

The Role of Thiols and Disulfides on Protein Stability

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Abstract: There has been a tremendous increase in the number of approved drugs derived from recombinant proteins; however, their development as potential drugs has been hampered by their instability that causes difficulty to formulate them as therapeutic agents. It has been shown that the reactivity of thiol and disulfide functional groups could catalyze chemical (i.e., oxidation and beta-elimination reactions) and physical (i.e., aggregation and precipitation) degradations of proteins. Because most proteins contain a free Cys residue or/and a disulfide bond, this review is focused on their roles in the physical and chemical stability of proteins. The effect of introducing a disulfide bond to improve physical stability of proteins and the mechanisms of degradation of disulfide bond were discussed. The qualitative/quantitative methods to determine the presence of thiol in the Cys residue and various methods to derivatize thiol group for improving protein stability were also illustrated.

INTRODUCTION

The use of proteins for therapeutic applications has increased dramatically in the last several decades. There has been a tremendous increase in the number of approved drugs derived from recombinant proteins and monoclonal antibodies since the approval of recombinant insulin in 1982 (Fig. 1) [1]. In addition, the number of approved indications is almost four times the number of approved drugs in 2005. Sales of protein drugs in 2008 are estimated to be around 71 billion USD [2]. As a result, many more proteins are currently being investigated for use as therapeutic agents for various diseases (i.e., cancer, autoimmune diseases). Their development as potential drugs, however, has been hampered by difficulties in formulating them into therapeutic agents with long (i.e., 1–2 years) shelf lives. Most of the approved protein drugs that are commercially available are administered parenterally because they have very low oral bioavailability as well as the presence of proteolytic enzymes, which cause enzymatic degradation of proteins.

Chemical and physical instability can be a major hurdle for researchers in developing stable formulations of protein therapeutics. Factors that influence the physical and chemical instability of a protein can be divided into two categories: (1) intrinsic factors derived from the inherent physicochemical properties of the protein (e.g., primary, secondary, tertiary, and quaternary structures) and (2) the extrinsic factors derived from the environment of the protein, such as pH, temperature, buffer, ionic strength, and excipients. Because physical degradation (e.g., conformational changes, aggregation and precipitation, etc.) is one of the major problems in formulating protein therapeutics, many studies have been done to elucidate factors that influence the physical stability

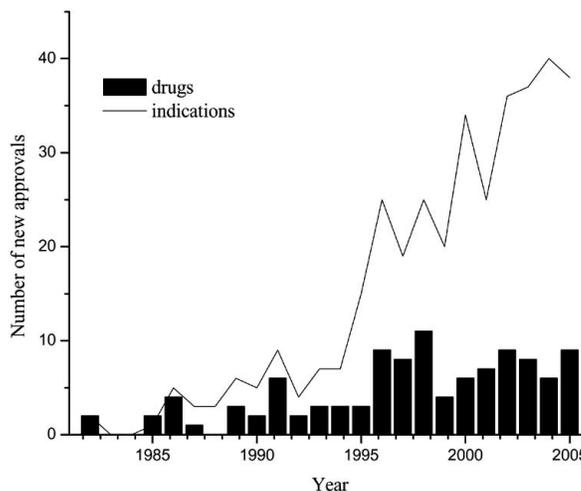


Fig. (1). Increasing trend in the use of recombinant proteins and monoclonal antibodies as drugs from 1982 to 2005. The solid bars indicate the number of protein drugs approved in each year. The solid line shows the increasing number of indications approved for the use of the protein drugs.

of proteins. Chemical degradation of proteins is observed due to the presence of certain functional groups (i.e., amide, carboxylic acid, hydroxyl, thioether) on the side chains of some amino acids (i.e., Asn, Asp, Ser, and Met); the chemical degradations mediated by these functional groups have been extensively studied and reviewed [3-12].

In this review, we focus on the role of Cys residues having a free SH group or in a disulfide bond(s) on the physical and chemical stability of proteins because these are almost always present in antibodies, receptors, hormones, and enzymes. The disulfide bond can be found in the form of intra- or intermolecular disulfide bond(s). The disulfide bond in

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peptides and proteins imposes local and/or global conformational rigidity on a protein, and the formation of non-native intramolecular **disulfide bond(s)** during the protein folding process may produce a misfolded protein that leads to aggregation and precipitation. Here, we have assimilated the relevant information on thiols and disulfides in proteins. We describe how protein **disulfide bonds** are produced in prokaryotic and eukaryotic cells, followed by the way that oxidative folding of reduced denatured proteins can be achieved *in vitro*, the effect of **disulfide bonds** on protein stability, various qualitative and quantitative methods used to assay protein thiols and disulfides, chemical degradation reactions of protein disulfides in solution and, finally, various methods that can be used to modify protein thiols so as to block their oxidation to disulfides.

DISULFIDE BOND FORMATION AS A POST-TRANSLATIONAL MODIFICATION OF PROTEINS

Native **disulfide bonds** in proteins are assembled during the oxidative folding process. In eukaryotes, this process occurs in the endoplasmic reticulum (ER) lumen [13], whereas in prokaryotes it takes place in the periplasm [14]. Thus, **disulfide bonds** are usually formed only in specific cellular compartments in healthy cells. Proteins synthesized by ribosomes are in reduced and unfolded form, and their oxidative refolding in eukaryotes is aided by a series of enzymes such as the endoplasmic reticulum oxidoreductin-1 (Ero1), protein disulfide isomerases (PDI) and Erv2 [13,15-17]. Many of these enzymes possess C-X-X-C (i.e., PDI) and C-X-C (i.e., Erv2) motifs that are involved in thiol-disulfide redox exchange reactions [18]. PDI directly oxidizes thiol groups in a protein via a series of thiol-disulfide exchange reactions by partnering with Ero1 or Erv2. The Ero1 enzyme, which is found inside the ER and is associated with the ER membrane, oxidizes PDI to form an Ero1-PDI mixed disulfide form. If the **disulfide bond** in the protein substrate is incorrectly formed, PDI catalyzes the reduction of these **disulfide bonds** and, subsequently, re-oxidizes the protein substrate to form the native **disulfide bond**. Thus, as a general rule, cytoplasmic proteins contain free thiols, while proteins in other compartments such as the ER can possess **disulfide bonds**. An exception to this rule is a family of thermophilic organisms that has been discovered to produce intracellular proteins with **disulfide bonds**. This is presumably due to the presence of protein disulfide oxidoreductase (PDO) in their cytoplasm, which oxidizes the cysteines to disulfides within intracellular proteins [19].

In bacterial cells, the oxidative folding machinery parallels the machinery in mammalian cells, and the oxidation reaction is catalyzed by the enzymes DsbA, DsbB, DsbC, and DsbD [14]. The **disulfide bond** A (DsbA) enzyme oxidizes thiol groups of the substrate protein to a **disulfide bond** in the periplasm with the simultaneous reduction of its own **disulfide bond**. Then, the reduced DsbA is reoxidized by DsbB. The oxidized state of DsbB is maintained by transferring electrons to ubiquinones (benzoquinone derivatives) and menaquinones (naphthoquinone derivatives), components of the respiratory electron transport chain system. When the substrate protein forms a non-native **disulfide bond**, DsbC enzyme reduces the non-native disulfides. This is followed

by the subsequent re-oxidation of the substrate by DsbA or DsbC to form the native disulfide. Finally, DsbD functions to keep DsbC in the reduced state, and the oxidized DsbD can be reduced by cytoplasmic thioredoxin.

PATHWAYS OF OXIDATIVE FOLDING OF PROTEINS FOR DISULFIDE FORMATION

Prior to the formation of a **disulfide bond**, the protein may or may not be folded into a native-like structure with the Cys residues in a favorable position to form a **disulfide bond** [20,21]. In the case of bovine pancreatic trypsin inhibitor (BPTI), the formation of all three **disulfide bonds** in BPTI occurs via stable intermediates with a native conformation containing one or two native **disulfide bonds** [20,21]. No significant amount of non-native disulfide intermediates was observed. However, the formation of only native-like intermediates does not always predominate; for example, hirudin, with three **disulfide bonds**, undergoes oxidative folding via heterogeneous intermediates with two or three non-native **disulfide bonds** [22]. These scrambled **disulfide bonds** undergo reduction followed by oxidative rearrangement to form the native protein structure. The reductive unfolding of this protein follows a pathway similar to that of the folding process, but in reverse. The *in vitro* oxidative folding of human macrophage colony stimulating factor β (rhm-CSF β) follows a pathway similar to that of hirudin. The rhm-CSF β protein is a dimer with three intermolecular **disulfide bonds** between the two monomers. Each monomer also contains three intramolecular **disulfide bonds** [23]. The oxidative folding of rhm-CSF β involves the formation of monomeric isomers with native and non-native intramolecular **disulfide bonds**; the monomer isomerization step is the rate-determining step in this process [23]. Upon disulfide shuffling, the protein forms a stable dimeric intermediate that possesses all of the intramolecular disulfides, including a non-native intermolecular **disulfide bond**. Rearrangement of the non-native **disulfide bond** creates the native oxidized rhm-CSF β .

Although the oxidative folding of different proteins may take different pathways, the process normally adheres to some general rules. **Disulfide bond** formation is usually favored at basic pH, and the presence of an oxidizing agent such as oxidized glutathione increases the rate of oxidative folding, whereas the presence of denaturants such as urea or guanidine hydrochloride (GdnHCl) hampers the folding process. These rules do not apply, however, to the oxidative folding of murine prion protein (mPrP 23–231), which has only one intramolecular **disulfide bond** at Cys179–Cys214 [24]. The formation of a **disulfide bond** in reduced mPrP is very slow at pH 8.0 in the absence of denaturant, but mPrP refolds properly in the presence of a denaturant and glutathione at pH 8.0 [24]. Contrary to intuition, the best pH for producing a disulfide in mPrP is 4–5. This observation can be explained by the fact that, at alkaline and neutral pH, the reduced and unfolded mPrP is present in a stable intermediate conformation with its Cys residues buried and separated from each other. The addition of glutathione, therefore, does not facilitate oxidation of Cys. The addition of denaturant and a pH of 4.0 facilitate the unfolding of the stable intermediate, bringing the two Cys residues closer together and allowing them to form a **disulfide bond**.

EFFECTS OF DISULFIDE BONDS ON PROTEIN PHYSICAL STABILITY

Intramolecular **disulfide bonds** are known to contribute to protein thermal stability. Reducing all five **disulfide bonds** inactivates the *Aspergillus niger* phytase due to conformational changes and/or unfolding [25]. Another example of the effect of **disulfide bonds** on stability is observed in the soybean Bowman-Birk inhibitor (BBI) [26,27]. The crystal structure of BBI has a bow-tie motif, with a trypsin-binding loop at one end and a chymotrypsin-binding loop at the other. In contrast to most globular proteins, it has exposed hydrophobic patches and charged residues containing bound water molecules in its interior [26,27]. Interestingly, BBI is remarkably stable against heat and chemical denaturants; this stability can be attributed to the seven intramolecular **disulfide bonds** within the protein's structure that "lock" it in its native conformation [26].

Introduction of a **disulfide bond** by Cys mutation has been shown to improve the physical stability of some proteins. In case of subtilisin E, mutations of Gly61Cys and Ser98Cys followed by the formation of the Cys61–Cys98 disulfide produced an active enzyme with a 4.5 °C increase in melting temperature and a half-life three times longer than that of the native protein [28]. In addition, there was no change in its enzymatic activity due to the engineered **disulfide bond**. Similarly, a Cys39–Cys85 **disulfide bond** was engineered into dihydrofolate reductase (DHFR) without a significant alteration in its conformation. The disulfide mutant had an improved stability with its ΔG° higher than that of the native protein by 1.8 kcal/mol [29].

At this point, it is necessary to discuss the thermodynamics behind the stabilizing effect of **disulfide bonds** on proteins. This stabilizing effect of disulfides is manifested by an increase in the melting temperature of the protein. The melting or unfolding transition of a protein is generally regarded as a reversible process between the native and the unfolded states. Differential scanning calorimetry (DSC) is usually employed to determine the reversible thermal unfolding of a protein. Superimposable DSC thermograms resulting from repeated scans of the same protein sample imply a two-state reversible unfolding process, which is depicted as $N \rightleftharpoons U$, where N represents the native state and U the unfolded state of the protein. This is also known as the "all or none" transition in statistical thermodynamics. In such a transition, the protein is either in the N state, with all its residues in the native conformation, or it is in the U state, where all its residues are in the unfolded conformation. The Gibbs free energy for this process is related to the equilibrium constant of this reaction by the following equation:

$\Delta G = -RT \ln K_{eq}$, where K_{eq} is the ratio of the folding (k_f) and unfolding (k_u) rate constants. So, $K_{eq} = k_f/k_u$. When a **disulfide bond** is introduced into a protein, there is likely to be a significant increase in the free energy of U . The transition state (TS) can either (i) have a large increase in its free energy similar to the unfolded state, in which case, the **disulfide bond** is lost in the TS or (ii) have a negligible change in its free energy. In this case, TS is more N -like and retains the **disulfide bond** [30]. In (i), the folding rate does not change much, but the unfolding rate decreases significantly. In (ii),

the folding rate increases while the unfolding rate remains constant. In either case, the ratio of k_f/k_u , which is equal to K_{eq} , decreases, leading to an increase in the free energy (ΔG) of the protein and making it conformationally more stable. The assumption is that there is little or no difference in the enthalpies (ΔH) of the disulfide-containing and reduced protein conformations.

It has been proposed that the decrease in the entropy of U can be predicted with the help of the following equation: $\Delta S = -2.1 - (3/2)R \ln n$, where n is the number of residues in the loop formed by the intramolecular **disulfide bond** [31]. Similarly, reduction of **disulfide bonds** or mutation of disulfide-forming Cys residues in a protein can result in increased entropy of U . Consequently, K_{eq} increases and ΔG of the protein decreases. This characteristic was observed in the 110 residue starch-binding domain (SBD) of *Aspergillus niger* glucoamylase, which possesses a **disulfide bond** between C3 and C98, creating a 94 residue loop between its N-terminus and the short loop between its seventh and eighth β -strands [32]. Two different mutants, C3G/C98G (GG) and C3S/C98S (SS), were constructed, and their stability was compared to that of the wild type by determining their unfolding temperature and other thermodynamic parameters using DSC and CD techniques [32]. The unfolding temperatures were 10 °C lower for the mutants (43 °C) compared to wild-type SBD (53 °C). The unfolding processes for SBD, GG, and SS were determined to be reversible, with GG and SS having a free energy lower than SBD by about 10 kJ/mol. The contribution from entropic destabilization was much larger than that from enthalpic destabilization.

When multiple **disulfide bonds** are introduced into a protein, they can act cooperatively to stabilize the protein. The overall increase in the free energy of a protein in that case is higher than the sum of the increases in free energies due to individual disulfide pairs. This phenomenon was observed in *Bacillus circulans* xylanase when **disulfide bonds** were introduced on the protein. In the case of the xylanase enzyme mutants, a single **disulfide bond** was first introduced at C100–C148 (S100C/N148C; DS1 mutant), C98–C152 (V98C/A152C; DS2 mutant), and C2–C188 (A1GC/G187, C188; cX1 mutant). These mutants have higher melting temperatures (T_m) than the wild type protein, with ΔT_m around 3.8–5.0 °C. When two **disulfide bonds** (C100–C148 and C2–C188) were implemented in the cDS1 mutant, the melting temperature was increased by 12.3 °C compared to that of the wild type; thus, the effects of the **disulfide bonds** are additive [33]. Although the cDS1 mutant has a higher melting temperature, the formation of two **disulfide bonds** reduces the enzymatic activity to 68%, suggesting that increasing the rigidity influences the catalytic activity and substrate-binding property of the enzyme.

It is difficult to predict whether increasing conformational rigidity upon addition of a **disulfide bond** will always improve the stability and/or activity of a protein. A good example of the effect of the location of a disulfide on structural stability and enzymatic activity is T4 lysozyme. Four mutants of T4 lysozyme with a **disulfide bond** at Cys9–Cys164, Cys21–Cys142, Cys90–Cys122, and Cys127–Cys154 were designed based on molecular modeling experiments [34]. The thermal stabilities of the oxidized mutants were compared to those of their corresponding reduced

mutants as well as to that of wild-type T4 lysozyme. The oxidized Cys9–Cys164 mutant has a melting temperature 6.4 °C higher than that of the wild type, but maintains its enzymatic activity. In contrast, the Cys21–Cys142 mutant has the best thermal stability but no enzymatic activity. Finally, the Cys90–Cys122 and Cys127–Cys154 mutants have lower thermal stabilities and enzymatic activities compared to that of the wild-type enzyme. In another case, Ser52, Ser53, Ser78, Ser79, and Ser80 in alkaline phosphatase (AP) were mutated to the respective Cys residue; a disulfide bond was formed between the new Cys residue and Cys67. All of the disulfide mutants had improved thermal stability but lower enzymatic activities than did the native AP [35]. The decreased enzymatic activity of all the mutants appeared to be due to the enhanced rigidity and lower substrate affinity of the active site imposed by the disulfide bond, which is located near the active-site residues. The location of the non-native disulfide bond affects the physicochemical stability and biological properties of the mutant proteins in a manner difficult to predict. Thus, disulfide bonds can have a favorable or unfavorable effect on protein stability.

To impose conformational rigidity, a disulfide bond has also been introduced in peptides to create cyclic structures (cyclic peptides) that stabilize specific secondary structures (i.e., β - and γ -turns). The formation of cyclic peptides in some cases enhances receptor-binding affinity and selectivity, as shown in opioid peptides [36]. Several cyclic peptides with a disulfide bond have been marketed as therapeutic agents, including Integrilin[®] [37,38] and oxytocin (Pitocin[®]) [39]. Integrilin[®] is a cyclic Arg–Gly–Asp (RGD) peptide that is used to treat thrombosis; it binds selectively to gpIIb/IIa receptors on the surface of platelets, thus blocking fibrinogen-mediated platelet aggregation [37,38]. Cyclic peptides also have been shown to be more stable to chemical and enzymatic degradation than are their parent linear analogs. The Asp-mediated chemical degradation of RGD peptides was suppressed by the formation of a cyclic RGD peptide with a disulfide bond; thus, the chemical stability of the cyclic RGD peptide is higher than that of the parent linear form [11,40,41].

FORMULATION OF CYS-CONTAINING PROTEIN

The presence of free thiol and disulfide bond in a protein may affect its physical stability due to a disulfide bond exchange and intermolecular disulfide bond formations that produce aggregates followed by protein precipitation. For example, covalent dimerization of human growth hormone (HGH) via a disulfide bond reduces the activity of HGH [42]. Addition of antioxidants such as glutathione, thioacetic acid, and cysteine in protein formulation has traditionally been successful in lengthening protein shelf-life. Recently, we have shown that the EC1 domain of E-cadherin with only one Cys residue can form a covalent dimer (EC1-s-s-EC1) upon standing in solution. Furthermore, the formation of this covalent dimer induces the physical degradation and precipitation of the protein. Prevention of covalent dimer formation by addition of dithiothreitol (DTT) to the solution of EC1 inhibits precipitation [43]. Derivatization of the thiol group of the Cys residue in EC1 with iodoacetamide and polyethylene-glycol maleimide improves the physical stability of the EC1 protein.

ANALYSIS OF FREE THIOLS AND DISULFIDE BONDS

Chemical Derivatization

The presence of a free thiol group in the Cys residues in a protein can be quantitatively determined by different methods, including the use of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) [44-47], maleimide (i.e., ThioGlo) [48], and bimane reagents (Fig. 2) [49,50]. The presence of a disulfide bond can be detected using the crabescen reagent [51]. Ellman's reagent (DTNB) was the earliest reagent widely used for estimating the number of thiol groups. The reaction between DTNB and thiol group(s) produces an equivalent amount of 5-thio-2-nitrobenzoic acid, the absorbance of which can be measured at 412 nm. However, it was found that the DTNB method may underestimate the concentration of thiol groups in a protein due to its incomplete reaction [48,52]. Addition of cystamine as an accelerator of the DNTB-thiol reaction improves the accuracy of thiol determination [47]. As an alternative reagent, 4,4'-dithiodipyridine (DTDP) has a performance similar to that of the DTNB/cystamine in determining thiol concentrations (Fig. 2) [47]. Upon reaction with the thiol group, DTDP releases 4-thiopyridine (4-TP), which absorbs at 342 nm. One drawback to using DTDP is that some proteins have inherent absorbance at 342 nm (due to the ligand metal charge transfer band during the chelation of thiol groups with some metals from the *f*-block of the periodic table), which can interfere with the quantitative measurement of 4-TP. A new derivative of Ellman's reagent, *n*-octyl-5-dithio-2-nitrobenzoic acid (ODNB) has been designed; as it is more hydrophobic than DTNB, this reagent can react with all thiol groups of a protein, including the buried ones, in a relatively short time (Fig. 2) [52].

Maleimide derivatives are also used to determine the number of thiol groups in a protein. ThioGlo[®] reagents (ThioGlo-1[®], $\lambda_{\text{Ex}} = 379$ nm and $\lambda_{\text{Em}} = 513$ nm) are maleimide derivatives of naphthopyranone fluorophores that emit fluorescence upon thiol alkylation (Fig. 2). ThioGlo-1[®] is forty times more sensitive than DTNB and other maleimide reagents. The protein-ThioGlo adduct does not require separation prior to quantitative determination due to the low fluorescence of the starting reagent. One caution is that the reaction should not be carried out at pH >8 because of the possibility of amine alkylation of the maleimide and adduct degradation. Possible disadvantages include the low water solubility of the ThioGlo reagent and the instability of protein-ThioGlo adducts, which may lead to inaccuracy in the quantitative determination of the thiol group.

Bimane derivatives (i.e., monobromobimane (mBBr), dibromobimane (bBBr) and monobromotrimethylammonio-bimane (qBBr)) have also been developed for quantitative determination of thiol groups of cellular proteins (Fig. 3). Bimane derivatives have no intrinsic fluorescence; upon reaction with thiol groups in proteins, they become highly fluorescent [49]. Both mBBr and bBBr can be used to measure the total cellular thiol content and qBBr can be used to measure the extracellular and membrane thiol content. Due to the presence of a charged group in qBBr, this molecule cannot permeate cell membranes; therefore, it may be used to measure selectively the extracellular thiols. mBBr can

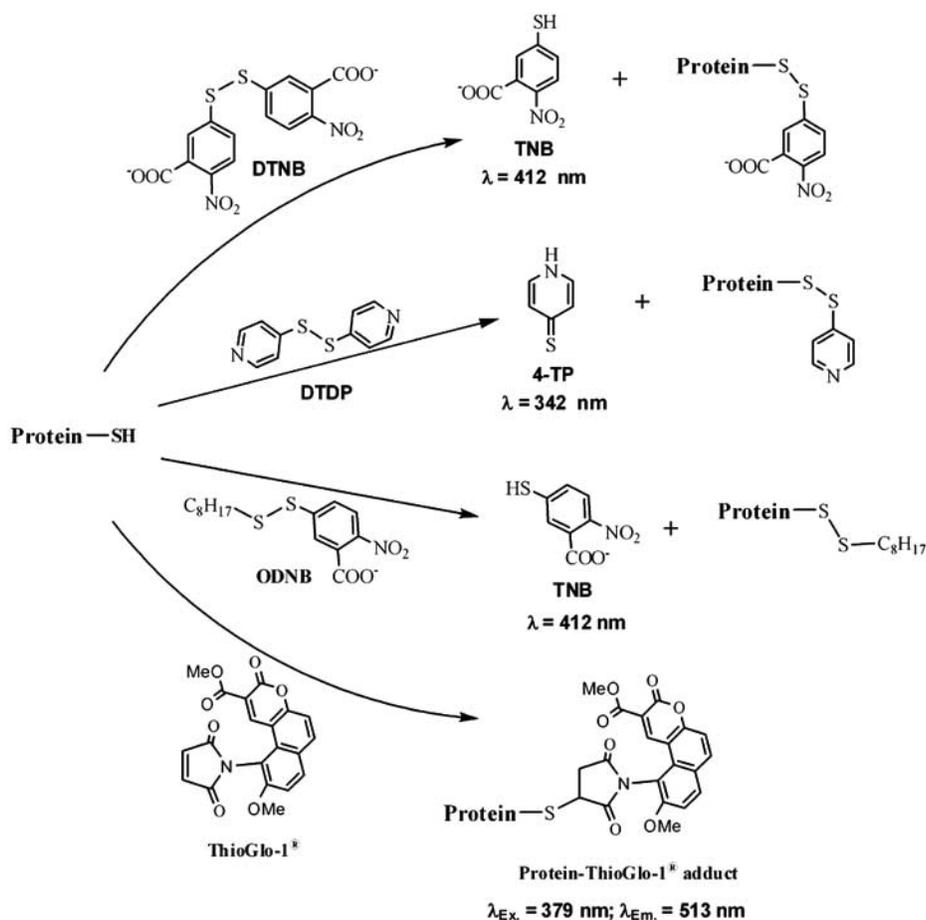


Fig. (2). Increasing trend in the use of recombinant proteins and monoclonal antibodies as drugs from 1982 to 2005. The solid bars indicate the number of protein drugs approved in each year. The solid line shows the increasing number of indications approved for the use of the protein drugs.

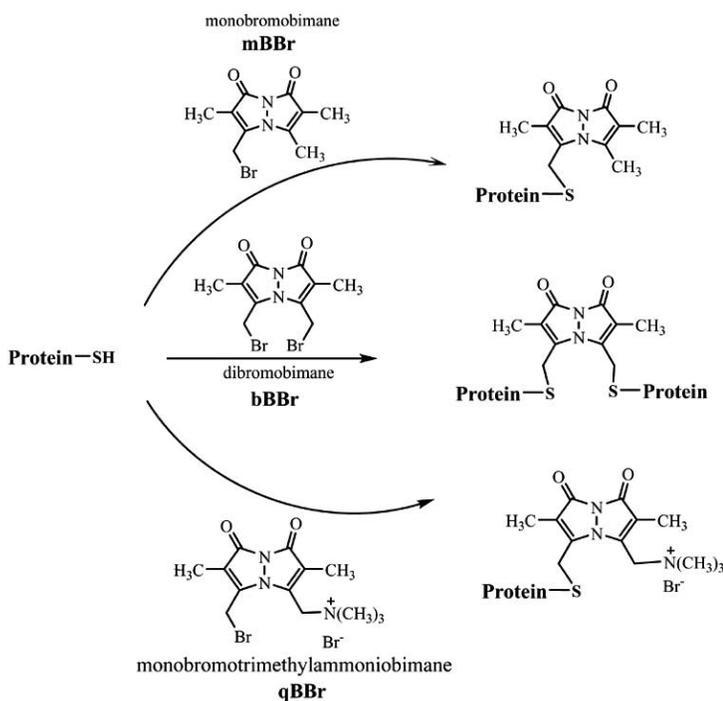


Fig. (3). Bimanes such as monobromobimane (mBBr) and dibromobimane (bBBr) are used to label protein thiols inside the cell as well as on cell surfaces. Monobromotrimethylammoniumbimane (qBBr) is used to label protein thiols that are present only on the cell surface.

penetrate cell membranes and react with thiols in the cells as well as the thiols in the extracellular space. Thus, the difference in the thiol content measured by mBBR and qBBR gives the amount of thiols inside the cells. One of the advantages of using these bimeane derivatives is that they are stable during exposure to air and irradiation as well as during general laboratory procedures. Bimeane has also been used to determine the intracellular levels of thioredoxin after isolating the modified protein using an antibody affinity Sepharose column. After elution from the column, the amount of thioredoxin can be determined using a fluorescence spectrometer [53].

NMR Methods

Chemical modification methodologies have been important tools for probing the redox status of Cys residues in proteins because they are widely applicable and permit rapid analysis. They do not, however, provide site-specific information or report on structural changes that accompany oxidation. Most methods used to identify the position of Cys residues involved in **disulfide bonds** are destructive, requiring denaturation and/or cleavage of the protein into fragments to conduct the analysis. Such methods are susceptible to artifacts that may arise as a result of altering the native conformation of the species during its examination. Moreover, in some cases, chemical modification may alter the solubility and/or conformation of the protein, making results difficult to obtain or interpret. High-resolution structure determination is the most accurate way of identifying specific disulfide linkages, but the process can be time consuming and resource intensive. Structure determination may be accomplished using X-ray crystallography or solution NMR. These methods reveal the exact position of free Cys residues and **disulfide-bonds**, as has been thoroughly demonstrated using thioredoxin [54-56] as well as numerous other proteins. Solution NMR also provides an alternative means of analyzing the oxidation state of Cys residues in intact proteins without the need to solve the high-resolution structure. Described below are a few, more rapid NMR approaches that have been used to identify the presence and position of free thiols and **disulfide bonds** in native, intact proteins.

The first reported NMR method for assessing the oxidation state of Cys residues in a protein was based on detection of ^{13}C -labeled backbone carbonyl carbon atoms in Cys residues [57]. The approach combined several selective, stable-isotope labeling schemes with mutagenesis of each Cys to assign the observed resonances to a specific position in the protein. The success of an individual assignment depends on the amino acid present on the C-terminal side of each Cys. The adjacent residues were labeled with ^{15}N so that selective detection of the ^{13}C -carbonyl in the Cys could be achieved based on the ^{13}C - ^{15}N connectivity to its neighbor [57]. The isotopic labels were incorporated using recombinant expression in *E. coli*, in which the bacteria were grown in media containing the selectively labeled amino acids. In cells some amino acids are metabolized such that nitrogen scrambling occurs readily, resulting in incorporation of ^{15}N into unintended residues. In this case, additional NMR spectra were collected to resolve ambiguity and assign the peaks to specific Cys residues [57]. Following peak assignment, the pro-

tein was reduced by adding increasing amounts of DTT to the sample, and the change in intensity of the ^{13}C carbonyl peaks in a 1D spectrum was monitored. The chemical shift position of the Cys ^{13}C is altered when a **disulfide bond** is cleaved, resulting in a decrease in the signal intensity for peaks involved in the bond, whereas no change is observed for free thiols. In the analysis of *Streptomyces* subtilisin inhibitor (SSI), the surface-accessible disulfide was rapidly reduced by DTT, but a buried disulfide was not affected [57]. To confirm that these internal Cys residues were covalently bonded, SSI was denatured and reduced. Following refolding, reoxidation then was monitored. The protein was found to refold, as its original spectrum was reproduced. The surface-accessible Cys residues rapidly regained their original chemical shift positions, as in the original titration. Moreover, it was obvious from this experiment that the two internal Cys residues also formed a **disulfide bond**, because the signals from these two residues were perturbed but slowly returned to their native chemical shift values.

More recently, a method was published for predicting **disulfide bonds** in proteins primarily from their $\text{C}\beta$ and secondarily from their $\text{C}\alpha$ chemical shift values [58]. After a substantial number of complete resonance assignments and protein structures that contained reduced and oxidized Cys were obtained by NMR and reported to the BioMagnetic Resonance Bank (BMRB), the chemical shifts for each atom in the Cys residues were analyzed using statistical methods to identify trends in the chemical shift values. A significant deviation in the chemical shift position was identified at the $\text{C}\beta$ position in Cys between the reduced and oxidized states, revealing that the measured value for the $\text{C}\beta$ is highly indicative of the oxidation state of each Cys. The chemical shift of $\text{C}\beta$ also is affected by secondary structure, and as such, the specific conformation may alter the chemical shift position, moving it outside of the expected range of values. The $\text{C}\alpha$ value can be used to guide interpretation of the redox state of a Cys residue when the $\text{C}\beta$ value alone is ambiguous. The $\text{C}\alpha$ chemical shift of each amino acid is strongly influenced in a predictable way by secondary structure [59-61]. The $\text{C}\alpha$ value serves as a point of reference for interpreting the effect of secondary structure on the chemical shift of the adjacent $\text{C}\beta$. Considering the chemical shifts of the $\text{C}\alpha$ and $\text{C}\beta$ resonances permits the oxidation state of each Cys residue in a protein identified. In proteins containing multiple **disulfide bonds**, however, determination of the specific pairings may require additional experiments akin to those described above. Using one uniformly ^{13}C , ^{15}N -labeled sample, complete assignment of the resonances can be performed to determine the chemical shift position of each Cys in the protein and, concomitantly, the redox state of each.

Reversible redox modulation of **disulfide bonds** is of great importance in protein folding and the regulation of enzyme activity, particularly in signal transduction. In order to directly investigate the function of Cys oxidation and reduction in individual proteins, a new NMR method was developed from which the redox potential for individual **disulfide bonds** could be determined [62]. Three intracellular proteins were studied during a titration of the protein sample with a thiol-modulating agent, and changes in the spectrum were followed using the ^1H - ^{15}N HSQC experiment. Because the modulation of a **disulfide bond** in proteins often results in

conformational rearrangement, selective labeling of the Cys was not required. Instead, the proteins were uniformly ^{15}N -labeled, which is inexpensive and provides redundant signals to support quantitative analysis. The initial titration was performed using glutathione [GSH/GSSG], but the assay is amenable to using any reducing agent. The choice of reducing agent and the concentration of the agent required for reduction both factor into the calculation of redox potential using the Nernst equation. The peak height of individual NMR signals for the reduced and oxidized species is plotted as a function of reducing potential [E (mv)] to identify the midpoint. While NMR assays may require higher protein concentrations or more time to complete than absorbance or fluorescence assays, they uniquely allow site-specific detection of free thiols and disulfide bonds in native, intact proteins and have the ability to determine the redox potential of each disulfide pairing.

DEGRADATION OF DISULFIDE BONDS

Chemical degradation of disulfide bonds has been studied in both peptides and proteins with most of the disulfide bond degradation observed under neutral and basic conditions [11,63]. Degradation of disulfide bonds can be classified into three major pathways: (1) direct attack on the sulfur atom by the hydroxyl anion, opening the disulfide bond to form sulfenic acid/thiolate anion (Fig. 4); (2) a β -elimination reaction in which the α -proton of the Cys residue is abstracted (which produces dehydroalanine/persulfide (Fig. 5)); and (3)

the α -elimination caused by the hydroxyl ion attacking the β -proton of the Cys residue to produce the thiolate/ thioaldehyde (Fig. 6) [64].

The hydroxyl attack of the disulfide bond produces sulfenic acid and thiolate anion (1, Fig. 4). The thiolate anion 1 can proceed to attack another disulfide bond on another protein molecule to produce a thiolate dimer intermediate 2 (pathway a, Fig. 4). This intermediate (2) undergoes three possible reactions as shown in pathways b, c, and d. In pathway b, intramolecular nucleophilic attack of the sulfenic acid by the thiolate anion produces dimer 3 with two intermolecular disulfide bonds. Intermediate 2 can form sulfenic acid dimer 4 by abstracting a proton from water (pathway c). Higher molecular weight oligomers such as trimer 5 can be produced via intermolecular disulfide bond reactions utilizing pathway d. The thiolate anion 1 can also undergo an intermolecular reaction between the sulfur atom of the sulfenic acid group and a disulfide in another molecule to produce an intermediate dimer 6, as shown in pathway e. The resulting thiolate 6 oligomerizes by reacting with another protein molecule to generate a trimer 7 (pathway f); further intermolecular reactions lead to higher molecular weight oligomers. Intermediate 6 can also react with water to form a dimer molecule (8) with two free thiols.

The second degradation pathway is via β -elimination through the hydroxyl anion abstraction of the acidic α -proton of the Cys residue to yield a dehydroalanine-persulfide 9

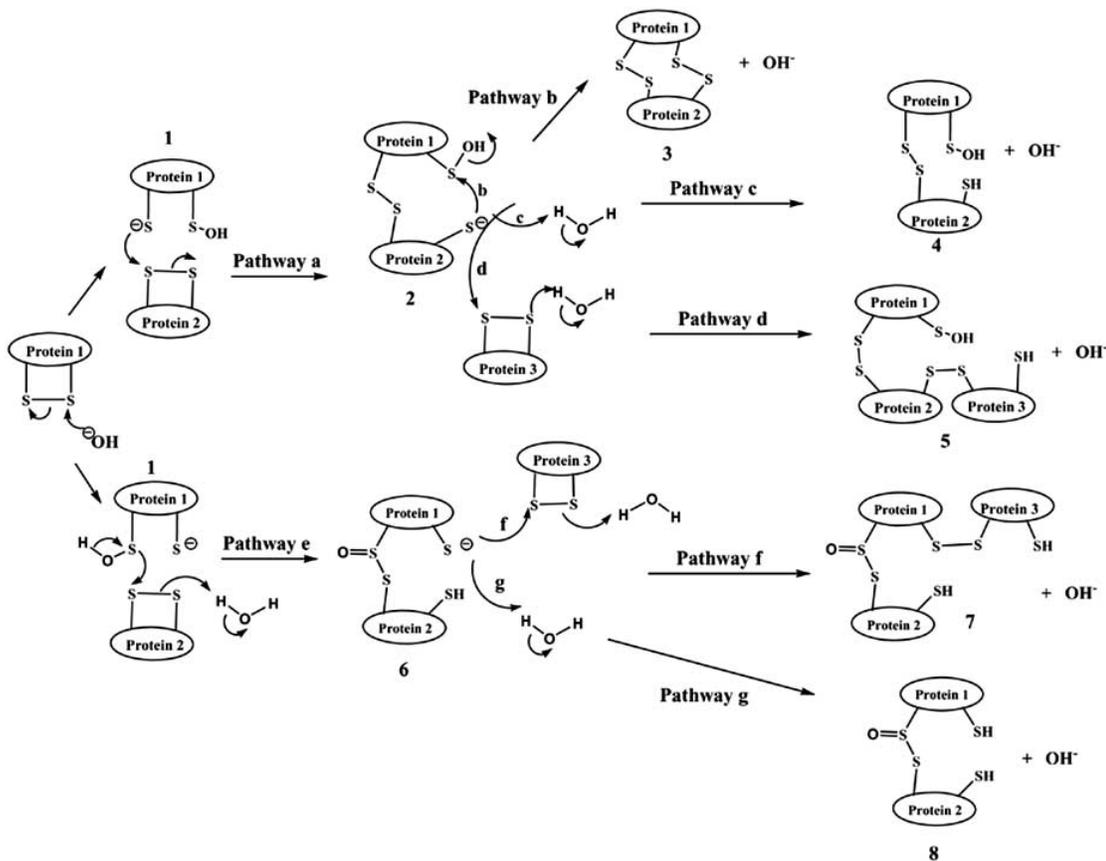


Fig. (4). The mechanisms of degradation of disulfide bonds in proteins upon incubation under neutral and basic conditions. The degradation of the disulfide bond involves a direct attack on the sulfur atom by hydroxyl anions.

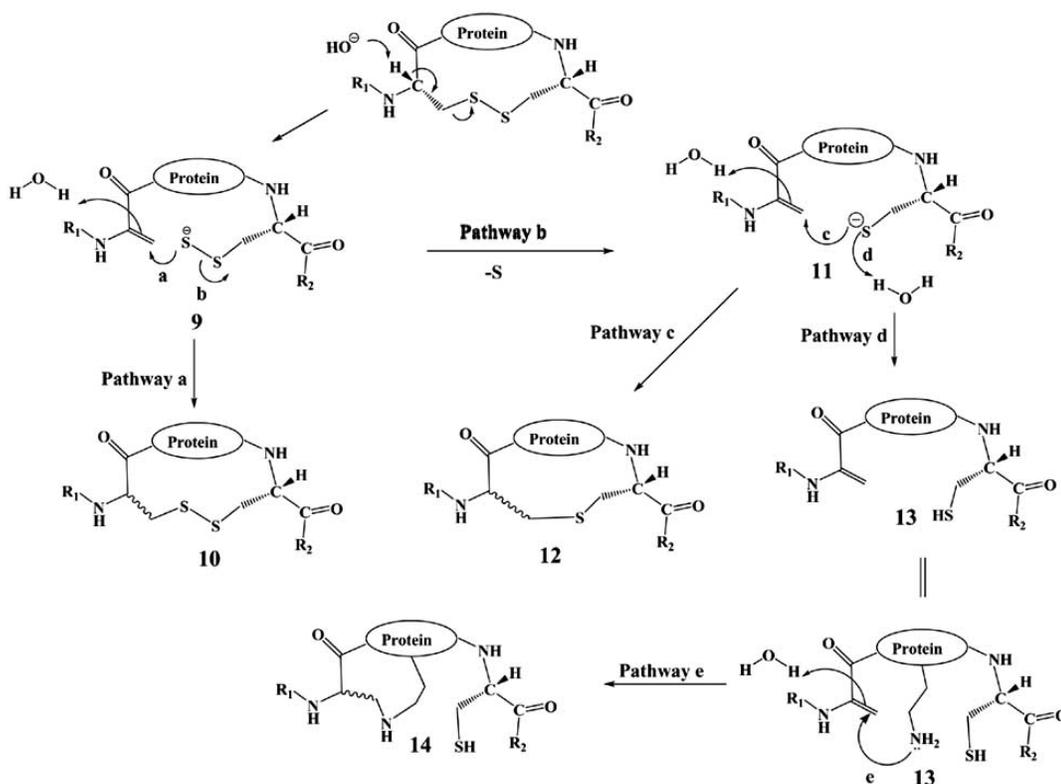


Fig. (5). The degradation reactions of a disulfide bond via β -elimination reaction under neutral and basic conditions. In this case, the α -proton of the Cys residue is abstracted by the hydroxyl anion followed by breakage of the C-S bond.

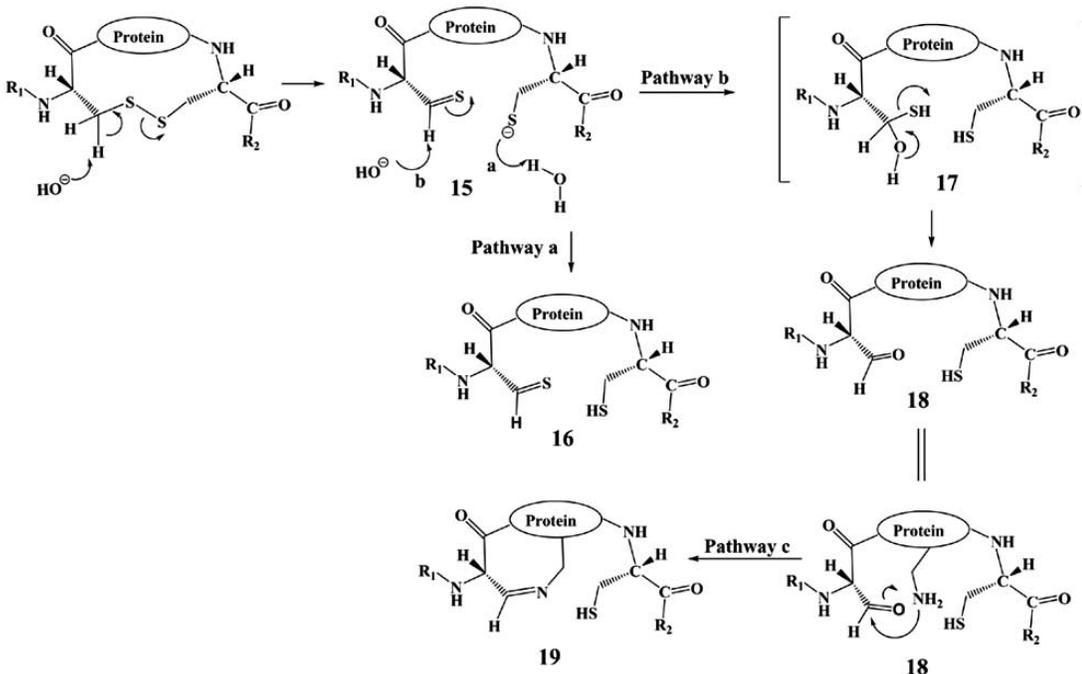


Fig. (6). The mechanism of degradation of a disulfide bond through the α -elimination reaction under neutral and basic conditions involving abstraction of the β -proton of the Cys residue by a hydroxyl anion to produce a thioaldehyde and a thiolate anion.

(Fig. 5). The disulfide bond can be reconnected by nucleophilic attack on the dehydroalanine by the persulfide anion (pathway a, Fig. 5). The resulting products (10) form a racemic mixture at the $C\alpha$ of the Cys residue (10, Fig. 5). The persulfide 9 can undergo a sulfur extrusion reaction via pathway b to yield the thiolate anion 11. The thiolate anion

in 11 reacts with the alkene functional group of the dehydroalanine to generate the thioether 12; this reaction can also take place in an intermolecular fashion to produce oligomers. A similar reaction has been observed in an IgG1 monoclonal antibody [65] and cyclic peptides containing a disulfide bond [11,63]. In the IgG1, this thioether bond was observed at

C233, which links the heavy chain to the light chain [65]. This modification was identified by LC-MS tandem mass spectrometry. Abstraction of a proton from the solvent by the thiolate anion **11** produces dehydroalanine-thiol **13**. There is a possibility that the amino group from either the Lys or the N-terminus of the protein may react with the alkene group of the dehydroalanine to produce a secondary amine **14**, which was previously observed in the degradation of cyclic peptides [11,63].

Finally, the α -elimination reaction proceeds via the abstraction of the β -proton of the Cys residue followed by **disulfide bond** breakage to produce thiolate/thioaldehyde **15** (Fig. 6). Protonation of the thiolate anion yields thiol/thioaldehyde **16** (pathway a). In the presence of the hydroxyl anion, the thioaldehyde group can be converted to an aldehyde **18** via intermediate **17** (pathway b). An amino group (i.e., Lys or N-terminus) that is in close proximity to the aldehyde group in **18** may also produce an imine product **19**. The reaction to produce imine can occur via bimolecular reactions to create oligomers.

Intermolecular covalent bond reactions (disulfide, thioether, amine and imine in Figs. 4-6) producing oligomers can lead to the association, aggregation and precipitation of proteins. For example, intermolecular disulfide formation causes aggregation of nascent thyroglobulin (Tg) protein. Monomeric Tg is a precursor to the synthesis of thyroid hormone. It contains more than 100 Cys residues and can produce aggregates through the formation of non-native intermolecular **disulfide bonds** due to the oxidizing environment in the endoplasmic reticulum of thyroid cells [66]. During the transport of Tg to the Golgi complex, the aggregates of Tg undergo disulfide reduction and dissociation to form monomers with assistance from molecular chaperones. This suggests that the incidence of Cys residues in the protein as well as the redox status of its environment are important factors that contribute to its disulfide-mediated aggregation. Intermolecular **disulfide bond** reactions in α - and β -crystallin induced by X-ray irradiation were shown to be partly responsible for cataract formation in the rabbit eye lens. It is interesting to note that the reaction between prednisolone and the amino group(s) of crystallin alters crystallin conformation to expose the buried thiol groups [67,68]. These exposed thiol groups then undergo oxidation to form intermolecular disulfides, which cause protein aggregation and precipitation [67]. It was made clear in a subsequent study that the crystallin that underwent conformation change on reaction with prednisolone was α -crystallin [69]. In most cases, the **disulfide bond** can be reversed by reducing agents (i.e., DTT). Intermolecular **disulfide bond** formation has been suggested to induce the aggregation of BSA and DTT has been shown to suppress the aggregation of BSA [70].

CHEMICAL MODIFICATION OF CYS THIOLS OF PROTEINS

The thiol group in Cys can undergo oxidation reactions to form sulfenic (R-S-OH), sulfinic (R-SO₂H), and sulfonic (R-SO₃H) derivatives as well as the **disulfide bond**. Thiol groups are one of the most reactive groups in proteins and can participate in side reactions with reagents used to manipulate other functional groups in a protein and the other solution

components. The presence of a free Cys residue may cause unwanted intramolecular disulfide scrambling and covalent oligomerization via intermolecular disulfide formation. To prevent these reactions, thiol groups can be derivatized with iodoacetate, iodoacetamide, 1,3-propane sultone [71], methyl methanethiosulfonate, methoxycarbonylmethyl disulfide [72], maleimide, tetrathionates, and dinitrophenyl alkyl disulfides (DNPSSR) [73] (Fig. 7). Human serum albumin, [Lys⁸] vasopressin, bovine insulin, and bovine pancreatic ribonuclease have been alkylated with 1,3-propane sultone to increase their solubility and stability to acid hydrolysis. Alkylating the free thiol of the tetanus toxoid with N-ethylmaleimide inhibits moisture-induced aggregation of the lyophilized toxoid [74].

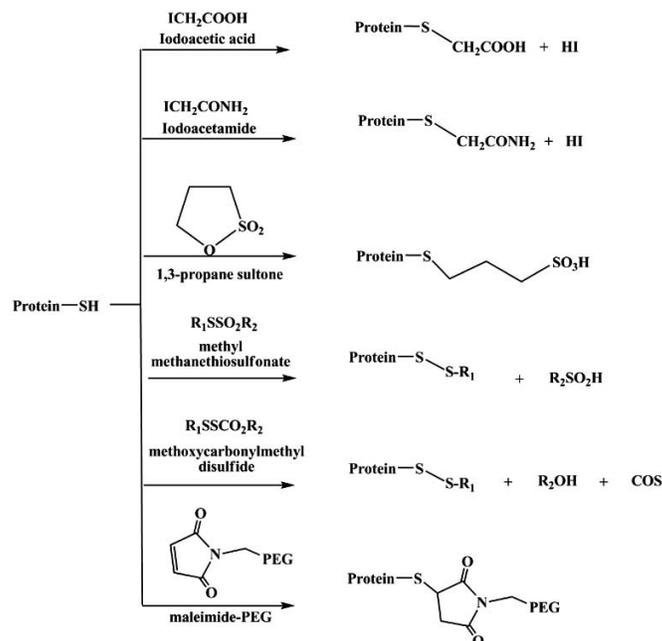


Fig. (7). Several common methods to derivatize the thiol group in proteins to prevent its reaction with other groups within the protein or with another protein. These derivatization reactions have also been used to improve the stability and solubility of proteins.

To avoid side reactions of the thiol group, Cys has frequently been mutated to Gly, Ala, and Ser residues in proteins. The C6A, C111S, and C6S/C111A mutants of human Cu-Zn superoxide dismutase (SOD) showed greater resistance to loss of enzymatic activity than did the respective native SOD enzymes upon thermal unfolding at 70 °C [75]. This corresponds to the fact that the mutants can reversibly refold after denaturation while the unfolded native SOD refolds incorrectly. Cys mutation studies have been used to determine the role of free and **disulfide-bonded** Cys residues in the function of many proteins.

Meprins, cell surface and secreted metalloendopeptidases, are oligomers made up of disulfide-linked multidomain subunits [76]. Mutations of C320A and C289A of mouse meprin- α both resulted in an increased susceptibility to proteolytic degradation and heat deactivation [76]. Although the mutants retained their catalytic activity toward a bradykinin peptide, they displayed decreased activity toward the protein substrate azocasein. It was found that the C320A

mutant formed only monomers while the C289A mutant formed dimers and oligomers, suggesting that Cys 320 but not C289 may be involved in inter-subunit disulfide formation [76]. Mutation of either Cys residue resulted in a change in protein structure, causing it to lose its enzymatic activity toward a protein substrate. Thus, Cys mutation is a technique that may not only shed light on the importance of the state of Cys residues (paired or reduced, intra- or inter-protein pairing) in a particular protein but may also help to distinguish the different roles of each Cys in the protein.

Cys residues of therapeutic proteins have also been PEGylated to improve their physicochemical stability, and pharmacokinetic and pharmacodynamic properties. PEGylated proteins usually have reduced glomerular filtration and immunogenicity. The molecular weight of these PEG reagents can range from 2 to 50 kDa. Since proteins generally contain fewer unpaired Cys residues compared to Lys residues, the PEGylation of a Cys residue is more selective than PEGylation of Lys residue. PEGylation of Lys residues gives a heterogeneous mixture of products with a distribution of conjugated products, which is difficult to control. The site-specific PEGylation of the Cys residue is normally carried out with PEG-maleimide, PEG-acrylamide, PEG-acrylate, PEG-vinyl sulfone, PEG-epoxide, or PEG-iodoacetamide. In a protein without a free thiol, a surface residue away from the active site can be mutated to a Cys residue followed by PEGylation. This method has been successfully used to improve the half-life of granulocyte colony-stimulating factor (G-CSF) [77] and the Q5C analog of interferon- α (IFN- α) [78], immunotoxin anti-Tac(Fv)-PE38 (LMB2) [79], and erythropoietin (EPO) [80]. When LMB2 was stabilized by PEGylation using 5- or 20-kDa PEG-maleimide, there was an increase in plasma stability (five- to eightfold) and anti-tumor activity (three- to fourfold) [79]. In this case, the animal toxicity and immunogenicity of LMB2 were significantly lower than that of the parent toxin. A native **disulfide bond** can also be conjugated with PEG by reaction with PEG-monosulfone; the product forms a three carbon bridge with the two sulfur atoms from the native disulfide bond. Interferon α -2b, anti-CD4⁺ antibody, and L-asparaginase also have been PEGylated in this manner [81]. In the case of interferon α -2b, the PEGylated product maintains its biological activity as well as its tertiary structure [82]. For an in-depth discussion of PEGylation, readers are directed toward excellent recent reviews [80-84].

CONCLUSIONS

Thiol groups and disulfides have important roles in the stability and solubility of proteins; however, these functional groups can be reactive under certain conditions, which may lead to chemical and physical instability of the proteins. In many cases, intramolecular and intermolecular disulfide bond exchanges could lead to the physical instability of the protein. The formation of non-native disulfide bond(s) may produce intermediate(s) that promote protein aggregation and precipitation. Thus, **it may be necessary to prevent intramolecular and intermolecular reaction of thiol groups in proteins**. In addition, thiol groups can be utilized to make **PEGylated protein** to improve protein stability *in vitro* and *in vivo*. It is expected that the efforts to understand the roles of thiols and disulfides in protein structure may lead to new

methods to increase success in formulating proteins as therapeutic agents.

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