Cysteine is present in a large number of natural and synthetic (bio)molecules. Although the thiol side chain of Cys can be in a free form, in most cases it forms a disulfide bond either with a second Cys (bridge) or with another thiol, as in the case of protecting groups. Efficient reduction of these disulfide bridges is a requirement for many applications of Cys-containing molecules in the fields of chemistry and biochemistry. Here we review reducing methods for disulfide bonds, taking into consideration the solubility of the substrates when selecting the appropriate reducing reagent.

1. Introduction

Peptides and proteins are formed by amino acids linked mainly by amide (peptide) and disulfide bonds. Although the latter are much less frequent, they confer unique physical and chemical assets to these biomolecules. The disulfide bonds are formed by the linkage of cysteine residues through their side chains. Interestingly, in spite of its key role, cysteine is one of the least abundant amino acids (Figure 1A) in all organisms, yet is a highly conserved residue in the functional sites of proteins. However, not all cysteine residues in living organisms are found in protein sequences as this amino acid is also one of the constituents of glutathione, the main redox regulator in cells. Indeed, high concentrations of glutathione are found in prokaryotic and eukaryotic cells. Glutathione is a triamino acid containing the linkage of cysteine residues through their side chains.

Glutathione is the predominant representative of the so-called low-molecular-weight (LMW) thiols. The members of this family of molecules act as redox buffers to protect cells against reactive oxygen species and nitrogen species, as well as electrophilic species, and even some antibiotics, etc. LMW thiols are structurally diverse, some of them, such as glutathione, homocysteine, coenzyme A and lipoic acid, are common in all organisms; others are organism-specific, such as mycothiol in actinomycetes, ergothioneine in fungi and mycobacteria, and bacillithiol in Firmicutes (Figure 2).

The disulfide bridges formed by two cysteine residues upon oxidation are essential to stabilize the tertiary structure of proteins and peptides, while at the same time conferring rigidity. The number of disulfide bonds in a single molecule can vary, and these bonds can be intra- and inter-molecular. This can be illustrated by the following examples: 1) human defensins (HDs), which are antimicrobial peptides (AMPs) component of the innate immune system, are small proteins (or large peptides) of up to 35 amino acid residues consisting of a triple β-sheet structure stabilized by three disulfide bonds (Figure 3A); 2) insulin is a hormone formed by two peptide chains linked by two disulfide bridges: chain A of 21 amino acid residues and chain B of 30 residues. Additionally, insulin has an intra-molecular disulfide bond in its A chain (Figure 3B); 3) antibodies are constituted by two heavy chains (Figure 3C, dark purple) and two light chains (Figure 3C, light purple), which contain intramolecular disulfide bonds and are linked by intermolecular disulfide bridges. The disulfide bond structure present in antibodies is evolutionarily highly conserved. However, two types of disulfide bridge can be distinguished in monoclonal antibodies (mAbs), namely the classical bridge, which is conserved, and the nonclassical bridge, which varies depending upon the mAb.

Nowadays, mAbs are strategic for pharmaceutical research and have further extension to clinical research. Disulfide bridges play a crucial role in the maintenance of mAb structure and function. However, because of the increasing interest in the development of antibody-drug conjugates (ADCs), there is a need for reducing agents able to chemoselectively release some free thiols in order to allow conjugation without modification of their functionality.

Furthermore, in addition to ADCs, many drugs on the market are cysteine-containing peptides. Thus, in the last three years, the FDA has approved Etelcalcetide for secondary hyperparathyroidism, Plicanatide for chronic idiopathic constipation, and Lu177 DOTA-TATE and Ga 68 DOTA-TOC for PET imaging – all three containing disulfide bridges. In addition, many other candidates that are in clinical and preclinical trials have one or more disulfide bridges.
The synthesis of multi cysteine-containing peptides may require the concourse of more than one type of cysteine protecting group for the purpose of regioselective chemistry. Examples of these distinct cysteine protecting groups are: 1) trityl (Trt), methyltrityl (Mtt), methoxytrityl (Mmt), which can be removed by a low concentration of trifluoroacetic acid (TFA); 2) diphenylmethyl (Dpm), which requires almost neat TFA for removal; 3) acetoamidomethyl (Acm), which is removed with I$_2$ or Pd(0)-catalyzed reactions; 4) phenylacetoamidomethyl (Phacm), which is enzymatically removable; 5) S-4,4'-dimethylsulfinylbenzhydryl (Msbh), which contains sulfoxide groups and is a safety-catch protecting group because it is stable to TFA, and after reduction of the sulfoxides to sulfides, it is a TFA-removable protecting group; and 6) S-tert-butyl (StBu) and S-(2,6-dimethoxythiophenol (S-Dmp), which are removed by reducing agents. If StBu/S-Dmp are used in combination with other kinds of protecting groups in a regioselective manner, StBu/S-Dmp should be removed first. If not, their removal in the

Sinenhlanhla N. Mthembu obtained her M.Sc. in pharmaceutical chemistry at the University of KwaZulu-Natal (South Africa). She is currently pursuing her Ph.D. in pharmaceutical chemistry at the same university. During her M.Sc. she worked on the synthesis of novel disulfide based reducing agent (DABDT). Her current work involves the development of novel strategies for the synthesis of disulfide-containing peptides by solid-phase peptide synthesis (SPPS).

Dr. Anamika Sharma is a Senior Post-doctoral fellow at the University of KwaZulu Natal (South Africa). She obtained her M.Sc. in general chemistry and Ph.D. in organic chemistry at the University of Mysore (India). She worked in the area of synthetic chemistry as a Research Associate at Syngene International (Bangalore, India). Her Ph.D. involved classical solution peptide synthesis and bioconjugation. Afterwards, she worked as Assistant Professor at Techno India (Kolkata, India). Her research includes the investigation of protecting groups for amino acids in solid-phase peptide synthesis and also broad areas in medicinal chemistry.

Fernando Albericio’s career has taken him through Europe, the USA, Latin America, and Asia to Africa (presently Research Professor at the University of KwaZulu-Natal (South Africa)). His research covers practically all aspects of peptide synthesis, as well as the synthesis of small molecules with therapeutic activities, especially against tuberculosis, infectious diseases, and cancer. His group is also involved in developing new strategies for drug delivery. He is involved in the third mission of the University: the transference of knowledge and technology to society. He was co-founder and general director of the Barcelona Science Park and is co-founder of BioDurban.

Beatriz G. de la Torre obtained her Ph.D. from the University of Barcelona (Spain). After a

The abundance of cysteine in different organisms. B) Structure of glutathione.
presence of other disulfide bridges leads to the reduction of this disulfide bridge too. Removal of StBu/S-Dmp is recommended while the peptide is still anchored to the resin. In this regard, the solid-phase reaction facilitates the removal of the excess of reducing agents and also allows the use of acid-labile protecting groups, which are removed during the global deprotection and cleavage of the peptide from the resin. In this context, it is important to have access to reducing agents compatible with organic solvents, which are the most suitable solvents for a broad range of solid supports used in SPPS.

During the last several decades, disulfide reducing agents have been widely used in many molecular biology protocols to stabilize free sulfhydryl groups and/or to reduce disulfide bonds in proteins and peptides. In the present review, we have classified the most widely used disulfide reducing agents into two groups on the basis of their chemical nature. One group is formed by thiol derivatives, which include monothiols and dithiols, while the second is formed by phosphine derivatives.

2. Reducers Based on Thiols

Thiols allow easy and specific reduction of disulfide bonds and are therefore the most widely used reagents for this purpose. The reaction is essentially a disulfide exchange achieved by means of a $S_	ext{N}2$ nucleophilic substitution (Figure 4A). This

Figure 2. Naturally occurring LMW thiols.

Figure 3. Inter- and intramolecular disulfide bridges. A) Human defensins, B) insulin and C) monoclonal antibody.
reaction, although simple, is limited by several factors. First, the thiol of the reducing agent must be deprotonated, thereby leading to the thiolate, which is a much better nucleophile. Thus, the process requires base catalysis and is pH dependent. The $pK_a$ of the thiol will be pivotal for the success of the reaction. For instance, at physiological pH, the more acidic the thiol is, that is, the lower the $pK_a$, the higher the amount of thiolate generated (Figure 4B).

Once the thiolate ($R_1-S^-$) is present in the reaction media, the nucleophilic attack occurs on the most electrophilic sulfur on the disulfide, in other words, on the least acidic thiol in the mixed disulfide. The most acidic thiol is then is released. In the scheme shown in Figure 4A $pK_a(R^2-SH) > pK_a'(R^1-SH)$.[11]

In addition, as in any $S_N2$ mechanism reaction, two more factors, namely steric hindrance and the solvent, affect the reaction rate and success of the process. Regarding the former, the disulfide must be as unhindered as possible, since the reaction becomes difficult when a tertiary thiol is part of it. With respect to the solvent, aprotic polar solvents work better because hydrogen bonding with the thiolate are not possible.

In general, there are two main drawbacks to the use of thiols as reducing agents. First, they have to be removed from the media when the sulfhydryl released is to be quantified or is to be involved in further reactions. Second, they are foul-smelling and toxic.

### 2.1. Monothiol

Monothiols have been widely used for the reduction of disulfide bonds and to keep sulphydryl groups in their form. Among the most used monothiols in the laboratory it worth mention glutathione, the reducer by excellence of the living organisms, cysteine and lipoic acid. However, $\beta$-mercaptoethanol ($\beta$-ME)[12] is unquestionably the monothiol most extensively used. The presence of the hydroxyl group confers several advantages to this molecule. In this regard, this group makes the molecule water-soluble, reduces the thiol $pK_a$ (9.6) one-unit with respect to ethanethiol (10.6), thereby favoring thiolate formation, and lowers volatility. However, $\beta$-ME is still foul-smelling.

As mentioned above, the thiol-disulfide interchange is regulated by several equilibria. When monothiols are used as reducing agent, at least two molecules of monothiol are needed to drive the reaction to completion. However, to ensure that the reaction does not stop in the formation of the mixed disulfide (Scheme 1, blue-red molecule), a large excess of the reductive thiol (Scheme 1, red) should be used. In this scenario, equilibria [1] and [2] are pushed to generate the two desired free thiols (Scheme 1, blue).

In the case of $\beta$-ME, reductions are usually carried out in high molar excesses to ensure completion of the reaction. It should be noted that $\beta$-ME is an inexpensive chemical and the only byproduct of this process is the oxidized $\beta$-ME, however, its disadvantage is the high toxicity.[14]
2.2. Dithiol

The first revolutionary introduction of a new reductive thiol was in 1963 by Cleland.\(^1\) He designed a diol molecule that adopted a stable cyclic structure in its oxidized form. To this end, Cleland synthesized two epimers of 1,4-dimercaptobutane-2,3-diol, known as dithiothreitol (DTT) and dithioerythritol (DTE; Figure 5).

Compared with \(\beta\)-ME, reduction using DTT or DTE was faster. In addition, because these compounds are solid, they are less odorous. Of DTT and DTE, the former is widely accepted as a reducing agent in the field of chemistry and biochemistry, mainly due to its ease of synthesis.\(^1\) The stability of the cyclic six-membered oxidized product formed drives the shift in the equilibrium forward, thereby avoiding the use of excess reducing agent and thus contrasting with \(\beta\)-ME.

The high stability of cyclic oxidized DTT implies that the reduction does not render the mixed disulfide species (Scheme 1). However, DTT has two limitations: 1) as a reducing agent, it is nonfunctional below pH 7 because the \(pK_a\) of the two thiol groups are 9.2 and 10.1, respectively, and 2) it is unstable in solution, having a very short half-life.\(^1\) For instance, its half-life is around 10 h at pH 7.5 but drops to 1.4 h at pH 8.5. Therefore, DTT solutions should be freshly prepared.

Owing to the limitations of DTT, the past few decades have witnessed efforts to identify a potential reducing agent based on a similar strategy and characterized by the capacity to reduce disulfides efficiently in neutral pH and with a reducing potential in the range of DTT. In this regard, special mention is given to Whitesides’ group, who developed several such agents.

More recently, Raines and co-workers have also worked on the use and development of disulfide reducing agents. The most significant one is (2\(S\))-2-amino-1,4-dimercaptobutane (DTBA), derived from aspartic acid (Figure 7).\(^2\) The \(pK_a\) value of the sulfhydryl groups in DTBA are one unit lower than in DTT, thereby making it a better reductant at lower pH. In addition, although the disulfide reduction potential of DTBA is slightly less than that of DTT, in general, it is faster. The weakness of DTBA...
lies in the same point as its strength, namely the presence of the amino group. As DTBA is protonated at physiological pH, its reduction ability is affected by unfavorable electrostatic interactions in the case of the cationic active sites of enzymes. To overcome this drawback, they designed another reducing agent, 2,3-bis(mercaptomethyl)pyrazine (BMMP; Figure 7).

This molecule showed lower thiol $pK_a$ values than the previous one and can therefore reduce disulfide bonds over a wider pH range.

The reducing agents discussed so far were developed for the reduction of proteins and peptides in aqueous media. However, an important niche to be addressed is the reduction of disulfide bridges supported in solid-phase, mainly as protecting groups of cysteine-containing peptides. In this case, the scenario differs considerably because the reducing agent should be soluble in organic solvents, compatible with the solid support, and non-malodorous, nontoxic, and stable to air oxidation, etc. With this in mind, our group followed a strategy to convert one of the water soluble reducing reagents already described into one that is soluble in organic solvents. As in case of DTBA, we prepared the new reducing agent starting from aspartic acid, but we introduced nonpolar electron-withdrawing moieties in the amino function. Under this premise, we obtained 2-(dibenzylamino)butane-1,4-dithiol (DABDT; Figure 8), which showed good solubility in a wide range of organic solvents, higher stability to air oxidation than DTT in solution, and good reducing ability in solution and solid-phase.

3. Reducers Based on Phosphines

Tertiary phosphines have long been known as disulfide reductants. Triphenyl phosphine was the first to be evaluated. Although it was able to reduce aromatic disulfides, it failed to reduce aliphatic disulfides because of its low nucleophilicity. When triphenyl phosphine was substituted by the more nucleophilic tributyl phosphine, all kinds of disulfide bonds could be reduced.

The nucleophilicity of the phosphine in the reduction process is crucial since, as in the case of thiol-based reducing agents, the reaction involves a $S_2$ mechanism. The reaction proceeds with initial nucleophilic attack of the phosphorus on one of the sulfurs of the $S-S$ bond, with the formation of thiophosphonium carbocation and the release of the first sulfhydryl. This step is reaction rate-determining. The phosphonium ion then undergoes hydrolysis to afford the phosphine oxide and the second sulfhydryl (Scheme 2). This second step only occurs in the presence of water, but once it happens the process is irreversible because of the stability of the phosphine oxide formed. This irreversibility avoids the need for excess reagent, thereby making phosphines a better choice than thiols as disulfide reducers.

Although there are several reports on the application of tributyl phosphine to reduce disulfides, the use of this agent was never extended because of its poor water solubility and, being a liquid, its high volatility makes it highly toxic with unpleasant smell. In addition, its tendency to undergo rapid air oxidation poses a significant challenge for practical applications.

![Scheme 2. Mechanism of disulfide reduction by phosphines. i) Formation of thiophosphonium cation, and ii) hydrolysis of the cationic intermediate to form the phosphine oxide.](image-url)
oxidation limits its application as a common disulfide reducing agent.

Among water-soluble tertiary phosphines, tris-(hydroxymethyl) phosphine (THMP) and tris-(2-carboxyethyl)phosphine (TCEP; Figure 9) were slightly used in the past. The former has several drawbacks. It is an odorous liquid and could generate byproducts such as formaldehyde during protein reduction. Instead, the second was not widely adopted because of its elevated cost and lack of commercial availability. However, since 1991, when Whitesides and co-workers introduced an easy synthetic procedure, TCEP became commercially available as hydrochloride (TCEP·HCl).

TCEP·HCl has multiple advantages over other disulfide reducing agents. It is a crystalline solid, non-volatile, odorless, and water-soluble, and it is not susceptible to oxidation and can therefore be manipulated in air. From the reactivity point of view, it has a greater reducing capacity than DTT, the reduction reaction is faster, and it can be run in a wide pH range. Thus, the properties of TCEP led to it becoming one of the most extensively used disulfide reducing agents.

In addition to TCEP, the reducing ability of tris(3-hydroxypropyl)phosphine (THPP; Figure 9) has been demonstrated. THPP has similar properties to TCEP and the advantage of being efficient not only in aqueous media but also in buffered aqueous-organic media. Moreover, THPP shows higher resistance to air oxidation than TCEP, a property that makes it a friendly reducer in open laboratory conditions.

Despite all the advantages of phosphine-based reducing agents mentioned above, they cannot be considered universal reducing agents. When their use became more common, they were considered to be innocuous for further conjugation of the released sulfhydryl. Nevertheless, it has been demonstrated that TCEP and THPP can react with the maleimide linker (by Michael addition) and iodoacetamide (by bimolecular nucleophilic substitution, $S_n2$) both commonly used in thiol-based bioconjugation. In case of maleimide, TCEP produces a stable ylene adduct that is unreactive to thiols, whereas THPP makes the maleimide unreactive by reduction to succinimide (Figure 10).

Therefore, to eliminate these side reactions, it is advisable to remove the reducing agent before conjugation. These agents can be removed using various approaches, including dialysis, immobilized TCEP resin, and column chromatography. However, all these methods have limitations. Recent advances involve the quenching of excess TCEP and THPP by either treatment with 4-azidobenzoic acid or water-soluble PEG-azides by Staudinger reaction, hence circumventing the purification step in maleimide-based bioconjugations.

In addition of the previous side-reactions, TCEP could also modified cysteine-containing peptides. Thus, non-desired desulfurization of cysteine generating the alanine residue has been observed during native chemical ligation (NCL) and during the sample preparation (denaturalization) for protein character-
ization by mass spectrometry.[37] In the later, it has also been described cleavage generating protein fragments.[38]  

4. Miscellaneous

A wide range of other reducing agents have also been evaluated for the reduction of disulfide bridges.[29,30] These methods, however, find application mainly for organic disulfides and have limited application in cysteine disulfide reduction. Furthermore, these methods have prolonged reaction times, higher temperatures and even reflux, dry conditions and sometimes they are inefficient towards sterically hindered disulfides, thereby limiting their applicability in peptide/protein chemistry.[30] Some of those that have been used in the reduction of the disulfide in proteins are shown in Table 1.

5. Concluding Remarks

The oxidation and reduction of cysteine-containing biomolecules play a key role in a broad range of scientific fields, from understanding biochemical pathways to drug discovery. As these biomolecules show great diversity, there is no universal reducing agent available. An appropriate reducing agent should be selected for each particular case. In general, dithiol reducing agents, whose oxidized form is a cycle, are the most useful. DTT and DTBA are suitable for aqueous solvents. The recently described DABTD, which is compatible with organic solvents, is appropriate for protected peptides anchored to the resin. Phosphine-based reducing agents are indicated for releasing free thiols that are to be further conjugated to other molecules. Phosphine interferes less with the next reaction than thiols.

Acknowledgements

This work was funded in part by the National Research Foundation (NRF) and the University of KwaZulu-Natal, MINECO, (RTI2018-09381-B-100), and the Generalitat de Catalunya (2017 SGR 1439) (Spain).

Conflict of Interest

The authors declare no conflict of interest.


Manuscript received: February 16, 2020
Revised manuscript received: March 19, 2020
Accepted manuscript online: March 20, 2020
Version of record online: ■ ■ ■ ■
MINIREVIEWS

Breaking bridges: The efficient reduction of disulfide bridges between two cysteine residues or a cysteine and a protecting group is a requirement for many applications of cysteine-containing molecules in the fields of chemistry and biochemistry.

S. N. Mthembu, Dr. A. Sharma, Prof. F. Albericio*, B. G. De la Torre*

1 – 10

Breaking a Couple: Disulfide Reducing Agents

Breaking a couple: disulfide reducing agents (@FAlbericio, de la Torre @UKZN, @UniBarcelona)

Share your work on social media! ChemBioChem has added Twitter as a means to promote your article. Twitter is an online microblogging service that enables its users to send and read short messages and media, known as tweets. Please check the pre-written tweet in the galley proofs for accuracy. If you, your team, or institution have a Twitter account, please include its handle @username. Please use hashtags only for the most important keywords, such as #catalysis, #nanoparticles, or #proteindesign. The ToC picture and a link to your article will be added automatically, so the tweet text must not exceed 250 characters. This tweet will be posted on the journal’s Twitter account (follow us @ChemBioChem) upon publication of your article in its final (possibly unpaginated) form. We recommend you to re-tweet it to alert more researchers about your publication, or to point it out to your institution’s social media team.

ORCID (Open Researcher and Contributor ID)

Please check that the ORCID identifiers listed below are correct. We encourage all authors to provide an ORCID identifier for each coauthor. ORCID is a registry that provides researchers with a unique digital identifier. Some funding agencies recommend or even require the inclusion of ORCID IDs in all published articles, and authors should consult their funding agency guidelines for details. Registration is easy and free; for further information, see http://orcid.org/

Sinenhlanhla N. Mthembu
Dr. Anamika Sharma
Prof. Fernando Albericio
Beatriz G. De la Torre  
http://orcid.org/0000-0001-8521-9172