11
Role of Disulfide Bonds in Peptide and Protein Conformation

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11.1
Introduction

The native structures of proteins and peptides are stabilized by a number of interactions that dictate directly or indirectly the folding, conformation, and flexibility of the molecule. Most of these interactions, such as hydrogen bonds and hydrophobic interactions, are noncovalent and relatively weak. Covalent interactions, on the other hand, are generally stronger, and are thought to exert a greater influence on stability and conformation, particularly in peptides, which tend to have fewer and weaker hydrophobic interactions because of their smaller size. The most common example of a covalent interaction is the disulfide bond, formed between the sulfur atoms of two cysteine residues [1]. Disulfide bonds are typically present in extracellular proteins and peptides, such as growth factors, hormones, enzymes, and toxins, and have also been found in several thermostable intracellular proteins of archaeal microbes [2]. They can play a role in covalently linking subunits in protein complexes (e.g., the heavy and light chains of antibodies or the two peptide chains of insulin) [3]. Structurally, the strong covalent links formed by disulfide bonds are thought to confer additional conformational stability on proteins such as keratin that have a high disulfide content [4]. Disulfides also play a catalytic role in enzymes such as thioredoxin, which acts as a cellular redox sensor via the oxidation status of its thiol groups [5–7]. Many disulfides in proteins appear to have no direct functional role, rather their main purpose is to maintain the conformation of the protein. In certain cases, conformational changes associated with the reduction and oxidation of these bonds may allow a protein to switch between different functions [8, 9]. The relationship between disulfide bonds and the conformation of peptides and proteins is thus an intriguing one. This chapter provides a broad overview of the roles of disulfide bonds in peptides, focusing on their roles in the folding and stability of proteins, as well as how different disulfide bonding patterns and connectivity give rise to different protein topologies and conformations, and ultimately a diverse range of protein functions. An understanding of these roles also has important applications in the field of protein engineering and drug design.

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11.2 Probing the Role of Disulfide Bonds

Several strategies have been applied to probe the roles of disulfide bonds. Typically, the disulfide bond under study is removed by replacing the selected cysteine pair with alanine, aminobutyric acid, or serine residues, and the effects on folding, stability, conformation, and activity of the peptide are monitored [10, 11]. The effects of removing a disulfide bond upon protein folding are often studied in refolding experiments using folding buffers, the most common being a mixture of oxidized and reduced glutathione, which allows the disulfides to exchange reversibly between their oxidized and reduced forms [12, 13].

Protein stability can also be measured in reductive unfolding experiments, where the protein is subjected to a strong reducing agent such as dithiothreitol (DTT) and both its unfolding kinetics and conformation are monitored as it unfolds through the progressive loss of disulfide bonds [13]. Nuclear magnetic resonance (NMR), X-ray crystallography, circular dichroism, or fluorescence spectroscopy can be used to assess the consequences of removing individual disulfide bonds upon protein structure and function. NMR spectroscopy can provide additional information on the conformational flexibility and stability of the protein [14]. Other studies have focused more on the evolution of disulfide bonds in stabilizing known protein folds, focusing on the prevalence, distribution, and position of these bonds in small disulfide-rich domains, as well as factors leading to different disulfide connectivities in various protein folds, such as conserved cysteine frameworks, amino acid sequences, and intercysteine loop size and composition [15, 16].

11.3 Contribution of Disulfide Bonds to Protein Stability

It is generally accepted that disulfide bonds enhance the thermodynamic stability of proteins, making them less susceptible to denaturation and degradation in the extracellular environment, and more resistant to extremes of temperature and pH. Stability here refers to the stability of the native fold to temperature or other denaturants rather than dynamic stability, which will be discussed below. Precisely how these disulfide bonds confer stability has been the subject of some debate. One explanation is that disulfide bonds reduce the conformational freedom of the protein in its unfolded state, reducing the entropy of this state and thus destabilizing it relative to the folded state [1, 17]. Another posits that disulfide bonds destabilize the unfolded state of the protein by sterically preventing an effective hydrogen bonding network from forming [18]. While such theories focus on destabilization of the unfolded state, it has also been argued that disulfides can destabilize the folded state of a protein by reducing its conformational freedom, taking into account that the folded state is not entirely static [19, 20]. Disulfide bridges in flexible regions decrease the native state entropy more so than bridges between rigid regions. These entropic effects are potentially countered by similar but opposite enthalpic effects: bridges

between rigid regions may cause torsional strain and local repacking, whereas those between flexible regions are more readily accommodated with little loss of favorable enthalpic interactions. Thus, native state entropic and enthalpic effects must be considered as well as unfolded state entropic effects. As noted by Betz [19], crystallographic data on novel disulfides introduced into subtilisin BPN′ [21, 22] indicate that disulfides are more readily accommodated by flexible regions rather than rigid ones, suggesting that enthalpic native state effects may dominate negative entropic considerations.

Loop sizes formed by native disulfides are usually large and their disulfide connectivities often result in disulfide bonds being formed between cysteines that are not sequential in the peptide sequence. These factors potentially lead to inefficient formation of the native disulfides, as seen in the cone shell peptide ω-MVIIA [23], as they are difficult to form based on random probability. Yet such linkages are often observed, especially in small peptides, as they contribute to the relative stability of the native fold by greatly reducing the conformational entropy of the unfolded peptide chain. Unfolding of a protein is usually measured by exposing the native protein to strong reducing conditions, such as DTT, and monitoring the loss of disulfide bonds and native structure. As expected, the most solvent exposed and unprotected disulfide is usually the most susceptible to reduction and the resulting partially reduced structure largely retains native structure. Further reduction of buried disulfides usually results in a loss of native conformation. In a statistical study of proteins in the Protein Data Bank (PDB [24]), the stability of proteins was correlated with chain length and the presence of disulfide bonds [25]; this study found that the number of disulfide bonds per residue was negatively correlated with the length of the protein chain in smaller proteins (less than 200 residues), but no correlation was observed in larger proteins. A positive correlation was found between the number of disulfide bonds and the favorable free energy that stabilizes the native state relative to its unfolded state, thus affirming the stabilizing effect disulfide bonds confer on proteins in general, and especially on short proteins with fewer hydrophobic interactions. Indeed, it is not uncommon for 20–50 residue peptides such as toxins to have three or more disulfide bonds stabilizing their structure. This stabilizing effect of disulfide bonds has been used to advantage in engineering protein scaffolds, as discussed below.

11.4 Role of Disulfide Bonds in Protein Folding

It is well established that the information required for the folding of a protein is encrypted within its primary amino acid sequence [26–28]. The formation of disulfide bonds is often an integral component of the folding pathway of a protein. Folding yields and efficiency are often determined by the ability of a protein to form the native disulfide bonds, but what exactly determines the formation of these bonds is not always obvious, and is complicated by the presence of other interactions and by whether these interactions promote the preferential formation of native disulfides.

The mechanism of protein folding has been studied extensively, with some of the best-studied models being small proteins with multiple disulfide bonds, such as bovine pancreatic trypsin inhibitor (BPTI) and bovine pancreatic ribonuclease A (RNase A) [12]. The isolation of stable disulfide intermediates in the folding pathway of these polypeptides has led to a more comprehensive understanding of the role of disulfides in driving protein folding, as well as their overall importance relative to other interactions [29, 30]. Several theories have been proposed regarding the role played by disulfide bonds in protein folding. One generally accepted model is a stepwise model in which a protein folds to its native structure via stable disulfide intermediates that are partially folded and form a native-like subdomain [31]. This was observed for BPTI, in which a stable intermediate containing a single disulfide bond between residues Cys30 and Cys51 was identified as a stable intermediate in the folding pathway, and was shown to adopt a partially native conformation comprising the structurally crucial α-helical and β-sheet regions of BPTI, but with the N-terminal region remaining unfolded [32]. Subsequently, the partially folded intermediate serves as a template to direct formation of the remaining disulfides or causes steric inhibition to prevent the formation of non-native disulfides, allowing the protein to fold into its native conformation [31, 33].

In the 25-residue peptide ω-MVIIA, by contrast, no singly or doubly disulfide-bonded intermediates formed preferentially during the initial phase of folding, pointing towards the lack of conformational specificity in driving native disulfide bond formation [23, 34]. However, once two native disulfide bonds had formed, formation of the final disulfide was favored, suggesting that other native interactions (hydrophobic, electrostatic, etc.) became more prominently involved once the polypeptide chain was significantly restrained. Thus, the disulfides play an important role in driving this polypeptide into a more compact conformation, allowing subsequent interactions to complete the final stages of folding.

In contrast to the case of small peptides such as ω-MVIIA that lack a hydrophobic core, the exact role played by disulfide bonds in the folding of larger peptides and proteins such as BPTI and RNase A is complicated by contributions from a larger number of noncovalent native interactions. Indeed, such interactions are solely responsible for driving folding of proteins lacking disulfide bonds. An important question that arises is whether hydrophobic interactions or disulfide formation drive the folding of disulfide-rich proteins. Several protein folding models suggest that conformational sampling of the native structure, either locally or globally, allows the formation of disulfide bonds which then stabilize the native conformation [35–38]. Such a mechanism implies that disulfide bonds do not direct folding, but rather stabilize the native conformation. An alternative model proposes that disulfide formation drives protein folding [39]. Local changes induced by the formation of a single disulfide bond can direct the global folding of a protein, which often occurs cooperatively. Disulfide formation can bring about a direct global effect by bringing together residues that are far apart in the sequence and promoting the formation of a folding nucleus [40]. As proposed by Wedemeyer *et al.* for RNase A [13], many non-native disulfide intermediates form randomly during the initial phase of folding and these non-native species go through several rearrangements until a specific set of

native disulfide bonds is formed that are able to direct folding. Once this occurs, the resulting structure is folded such that the disulfides are protected from rearrangement by being buried within the structure; subsequently, the remaining disulfides are formed preferentially.

Less well understood is the role of disulfide bonds in protein folding *in vivo*. Several factors potentially affect the formation of disulfides and protein folding in the cell, as reviewed recently by Bulaj and Walewska [41]. The presence of folding catalysts and molecular chaperones can have a strong influence on the folding of a protein into its native state. In particular, the enzyme protein disulfide isomerase is responsible for oxidative folding in the endoplasmic reticulum of the cell, playing an important role in the formation of natively paired disulfide bonds [42, 43]; this enzyme is present in organisms such as the cone snails that elaborate disulfide-rich peptides [44]. Other factors such as precursor sequences may also be involved in oxidative folding in the cell. Precursor sequences had little effect on the folding of ω-conotoxin MVIIA and potato carboxypeptidase inhibitor [45, 46], but studies on other propeptides demonstrated a direct involvement of the precursor sequence in folding. The peptide hormone guanylin is an interesting example with relevance to disulfide-coupled folding as its prosequence contains a disulfide bond in addition to the two native disulfide bonds of the mature hormone [47]. Lauber's group demonstrated that the disulfide bond in the prosequence was necessary for stabilizing the tertiary structure of the prohormone, which in turn favors the formation of the native disulfide bonds, as opposed to the cysteine residues of the prosequence being involved in the formation of non-native disulfide intermediates during the oxidative folding of proguanylin [47]. In bacterial cells, the enzyme DsbA catalyzes the oxidative folding of proteins by initiating disulfide bond formation. The mechanism of this enzyme was described recently by Kadokura *et al.* [48], who found that the oxidative folding process was even affected by whether the protein was translocated cotranslationally or post-translationally.

11.5 Role of Individual Disulfide Bonds in Protein Structure

The structure of the disulfide linkage itself plays an important role in protein structure as a consequence of the covalent geometry of the sulfur–sulfur bond. The covalently linked sulfur atoms are 2.0 Å apart and the C–S–S–C dihedral angle is preferably in the 90–100° range [1]. As summarized by Richardson [1], the disulfide bond adopts preferred conformations based on its cysteine side-chain χ angles, with most disulfides having either a left-handed spiral conformation ($\approx\chi_1 = -60°$, $\chi_2 = -90°$, $\chi_3 = -90°$, $\chi_2' = -90°$, $\chi_1' = -60°$) or a right-handed hook conformation ($\approx\chi_1 = -60°$, $\chi_2 = +120°$, $\chi_3 = +90°$, $\chi_2' = -50°$, $\chi_1' = -60°$) (Figure 11.1). The C^{α} distances between the two connecting cysteines are also constrained to less than 6.5 Å in most cases as a result of the rigid torsion and preferred conformations of the disulfide bond [1]. Thus, disulfide bonds have a significant influence on protein structure in their immediate vicinity.

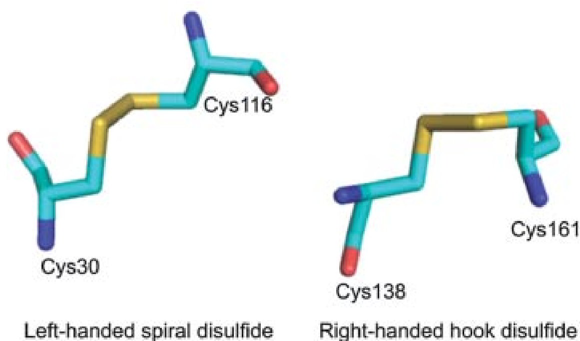

Figure 11.1 Two major conformations of disulfide bonds: left-handed spiral formed by the Cys30–Cys116 disulfide of hen egg white lysozyme (PDB ID: 1LZ1) and right-handed hook formed by the Cys138–Cys161 disulfide of carboxypeptidase A (PDB ID: 5CPA) Disulfide bonds are colored yellow. (Figure prepared using PyMOL; www.pymol.org).

Disulfide bonds are generally well conserved in protein families with similar folds, yet several mutational studies in proteins containing multiple disulfide bonds have shown that individual disulfides are not always necessary for maintaining the native fold. Removing a specific disulfide bond may have minimal effects on the structure and function of the protein. The crystal structure of BPTI lacking the Cys14–Cys38 disulfide bond, for example, was almost identical to the native structure [49]. The Cys13–Cys33 disulfide-deficient analog of the scorpion toxin charybdotoxin (ChTx) was also reported to adopt a native conformation [50]. In many cases, deletion of a disulfide bond was accompanied by local changes in structure but the global fold remained intact. Removing the Cys6–Cys20 disulfide bond in epidermal growth factor (EGF) [10] or the Cys1–Cys9 disulfide bond of the μ-conotoxin μ-KIIIA [51] resulted in local conformational changes in the N-terminal region but preservation of the overall fold. Disulfide deletion can sometimes alter local structural features of a protein. In the scorpion toxin α-KTx6, removing the fourth disulfide bond caused a local change in its two-stranded β-sheet from a twisted to a nontwisted conformation [52], while, in insulin-like growth factor I, removing the Cys47–Cys52 disulfide bond resulted in the local unfolding of a proximal α-helix [53].

Other studies, by contrast, have shown that deleting a specific disulfide bond results in significant changes in structure. For example, NMR analysis of disulfide-deficient analogs of ShK toxin indicated a drastic loss of native structure for each of the three disulfide-deficient analogs, with the analog missing the Cys3–Cys35 disulfide bond adopting a well-defined but altered structure [54] (Figure 11.2). Similarly, a loss of native structure was reported upon elimination of the Cys3–Cys13 disulfide bond of α-conotoxin GI [55] and the Cys44–Cys59 disulfide bond of enterotoxin B [56].

In small proteins and peptides with multiple disulfide bonds, the most solvent-exposed disulfide can usually be removed without a drastic loss in structure, but disulfides that are buried tend to be more crucial in maintaining the native fold of the

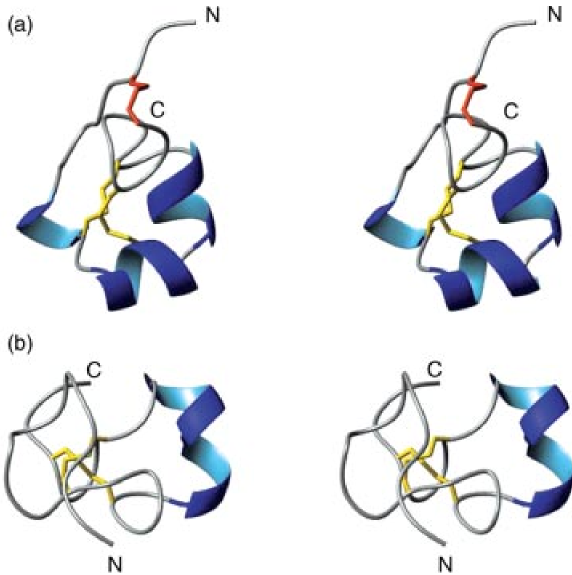

Figure 11.2 Stereoviews of (a) ShK (PDB ID: 1ROO) and (b) [Abu3,35]ShK$_{12-28,17-32}$ (PDB ID: 1C2U). Cys12–Cys28 and Cys17–Cys32 disulfide bonds are shown in gold, and the Cys3–Cys35 disulfide bond of ShK is shown in orange. The two molecules were superimposed over backbone heavy atoms of residues 14–24 and are shown in the same orientation.

structure [54, 57]. However, in most cases the removal of two or more disulfides results in a complete loss of native structure.

Less well studied are the conformational changes associated with the reduction of disulfides in extracellular proteins upon entry into the reducing environment of the cell [58]. It is thought that disulfides in these proteins act as redox switches rather than as stabilizers of native structure [59]. These disulfides are thought to adopt a strained conformation, storing potential energy that is released upon cleavage of this bond following cell entry [58]. The release of energy results in a conformational change in the protein which may be necessary for its function. In other redox-sensitive proteins such as Hsp33 in *Escherichia coli*, cysteine residues are oxidized to form disulfide bonds in response to oxidative stress. In this manner, the functions of such proteins are regulated by the conformational change associated with formation of these bonds [8].

11.6
Disulfide Bonds in Protein Dynamics

The conformation of a globular protein is intimately linked to its biological activity and it is expected that changes in conformation will have functional consequences. However, disulfide deletion studies have shown that this does not always hold true. Removal of a disulfide bond may affect the biological activity, but not the overall fold

of a protein. For example, deletion of the 6–20 disulfide bridge in EGF resulted in a loss of activity despite retention of a native-like fold [10]. The conotoxin μ-KIIIA provides an intermediate example, with activity being retained on the voltage-gated sodium channels $Na_V1.2$ and $Na_V1.4$ following removal of the first disulfide bond, and the structure also remaining native-like [51], but the selectivity profile for the other channel subtypes being different and the binding kinetics to the $Na_V1.2$ and $Na_V1.4$ channels faster. In contrast, there are several examples of disulfide-deficient analogs retaining their activity despite the loss of native structure. Deletion of the Cys3–Cys11 disulfide bond in endothelin-1, for example, resulted in a switch from a "cyclic" to "linear" conformation, but with retention of biological activity [60]. Similarly the Cys3–Cys35 disulfide-deficient analog of ShK retained its potassium channel blocking activity even though its structure was significantly different from that of the native toxin [54]. Such examples reflect the fact that flexibility and conformational stability also play important roles in activity, and that disulfide bonds contribute significantly to these physical attributes of a protein. Conformational stability here refers to dynamic stability of the protein as opposed to thermodynamic stability discussed above.

NMR is a powerful method for determining the stability of peptide or protein structures [14, 61]. In addition, NMR relaxation parameters can be measured to quantify conformational flexibility [62–65]. In barnase, a small globular protein lacking native disulfide bonds, two disulfides were introduced, leading to a local decrease in flexibility of the protein [66]. Similarly, a surface disulfide bond engineered into the cleft region of cytochrome b_5 dampened the dynamics of this enzyme [67]. NMR relaxation measurements carried out on oxidized and reduced forms of the *E. coli* enzymes thioredoxin [68] and glutaredoxin-1 [69] indicated that in both cases the disulfide bonds restricted the internal motions in these proteins. In BPTI, removal of the Cys14–Cys38 disulfide bond had only a minor effect on the backbone dynamics in wild-type BPTI, but a significant effect when removed in the Y35G mutant [70]. This observation highlights the cooperation between disulfide bonding and hydrophobic interactions in the dynamic stability of a structured polypeptide such as BPTI.

Intrinsically unstructured proteins (IUPs), a class of proteins that generally lack disulfide bonds, are unstructured and highly dynamic [71]. The 221-residue merozoite surface protein 2 (MSP2) is an IUP with a single disulfide bond near the C-terminus. NMR relaxation studies measuring the backbone dynamics of MSP2 indicated local motional restriction in the region around this disulfide bond, in contrast to the largely unstructured bulk of the protein [72]. Beyond this local restriction, however, the disulfide bond was predicted to contribute no further to the local structure of the C-terminal region.

In μ-KIIIA, molecular dynamics simulations were used to characterize conformational flexibility resulting from the removal of individual disulfide bonds [51]. The molecular dynamics simulations indicated that native μ-KIIIA explored less conformational space and was less flexible compared with its disulfide-deficient analogs, suggesting that the disulfide bonds impart conformational stability to the toxin. In relation to binding activity, increased conformational flexibility of the Cys1–Cys9 and

Cys2–Cys15 disulfide-deficient analogs may enable them to sample a larger volume of conformational space and facilitate eventual binding to the channel, explaining their retention of binding affinity for sodium channels. Indeed, the increased flexibility of the analog lacking the Cys1–Cys9 disulfide may explain its increased binding kinetics to the $Na_V1.2$ and $Na_V1.4$ channel subtypes by enhancing its ability to associate with and dissociate from its target channel [51]. A similar outcome was reported in conkunitzin-S1, where the addition of a disulfide bond to the native toxin was thought to make the structure more rigid, decreasing the binding kinetics of the toxin to the Shaker potassium channel target without affecting the overall blocking activity against this channel [73].

11.7 Disulfide Bonding Patterns and Protein Topology

Beyond the mere presence or absence of disulfide bonds, the location of such bonds within a protein plays an important role in protein topology. Several studies have analyzed the relationship between disulfide bonding patterns and protein structure, typically drawing on the conservation and evolution of disulfide bonding patterns within a family of homologous proteins or proteins with similar folds to reveal these relationships [15, 16]. Other studies have focused on comparing proteins that may not necessarily be homologous in amino acid sequence, but have structures with similar disulfide topology [74, 75]. Such large-scale comparative studies of both protein sequences and structures provide a better understanding of the conservation of disulfide bonding patterns and the factors that determine these patterns. A recent study classified structurally about 3000 small disulfide-rich protein domains into different fold groups and explored the disulfide bonding patterns within each group [15]. There was generally a high level of conservation in disulfide bonding patterns within each fold group, but also several naturally occurring variations, giving insight into the roles of disulfide bonds in maintaining these folds.

11.7.1 Conservation and Evolution of Disulfide Bonding Patterns

In a recent analysis examining the conservation of disulfide bonds within homologous protein domains, only 54% were found to be conserved, indicating a poor relationship between sequence identity and disulfide bond conservation [16]. Conserved disulfide bonds are presumed to play important structural or functional roles. It is not uncommon to observe conserved disulfide bonding patterns among non-homologous proteins with similar folds. Conversely, proteins with high sequence identity do not always share a conserved disulfide bonding pattern. For example, disintegrins are a group of homologous proteins from snake venoms that display a high level of sequence similarity, and yet adopt different disulfide bonding patterns and topologies. In general, similar bonding patterns result in similar topologies, although different topologies can sometimes be observed. Obtustatin, for example,

adopts a more compact fold compared to the extended conformations of echistatin and other disintegrins, despite having a similar disulfide bonding pattern [76, 77]. On the other hand, the disintegrin salmosin displays a disulfide bonding pattern and protein fold that differ from other disintegrins [78]. The disintegrin family has been divided into five different groups based on the length of the amino acid sequence and the number of disulfide bonds present. It was hypothesized based on the conserved cysteine residues in each subgroup that a selective loss of disulfide bonds occurred during evolution, resulting in structural diversity within the disintegrin family [79].

11.7.2 Conservation of Disulfide Bonds

Cysteine residues linked in disulfide bonds are often conserved or mutated in pairs [17]. One common variation observed within a fold group is that some members have one more or one less disulfide bond compared to other family members. Conkunitzin-S1, which belongs to the Kunitz domain family of proteins, possesses only two of the three disulfide bonds normally conserved in other members of this family (BPTI, dendrotoxins) [73], yet its structure is essentially identical. The converse is observed in the inhibitory cystine knot (ICK) family [80], where the spider toxins robustoxin, ω-agatoxin IVB, and μ-agatoxin I all have an additional disulfide bond not seen in most other members of this structural family [81]. The additional disulfide bond in robustoxin [82] links a loop between the first two β-sheets of the ICK fold to the C-terminal end, while in ω-agatoxin IVB and μ-agatoxin I the additional bond links a β-hairpin between the two β-sheets of the fold (Figure 11.3).

In some families, only one half of a cysteine pair making up a disulfide bond may be conserved. Among the thrombospondin repeat (TSR) domains, two out of the three disulfide bonds are conserved in most of its members [84], but the third disulfide bond varies in that one of the cysteine residues contributing to the disulfide bond is located in a different position in the amino acid sequence. Structurally, however, this "migrated" cysteine residue lies in a similar spatial region. Based on the unique disulfide bonding pattern observed across this family, Tan *et al*. classified the TSRs into two groups and functional differences have been demonstrated between these two groups despite their similar folds.

11.7.3 Cysteine Framework and Disulfide Connectivity

The cysteine framework (arrangement of cysteine residues in the amino acid sequence) is often conserved within similar fold groups, even though other amino acids may vary considerably. Indeed, the high level of conservation of cysteine frameworks has provided a basis for classifying certain groups of proteins such as the conotoxins [85, 86]. Within conotoxin superfamilies that have similar cysteine frameworks, additional diversity is often generated through the different bonding patterns of the cysteine residues, leading to several different disulfide scaffolds for a particular cysteine arrangement [86]. For instance, the α-conotoxins and the

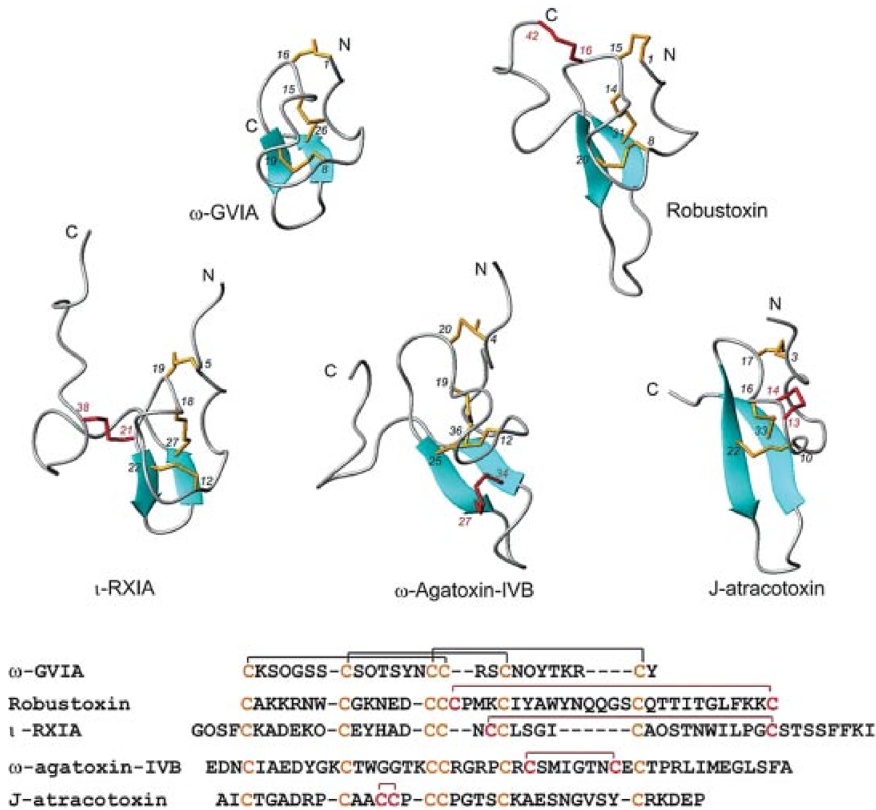

Figure 11.3 Structures and sequences of peptides with the ICK fold. Structures were aligned based on the disulfide bonding pattern of GVIA as in the sequence alignment shown. Additional disulfide bonds in the peptides with four disulfide bonds are highlighted in red. β-sheets were determined based on the ribbon macro of MOLMOL [83].

χ/λ-conotoxins have different disulfide connectivities even though they share the same (-CC-C-C-) cysteine framework, resulting in different conformations and biological targets [87]. Such a difference could be due to the presence of proline residues in the sequence, as discussed below.

The ι-conotoxin RXIA and the spider toxin J-atracotoxin share the same cysteine framework (-C-C-CC-CC-C-C-), but have different disulfide connectivities [88] (Figure 11.3). On the other hand, different cysteine frameworks can sometimes support the same pattern of disulfide connectivities. Robustoxin [82] and versutoxin [89], despite having a different cysteine framework from ι-RXIA, share the same disulfide bonding pattern and are structurally more similar to ι-RXIA than is J-atracotoxin, even though the latter shares a similar arrangement of cysteine residues with ι-RXIA [88].

The number and type of residues between cysteine residues can be an important factor in determining disulfide connectivity and overall fold. For example, the

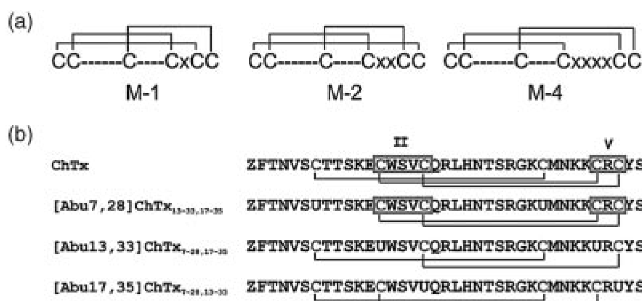

Figure 11.4 (a) Disulfide connectivity patterns of the M-superfamily branch of conotoxins (M-1, M-2 and M-4). X in the third intercysteine loop represents any residue. (b) Amino acid sequences of the scorpion toxin ChTx and its three disulfide-deficient analogs in which each of the three cysteine pairs was individually replaced with Abu (aminobutyric acid). Z represents pyroglutamate and U represents aminobutyric acid in the sequences above. Conserved intercysteine loop spacings in the second and last intercysteine loops of ChTx and the first disulfide-deficient analog are highlighted in the box.

M-superfamily of conotoxins, which has the cysteine framework (-CC-C-C-CC-) [85], has been subdivided into five groups (M-1 to M-5) based on the number of residues between the fourth and fifth cysteines [90, 91]. The structures in the M-1 (C1–C5/C2–C4/C3–C6), M-2 (C1–C6/C2–C4/C3–C5) and M-4/5 (C1–C4/C2–C5/C3–C6) branches have distinctive disulfide connectivity patterns (as indicated in parentheses) (Figure 11.4a), suggesting that the number of residues in the last cysteine loop might determine the disulfide connectivity in this family [92, 93]. Unsurprisingly, tertiary structures differ among these groups. The M-1 conotoxin mr3e possesses a double-turn motif while the M-2 conotoxin mr3a adopts a triple-turn motif and folds into a more globular structure compared to mr3e [92].

Similarly, it has been proposed that intercysteine spacings could be responsible for the selective formation of specific disulfide bonds in the scorpion toxin ChTx [50]. Separation of the cysteines in the second and last intercysteine loops of ChTx by three and one residues, respectively, (C · · · CxxxC · · · C · · · CxC) was proposed to direct preferential formation of native disulfide pairings by disfavoring certain cysteine pairings (Figure 11.4b). Cysteine mutational studies suggest that the highly conserved intercysteine spacing of the second and last intercysteine loops is not essential for maintaining the α/β scorpion fold of ChTx, as the native fold was achieved in a disulfide-deficient analog lacking the conserved intercysteine spacings. Rather, the intercysteine spacings seem to contribute to the folding efficiency of the toxin into its native fold, evident from the higher yield of structures with native disulfide connectivity obtained in the disulfide-deficient analog ([Abu7,28]ChTx$_{13–33,17–35}$) having the conserved spacings CxxxC and CxC [50].

The nature of the residues in each intercysteine loop, as well as the number, plays a role in directing disulfide connectivities. In particular, the presence of proline, which is known to favor a turn in conventional folding patterns, seems to affect the final disulfide bonding pattern of some proteins. This was demonstrated in the

α-conotoxin ImI, in which substitution of the conserved proline in the first inter-cysteine loop resulted in a non-native "ribbon" disulfide bonding pattern and conformation, as opposed to the native globular fold [87]. Similarly, mutation of two proline residues in maurotoxin resulted in a rearrangement of disulfide bond pairings accompanied by slight changes in conformation [94]. Zhang and Snyder showed that substituting amino acids other than proline had negligible effects upon the disulfide pattern and conformation [95].

11.7.4
Non-Native Disulfide Connectivities

It is evident that disulfide connectivity plays an important role in the conformation of a peptide or protein. This has been highlighted by studies examining the conformations of peptides containing non-native disulfide connectivity patterns. Several studies have focused on the α-conotoxins, which have two disulfide bonds with a C1–C3, C2–C4 connectivity pattern and adopt a globular fold [96]. In the conotoxin α-GI, however, two additional non-native disulfide bond isomers can be formed, the "ribbon" isomer (C1–C4, C2–C3 disulfide connectivity) and the "beads" isomer (C1–C2, C3–C4), each adopting a different fold from that of the native [96].

The role of disulfide connectivity in protein conformational flexibility is less clear cut. In general, the ribbon and bead isomers are expected to display greater flexibility, as the structures of these isomers are not as tightly folded as the globular isomer [96]. This was demonstrated for the disulfide isomers of α-conotoxins GI and AuIB, in which solution structures of the ribbon and bead isomers were less well-defined compared with the native globular isomer [97]. Yet the opposite was reported for α-conotoxin BuIA, wherein the native globular isomer adopted multiple conformations in solution but the ribbon isomer had a well-defined conformation [98]. Intriguingly, the conformationally flexible ribbon isomer of α-AuIB was more potent than native [97], but the well-defined ribbon isomer of α-BuIA was inactive [98].

The disulfide connectivity pattern for the somatomedin B domain of human vitronectin has also been studied in great detail. This 35-residue domain is unique in that its four disulfide bonds are closely arranged in the center of the domain, replacing the typical hydrophobic core found in larger proteins [99]. However, the close proximity of these four disulfides within the core of this domain has made it difficult to determine their exact connectivities, leading to different connectivities being proposed by different groups [100–103]. Intriguingly, the different disulfide bonding patterns reported in the literature were compatible with the same fold of the somatomedin B domain. Kamikubo *et al.* explored this further by computing conformational energies of alternate disulfide-bonded forms during structure calculations [101]; the different disulfide bonding patterns proved to have comparable energies and were all compatible with the NMR-derived structure. The alternative disulfide connectivities were subsequently disproved experimentally by studying the functionality and stability of synthetic somatomedin B peptides with alternate disulfide bonding patterns [99]. Only one of the three previously proposed disulfide

connectivity patterns produced an active and stable structure and was taken to be the native disulfide bonding pattern.

11.8 Applications

Studies of the role of disulfide bonds in protein stability and folding have led to a better understanding of the relationships between protein structure and disulfide bonding patterns, and the experience gathered in these studies has been useful for predicting protein structure and folds by computational means [74, 75]. Several approaches have been described to predict disulfide connectivity and protein structures from protein sequences with increased accuracy [104–108].

Disulfide bonds are also important in peptide and protein engineering. The loss of disulfide restraints must be taken into account when attempting to minimize a peptide structure in which these disulfides contribute to its overall stability. In minimized analogs of ShK, for example, lactam bridges were utilized to compensate for the removal of the disulfides [109]. In disulfide bridge-based PEGylation [110], a solvent-accessible disulfide is reduced to release two free cysteine thiols across which the poly(ethylene glycol) (PEG) group can then be attached to the protein using thiol-selective chemistry. In this strategy, designed to make the protein therapeutically more bioavailable, it is important that the protein structure be maintained following reduction of the disulfide bond. In protein engineering, disulfide bonds may be added to commercially important proteins or therapeutics to increase the thermodynamic and proteolytic stability of the protein [111]. For example, Matsumura *et al.* demonstrated that introducing disulfides at strategic locations of T4 phage lysozyme could significantly stabilize the protein against thermal unfolding [112, 113]. Disulfide bonds can be added as cyclic constraints to conformationally constrain linear peptides that would otherwise be flexible in solution. Such a modification, by limiting conformational freedom, represents a valuable strategy for enhancing receptor selectivity and increasing binding affinity. This approach has been used to optimize linear peptides targeting the opioid receptors [114]. Introduction of a disulfide restraint to an opioid receptor peptide increased its selectivity for the μ-opioid receptor, presumably by locking the peptide into a conformation active for the μ receptor but not the δ and κ subtypes. A similar observation was reported for β-melanocyte-stimulating hormone-derived melanocortin-4 receptor (MC4R) peptide agonists, in which disulfide cyclization improved selectivity and potency for the MC4R over the MC1, 3 and 5 receptor subtypes [115]. Some disulfide-stabilized frameworks such as the highly stable ICK motif, which can accommodate variable intercysteine amino acid sequences while maintaining its overall fold, have potential applications as protein scaffolds for drug design or engineering novel polypeptides [81, 116].

Although disulfides can be incorporated to increase protein stability, the disulfides themselves are susceptible to reduction in certain extracellular environments such as the blood [117]. To overcome this problem, disulfides can be replaced with disulfide

mimetics such as dicarba or diselenium bridges, which are less susceptible to degradation. One potential drawback of such strategies is the difficulty of reproducing the covalent geometry of the disulfide bond. Compared with the sulfur–sulfur bond in disulfide bridges, selenium–selenium bond lengths in diselenium bridges are slightly longer (around 2.02 Å) [117], while the carbon–carbon bond lengths in dicarba bridges are shorter (around 1.34 Å) [118]. In separate studies, diselenium and dicarba bonds were incorporated in the α-conotoxin ImI [117, 118]. For both substitutions, the conformation of the analog was close to that of the native structures, with slight local differences in the vicinity of the substituted residues arising from the different covalent geometries. Nevertheless, biological activity against the nicotinic acetylcholine receptor was retained for both modified peptides, supporting their future use in the design of stable scaffolds for drugs. Furthermore, disulfide substitution with diselenium bridges could also be an effective strategy for enhancing folding yield and kinetics, as recently demonstrated in ω-GVIA, in which diselenium-substituted analogs of ω-GVIA displayed a higher propensity to adopt the native fold and folded with a half-time approximately 5-fold shorter than native ω-GVIA [119].

In addition to stabilizing structural folds, disulfides may also be used as an effective means of covalently linking two or more subunits together. This approach was employed to attach cell-penetrating peptides (CPPs) to cargo proteins, facilitating the entry of these proteins into the cell for in-cell NMR studies [120]. The reduction-sensitive disulfide link was subsequently cleaved in the reducing environment of the cytoplasm, allowing the cargo protein to detach from the CPP and distribute uniformly around the cell [120].

Yet another useful application of disulfide bonds is the technique of "disulfide trapping" [121, 122]. This method takes advantage of formation of a disulfide bond between a pair of cysteine residues substituted in strategic locations in a protein with known structure in order to detect long-range backbone motions when the two cysteine residues collide with one another to form the disulfide bond. Patterns and rates of disulfide formation can be analyzed to obtain information about the trajectories and frequencies of backbone and domain motions [122].

11.9 Conclusions

Disulfide bonds play important roles in the conformation and stability of proteins and peptides. Studies of their roles through both experimental means and analysis of evolutionary patterns have led to a better understanding of how disulfide bonds contribute to various biological processes in nature such as folding and dynamic stability, and their importance in maintaining the native conformation of a protein. It is evident that the roles and importance of individual disulfide bonds vary across different protein folds, with some being more important for the proper function of a protein. The location of a disulfide within a structure may also influence the role it plays in stabilizing or folding the protein.

Gaps remain in our understanding of how disulfides contribute to protein folding and stability, leading to different theories being proposed. In particular, it is difficult to differentiate completely the relative contributions of disulfide bonds and other noncovalent interactions. It should also be noted that most studies are conducted *in vitro* rather than *in vivo* and may not accurately represent how disulfides stabilize a protein or assist its folding in the cell. Nonetheless, an understanding of how disulfide bonds contribute to the folding, stability, and function of a protein *in vitro* has important implications for the field of protein engineering and therapeutic design, particularly in achieving higher yields of product or designing a stable scaffold for therapeutic use. Several effective strategies have already been developed to either compensate for the loss of structure-stabilizing disulfides in protein minimization or incorporate disulfides or disulfide mimetics to stabilize protein scaffolds. With future advances in techniques and more in-depth studies, the roles of disulfide bonds will be understood even more clearly.

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