disulfide bond and a perthiol (RSSH) (Fig. 5, 5), or formation of a protein trisulfide bond and a thiol (RSH) (Fig. 5, 6).

This reaction is dependent on many factors, in particular, the presence of SH^-, and a pH at or above neutral. Some evidence indicates a reaction of this kind is responsible for at least some of the trisulfide formation that occurs in biopharmaceutical production (3, 22). Moreover, H_2S has long been known to form perthiols with protein disulfide, while reduced protein does not result in perthiols (12). Thus, the formation of trisulfide requires a reduction-oxidation cycle through a perthiol intermediate that requires fairly controlled redox conditions. Too oxidizing conditions will not allow the presence of H_2S; too reducing conditions will not allow perthiol formation (RWN and JRW, unpublished observations).

As seen in Figure 5, formation of trisulfide from H_2S requires oxidation. Trisulfide formation in a eukaryotic cell may therefore be favored in compartments where redox conditions are somewhat oxidizing, like the endoplasmic reticulum or mitochondria (52). On the other hand, trisulfide formation may be favorable in a protein even under reducing conditions, if the trisulfide is conformationally stabilized by the protein structure, as is seen for cytosolic disulfides (29). In any case, considerable H_2S flux and perthiol storage has been observed in cells (54), indicating that this prerequisite for trisulfide formation exists *in vivo*. A study of sulfide in mammalian tissues showed that bound sulfide increases in tissues after previous H_2S spiking (25). This shows that in the presence of high sulfide concentrations, mammalian tissues incorporate sulfide in a manner that requires reduction to liberate it. This suggests incorporation as trisulfide or perthiols. Several studies have concluded that H_2S most likely arises from perthiols or trisulfide, and H_2S is produced upon reduction of xanthine oxidase (36) and aldehyde oxidase (6). In addition, a trisulfide intermediate exists in the cystathionase–rhodanese complex after trans-sulfuration of cystine (59), and in the dimeric product of urea-denatured ferredoxin (48).

Of course the formation of trisulfide, as proposed in Figure 5, is not necessarily dependent on the HS^- concentration in the system, since the reaction of going from reactant 4 to product 5 in Figure 5 opens up a reverse reaction where persulfide containing peptides (*e.g*., glutathione perthiol or small molecule perthiols) would play as the sulfur donor to create the trisulfide product in Figure 5. Small molecule persulfides are known to interact with proteins (*e.g.*, in the case of "greening" when Coenzyme A persulfide binds to acyl-coenzyme A dehydrogenase) (66). In this reaction, coenzyme A reacts with a sulfur donor and forms coenzyme A persulfide. Upon binding of coenzyme A persulfide to coenzyme A dehydrogenase, a green product is formed due to the extra sulfur molecule in the bridging ligand (66). A reaction type as the above mentioned could form a mixed trisulfide product which upon attack from a thiol could result in protein trisulfide formation as illustrated by reactants 7 and products 8 in Figure 5.

That there are other possible pathways for tri- or polysulfides formation than the one illustrated in Figure 5 is clear from the SOD example. Hence, reaction with the SOD dimer and elemental sulfur has been seen *in vitro* (69). In this reaction, the thiol at C111 carries out the nucleophilic attack on the sulfur donor and creates a polysulfide leaving group. While

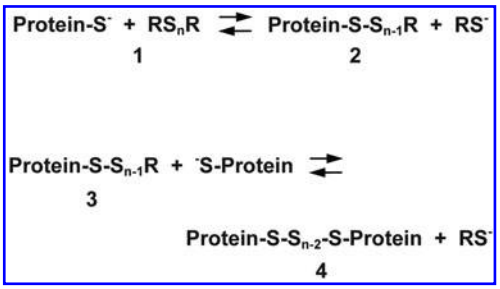

FIG. 6. Proposed polysulfide formation reaction in dimeric proteins. The formation of a polysulfide bond between two natively free cysteines in a SOD dimer may occur through a reaction where the free cysteines of the SOD monomer molecules make a nucleophilic attack on a small molecule polysulfide component **(1)**. A mixed polysulfide between the protein and the polysulfide component is created **(2)**. Another free protein cysteine then attacks the mixed polysulfide **(3)** whereby a polysulfide protein dimer is formed together with a thiol leaving group **(4)**.

elemental sulfur is unlikely to be found to any significant level in most living cells, it nevertheless shows that other polysulfides can act as neutral sulfur donors of formation of tri or polysulfides, as shown in Figure 6. This reaction also explains why treating SOD with sulfur-containing compounds only resulted in the perthiol SOD form if polysulfide compounds acted as sulfur donors (8).

Kinetic and thermodynamic considerations of trisulfide formation

In perthiols (RS_nH), the pK_a is decreased relative to normal thiol (17, 63), which makes perthiol a better leaving group than a thiol. This kinetically favors the disulfide-product in Figure 5.5, instead of the trisulfide product in Figure 5.6. As the kinetics probably favor disulfide formation over trisulfide formation, the trisulfide form must be more thermodynamically stabile than the disulfide form in proteins where trisulfide forms are found. A trisulfide could be favored over a disulfide in structures where an extra sulfur fills a void and generates at structure that is overall thermodynamically stabilized.

Ultimately, the position of the disulfide/trisulfide equilibrium depends to a large degree on the specific structural properties of the surroundings and the chemical environment (*e.g.,* pH of the solution will affect the leaving group properties of the perthiol).

Trisulfide formation in heterologously produced protein biopharmaceuticals

The nature of the reaction shown in Figure 5 prevents the formation of trisulfide under very oxidizing or very reducing conditions, except at pH extremes. This may explain, at least in part, why trisulfides, for the most, have been seen in recombinant proteins produced in *Escherichia coli* as the host organism. In the *E. coli* cytosol, disulfide-containing proteins are maintained in the reduced state. When the cells are disrupted, the recombinant proteins are released, together with all the other cell components, into an environment where thiols are oxidized. At the same time, degradation of cysteine results in release of sulfide, which can be detected by its odor quickly after disruption of an *E. coli* culture. This combination of oxidation and release of sulfide produces ideal conditions for trisulfide formation. That proteins for pharmaceutical purposes are normally subjected to much more stringent analysis than proteins used for scientific or technical purposes may be the reason that trisulfide formation, particularly in these proteins, has more often been noticed.

Trisulfide formation is difficult to control during fermentation, as shown by Gu *et al.*, who found that varying fermentation operating parameters gave tremendous variations in trisulfides (20). This is consistent with observations for hGH production, as reported by Jespersen *et al.*, who found that trisulfide in hGH vary from 5%–15% (30) during highly controlled pharmaceutical production. When trisulfide formation was forced by adding H_2S to mAb solutions, trisulfide bonds were found in up to 39% of the molecules in some mAb subtypes (20).

Monitoring of H_2S concentration and trisulfide formation during fermentation has, to the best of our knowledge, not yet been reported, but could provide information on whether changes in trisulfide formation reflect H_2S changes in fermentation broths. *In vitro* trisulfide formation is interesting and challenging for the protein-producing industry, because of the questions it raises about bioavailability and general function. However, the most exciting aspect of trisulfide proteins is the possibility that they exist and function *in vivo*.

The challenges to identifying trisulfide proteins are numerous. The molecular mass of the single sulfur atom may easily be mistaken for two oxygen atoms. In addition, extracting them from cells without reducing the trisulfide bonds is difficult. In addition, if the structure is not grossly altered, the overall chemistry of the protein is only minimally changed by the addition of the extra sulfur atom, and judging from the known trisulfide proteins, they are usually hard to detect with ordinary ion-exchange, C18 reverse-phase, or size-exclusion chromatography. With the exception of trisulfide-SOD, they also do not show any clear spectral changes. If the researcher is looking for post-translational modifications, the minor deviations in a spectrum or chromatogram might easily be mistaken for sample impurities. Even if these challenges are overcome, discovery of an *in vivo* trisulfide protein will still raise questions about whether it is an artifact generated during sample handling, since a disulfide plus an H_2S form perthiol, and an oxidation reaction might be sufficient to create a trisulfide protein. These may be some of the reasons that trisulfide proteins have not yet been identified *in vivo*, and why almost all descriptions of trisulfide proteins are from pharmaceutical production, where intense analysis is conducted on any deviation from the native protein. This bias consequently leads to failure to observe findings like trisulfide bonds, if the research is not specially aimed towards detecting post translational modifications.

Identification of Trisulfides in Proteins

None of the described trisulfide protein derivatives can be clearly differentiated from the native form by their spectral patterns, with the exception of the unusual absorption band at 325 nm that is seen for trisulfide-SOD, but not native SOD (8, 9, 14, 45). All other trisulfide proteins described to date

were distinguished from their native forms by various chromatographic techniques, where they showed small differences.

The trisulfide-sCT derivative was observed in a study in which native sCT was placed at 60°C and pH 5–6 for several days, leading to extensive degradation of native sCT. By reverse-phase HPLC, the trisulfide-sCT form was well separated from the native form (67). Trisulfide–hGH is somewhat more hydrophobic than the native form, which makes hydrophobic interaction chromatography (HIC) an effective method for detecting this trisulfide variant (1, 10, 30, 47). A slight increase in hydrophobic character also seems to be a feature of trisulfide–vasopressin (41). In IL-6, a trisulfide bond was found in a mutant version of IL-6, Δ22-IL-6, which was generated as a therapeutic candidate (7). The mutant lacks 22 amino acids at the N-terminus, and two cysteines known to be nonessential for function. However, a disulfide bond found in the wild-type protein is still present in Δ22-IL-6. Isoelectric focusing of Δ22-IL-6 showed a product that could be purified by various chromatographic techniques, including HIC (7). This suggests that the structure of the trisulfide derivative of Δ22-IL-6 overlaps with the disulfide form of Δ22-IL-6. The pI of the trisulfide-Δ22-IL-6 variant was 6.70, which was 0.14 units higher than for non-trisulfide Δ22-IL-6 (7).

Anion-exchange chromatography was used to separate the first reported trisulfide-mAb from the native form (49), but subsequent reports on trisulfide in mAb subgroups used samples that were only desalted by C4 reverse-phase-HPLC, prior to electrospray mass spectroscopy (20). The locations of reported trisulfide bonds in the five proteins discussed here are illustrated in Figure 4.

The most well-characterized trisulfide proteins are hGH and SOD. Pavlu and Gellerfors, Jespersen *et al.*, and Andersson *et al.*, showed by mass spectrometry that the compound that separates from native hGH by HIC-HPLC has an extra mass of 32 atomic mass units (amu). This mass increase was originally interpreted as the result of two additional oxygen atoms at the cystine (47), a possibility that might have been considered by the Jespersen group, as they clearly state in their discussion that oxygen cannot account for the change in the disulfide bond (30). By peptide mapping, Jespersen *et al.* and Andersson *et al.* found that the peptide with the extra mass gave a different RP-HPLC chromatogram than the corresponding peptide from native hGH. The relevant peptide contains the hGH amino acid sequence from 179–183 + 184–191, which contains one of the two hGH disulfide bonds (Fig. 4B) (1, 30, 47). Jespersen *et al.* showed that adding dithiothreitol or cysteine caused the higher-mass peptide fraction to lose the extra 32 amu, resulting in a peptide fragment with the same HPLC retention time as the peptide from native hGH (30). Adding a reductant to the undigested hydrophobic sample made it indistinguishable from the native hGH by HIC, indicating that the derivative was converted to native hGH (30). Finally, when the derivative was treated with a reductant, H_2S gas was detected by formation of dark lead sulfide on a lead acetate-soaked filter paper held above the sample (30). From these studies, the higher mass hGH derivate appears to contain an extra sulfur molecule covalently bound within the Cys182–Cys189 bond (30), as illustrated in Figure 4B. Recent advances in mass spectrometry allow detection of differences between the masses of two oxygen atoms (31.999 amu) and sulfur (32.066 amu) for the most common isotopes, which can rule out oxygen as the suspect atom.

No clear consensus structure for trisulfide bonds

Structurally, the reported trisulfide proteins do not have much in common. Figure 4 shows five published trisulfide proteins, with the cystines involved in the trisulfide bonds marked with red circles. With the exception of the trisulfide-hGH Cys52–Cys165 bond (Fig. 4B), a common feature appears to be that all reported trisulfide bonds are in parts of the proteins where the cystines are accessible. The best example of the easily accessible cystines is the multiple trisulfide bonds in the mAbs (Fig. 4E), where no intra-chain trisulfide bonds are formed, and trisulfide bonds are seen only in cystines between different mAb chains (20). Figure 4E shows the IgG2 molecule, which consists of two heavy chains forming a Y, and two light chains attached to the upper part of the heavy chains by trisulfide bonds. IgG2 is reported to contain only disulfide bonds, shown in yellow, between heavy chains, with trisulfide bonds only between light and heavy chains (20). The fraction of trisulfide derivatives varies for different mAbs (20), but all appear to be able to form more than one trisulfide bond per molecule (20,49).

A related case is seen for trisulfide-SOD formation, where the trisulfide/heptasulfide bond is found in cystines formed between two SOD molecules (Fig. 4A), and not in the intramolecular disulfide bond of SOD (14, 45, 69). In this particular case, the actual structure of the heptasulfide has been solved. The apparent structural challenges to formation of the equivalent trisulfide are obvious as the true nature of this form remains to be identified. Nevertheless, many of the considerations regarding formation of trisulfide would apply to heptasulfide as well.

Consequences for Protein Structure and *in vivo* Function

Trisulfide formation could be expected to have consequences for protein function, since adding an extra sulfur atom in a disulfide bond obviously causes a conformational change. However, in several cases, ligand proteins bind equally well to a receptor in the native or trisulfide form (1, 41). Gu *et al.* showed that antigen binding of a preparation of the mAb variant mAb1 with 39% trisulfide, was equal to that of mAb1 containing only 0.5% trisulfide (20). Similar results were obtained for mAb2 containing high and low concentrations of trisulfide (20). Likewise, both Andersson *et al.* and Thomsen *et al*. showed that formation of a trisulfide between Cys182 and Cys189 in hGH had no effect on receptor binding compared to native hGH (1, 60), although no biophysical analysis of the interaction was performed. These results are rather surprising, as the disulfide bond between Cys182 and Cys189 in hGH is close to the receptor binding site (15). The conformational change in the hGH peptide loop that contains the extra sulfur atom must therefore be minimal. This was studied by Andersson *et al*. and by Strandberg *et al.*, by NMR on the tryptic peptide fragment 179–183 + 184–191 (1), or the synthetically produced peptide (1, 58). Only minor conformational changes were seen in the disulfide and trisulfide forms of the peptide, with the trisulfide variant showing a less disordered structure, due to additional hydrogen bonds (1, 58). If the trisulfide bond creates a more stable peptide loop than the disulfide bond, then receptor binding to the trisulfide variant would be associated with less entropy loss than binding to the disulfide form. This could explain why receptor binding might be unaffected by a trisulfide-bond in hGH.

Similar results were seen with vasopressin, in which the trisulfide form and the native protein differed only marginally by NMR (41). The trisulfide variant of vasopressin shows a 3-fold reduction in substrate binding, compared to one subtype of vasopressin receptor, but not another, and the effect of the additional sulfur was not thought to have physiological consequences (41). The biological activity of the hormones oxytocin and deaminooxytocin was altered with the introduction of a trisulfide bond. The trisulfide analogs had 3.3-to-3.6-fold lower receptor binding, and 5%–40% biological activity of the disulfide versions (13).

As seen in Figure 4A, formation of the intermolecular polysulfide (heptasulfide and/or trisulfide) bond in the SOD dimer between two natively unpaired cysteines will clearly alter protein properties. Accordingly, the polysulfide variant shows slower unfolding, and higher resistance towards oxidation than the native SOD dimer (14). These features might be relevant to amyotrophic lateral sclerosis, since oxidation-mediated aggregation is one of several SOD modifications seen in this neurodegenerative disease (14,53).

In an IL-6-dependent cell assay, Breton *et al*. found that the trisulfide form of Δ22-IL-6 had only about 25% of the potency of the disulfide form (7), which is not unexpected, as the trisulfide derivative also has a somewhat more basic pI than the disulfide form, indicating significant chemical changes.

Mustafa and colleagues showed that S-sulfydration of glyceraldehyde-3-phosphate dehydrogenase actually increased the activity of the enzyme (43). The formation of the S-sulfhydrated enzyme could be carried out by cystathionine γ-lyase generated sulfide (43). This suggests that the activation of the enzyme by S-sulfhydration might take place *in vivo*.

Perspectives

Any disulfide-containing protein potentially contains a trisulfide bond, especially if the purified protein has a variant with a size increase of 32 amu. The small increase in molecular mass of 32 amu in a large protein may easily go unnoticed, so many well-characterized disulfide containing proteins could have trisulfide bonds. This is particularly the case in peptide mapping, since trypsin digestion is often carried out in conjunction with reduction. As complete reduction will release H_2S from trisulfide, simple H_2S formation assays can be conducted on the reduced purified protein, to identify possible trisulfide bonds. This may be particularly relevant in proteins isolated from an *E. coli* lysate where H_2S levels can be high.

Although trisulfide proteins have so far been found only *in vitro*, we believe that with improved techniques for thiol quenching *in vivo* (2,21), and rapidly evolving proteomic techniques (70), it is only a matter of time before trisulfide proteins are discovered *in vivo*. As shown above, the presence of H_2S appears to be a prerequisite for trisulfide formation. Considerable amounts of volatile as well as bound sulfide have been found in vertebrate tissues (25, 44, 64), including forms that are not extractable with acids (as iron-cluster sulfides would be). By treating with reducing agents, the bound sulfide can be liberated as H_2S from the tissue matrix (61). The origin of this sulfide pool has not been defined in detail and it is tempting to speculate that trisulfide proteins might be responsible for at least some sulfide sequestration. This hypothesis follows from the findings that some of the trisulfide proteins have a comparable activity to their native forms, and that trisulfide bonds might be readily formed at accessible disulfide bonds. This proposal also predicts that new trisulfide proteins might be discovered in organisms in environments with high amounts of sulfide, like anoxic sea beds and sulfur hot springs, or in systems where polysulfides are known to be produced (19).

Another possible function of sequestering sulfide as trisulfide might be to store H_2S for rapid release as a local signaling molecule. H_2S is a known gasotransmitter in synaptic signaling and smooth muscle (32). H_2S specifically and reversibly inhibits cytochrome c oxidase in the mitochondrial electron transport chain, and has been used to induce a hypometabolic, suspended-animation-like state in mice (5), a procedure that has clinical potential. Therefore, the possibility of H_2S sequestering by trisulfide proteins has medical as well as industrial implications.

If trisulfide bonds exist in *vivo*, formation and release may be an enzymatically catalyzed reaction. The enzymes that form or break trisulfide bonds might resemble known thiol-catalyzing enzymes such as thioredoxins and glutaredoxins, or sulfurtransferase proteins such as cystathionase or the rhodanese enzyme family. Whitfield *et al*. found that H_2S is rapidly metabolized in vertebrates including mammals (65). This has an impact on models of H_2S as a messenger molecule, but also suggests the presence of endogenous enzymes that sequester free H_2S within cells, possibly in the form of trisulfide, from which H_2S can be rapidly released as a local signaling molecule. Moutiez *et al*. found that the trisulfide molecule GSSSG could be reduced by glutathione reductase (40), suggesting that recognition of the trisulfide structure is possible by existing endogenous enzymes that catalyze thiol reactions.

The findings reviewed here suggest that trisulfide proteins might occur more frequently than previously thought from the limited number of reported findings. Not all bound sulfide is likely to involve trisulfide proteins, but the results described here suggest that incorporation of sulfide in covalent disulfide bonds may not be as rare as previously thought, even in mammals, and has industrial and clinical implications.

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Abbreviations Used

Δ22-IL-6 = mutant variant of interleukin-6 with the first 22 amino acids removed and Cys23 and Cys29 substituted with serine
GSH = glutathione
GSSG = glutathione disulfide
GSSSG = glutathione trisulfide
hGH = human growth hormone
IgG-2 = immunoglobulin-2
mAb = monoclonal antibody
NMR = nuclear magnetic resonance
RP-HPLC = reverse phase high pressure liquid chromatography
sCT = salmon calcitonin
SOD = superoxide dismutase

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