is essential for maintaining the biological activity. Ribonuclease, for example, loses almost all activity when the four disulfide bonds are reduced. The mechanism of reaction involves the cysteinyl and cystinyl residues in the disulfide bond formation by oxidation or reduction, conversion of a cystinyl residue to a cysteinyl residue and a sulfenic/sulfonic acid residue at alkaline pH, and decomposition to a dehydroalanine residue at alkaline pH (β-elimination reaction). Disulfide bond formation is often catalyzed by the presence of a mercapto reagent (e.g., DTT and cysteine) in millimolar concentrations (typically 1–10 mM). Controlled disulfide bond formation has gained much attention in the biopharmaceutical industry in connection with the in vitro folding of proteins expressed in *Escherichia coli*. Presence of divalent metal ions (typically Cu^{2+}) may result in the oxidation of cysteinyl residues by an ill-defined reaction mechanism. Cleavage of the disulfide bond is initiated by an attack on a sulfur atom by a nucleophile reagent (HS−, RS−, CN−, SO_3^-, or OH−). The reaction, which takes place at neutral to alkaline pH, consists of two steps, with a formation of a mixed disulfide as the intermediary step. The indicators of cysteinyl residues include intermolecular disulfide bond formation, resulting in aggregation; under reducing conditions, the disulfide bonds destabilize, resulting in the conversion of cystinyl to cysteinyl residues (in vitro refolding may be the only solution to reestablish the correct disulfide bonds), the presence of scrambled and structural altered forms, and smell of sulfur. Be careful when adjusting pH with high concentrations of NaOH. Locally high pH may facilitate β-elimination. Preventive actions against cysteinyl residue loss are described in Table 9.10.

TABLE 9.10

Preventive Actions Against Cysteinyl Residue Loss

Factor	Comment
pH	Minimum reactivity is expected in the pH range 3–7. The reactivity of the –SH group is at maximum above the pK_a (8.5), where the group is deprotonated. In strongly acidic media the reaction is expected to take place via a sulfenium cation by an electrophile displacement.
Protein concentration	The intramolecular disulfide bond formation is a first-order reaction and thus independent of protein concentration. Intermolecular reactions via the cysteinyl residue may be affected by the protein concentration (aggregation). The aggregation rate is favored by high protein concentration.
Temperature	The temperature is kept low (4°C–20°C) especially at pH above 9.5.
Time	The reaction is a function of time.
Conductivity	No data available.
Redox potential	Reducing conditions favor free cysteinyl residues. Oxidizing conditions favor disulfide bonds. The redox potential is a function of pH (60 mV/pH unit).
Co-solvents	Cysteine (nonanimal origin) is recommended as a reducing agent for large-scale operations. Divalent metal ions are removed by EDTA.

Source: *Handbook of Biogeneric Therapeutic Proteins: Regulatory, Manufacturing, Testing and Intellectual Property Issues*, Taylor & Francis Group, Boca Raton, FL, 2005.

Abbreviation: EDTA, ethylenediaminetetraacetic acid.