Review

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Folding of peptides and proteins: role of disulfide bonds, recent developments

Abstract: Disulfide-containing proteins are ideal models for studies of protein folding as the folding intermediates can be observed, trapped, and separated by HPLC during the folding reaction. However, regulating or analyzing the structures of folding intermediates of peptides and proteins continues to be a difficult problem. Recently, the development of several techniques in peptide chemistry and biotechnology has resulted in the availability of some powerful tools for studying protein folding in the context of the structural analysis of native, mutant proteins, and folding intermediates. In this review, recent developments in the field of disulfide-coupled peptide and protein folding are discussed, from the viewpoint of chemical and biotechnological methods, such as analytical methods for the detection of disulfide pairings, chemical methods for disulfide bond formation between the defined Cys residues, and applications of diselenide bonds for the regulation of disulfide-coupled peptide and protein folding.

Keywords: disulfide; folding; intermediate; kinetic; protein disulfide isomerase; regioselective; selenocysteine.

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List of abbreviations

1,5-DAN, 1,5-diaminonaphthalene; Acm, acetamidomethyl; ASA, solvent accessible surface area; BPTI, bovine pancreatic trypsin inhibitor; DMSO, dimethyl sulfoxide; DPAET, diisopropylaminoethane thiol; ECD, electron captured dissociation; EPL, expressed protein ligation; ETD, electron transfer dissociation; GSH and GSSG, reduced and oxidized forms of glutathione, respectively; Gu/HCl, guanidine hydrochloride; HPLC, high performance liquid chromatography; MeBzl, 4-methylbenzyl; NCL, native chemical ligation; PDI, protein disulfide isomerase; ST, heat-stable enterotoxin; ^{t}Bu, tertiary butyl; SeCys, selenocysteine; TCEP, tris-(2-carboxyethyl)-phosphine; TFE, trifluoroethanol.

Introduction

Since the elegant study reported by Anfinsen (1) on the folding of RNase A, numerous additional protein folding reactions have been reported in the interim half century. Anfinsen introduced a general rule for protein folding, called Anfinsen's dogma, 'all the information required for protein folding is contained in the amino acid sequence of a protein and the native conformation is thermodynamically stabilized'. Thus, under appropriate conditions, proteins spontaneously fold into their native conformation. Therefore, numerous attempts have been made to validate the thermodynamic control of protein folding, and to trap or regulate folding intermediates to overcome free energy control (2, 3).

Disulfide bonds are often found in many extracellular peptides and proteins, and the native pairings of disulfide bonds are essentially required for their biological activity (4). In general, disulfide bonds are thought to play a role in the thermodynamic stability of peptides and proteins by decreasing the entropy at their denatured state in folding. Disulfide-containing peptides and proteins show characteristic folding patterns because the half-lives of transiently produced folding intermediates are considerably longer than that of non-disulfide bonded proteins (2, 3, 5). Therefore, it is frequently possible to trap key intermediates during protein folding, separate, and analyze them by biophysical approaches, such as mass spectrometry and NMR, thus providing essential information related to the kinetic and thermodynamic control of protein folding. However, it is generally difficult to estimate the role of disulfide bonds and to regulate their formation in the disulfide-coupled folding of peptides and proteins.

In the latter half of the 20th century, the folding mechanism of proteins was mainly investigated via the analysis of folding intermediates with mis-bridged (non-native) or native disulfide bonds during the folding of proteins, such as RNase A and BPTI (2, 5). These studies resulted in the

development of an important principle related to protein folding from the standpoint of folding intermediates, such as off-pathway and on-pathway intermediates (5, 6). Thus, protein folding proceeds via specific processes, as predicted by Levinthal (7). However, a complete understanding of the tertiary structure of a protein and the role of intermediates in protein folding remain to be determined.

As considered above, disulfide bonds between Cys residues are an essential structural element of disulfide-containing peptides and proteins. Generally, disulfide-coupled protein folding is performed under thermodynamic control in the presence of chemical additives and redox reagents, such as glutathione (1). Several mis-bridged disulfide isomers and cross disulfide linked species are also observed during folding, as described above, and the rate-determining step is thought to be the disulfide-exchange reaction from non-native to native disulfide bonds in folding intermediates (8). However, the mechanism of the disulfide exchange reaction, especially for the cross disulfide linked species, has not been established in detail. Therefore, we herein discuss recent developments associated with the mechanism of formation of cross-disulfide species in disulfide-coupled protein folding.

In addition to chemical additives, several novel enzymatic catalysts of disulfide bond formation, such as protein disulfide isomerase (PDI), in living cells have been discovered and have been utilized in studies of *in vitro* protein folding. Recently, to understand the role of disulfide bonds in protein folding, PDI has been utilized to regulate *in vitro* protein folding, in addition to chemical additives (9–11). Some applications of PDI to protein folding are also discussed here.

Several techniques, including chemical methods, for the preparation of disulfide bonds and folding intermediates have recently been introduced, in attempts to understand and analyze the role of disulfide bonds, not only for the final native structure but also for kinetically trapped intermediates (12). Techniques for the regioselective formation of disulfide bonds of peptides permit the conformations of not only native but also non-native disulfide bonded species to be studied. In addition, the development of chemical methods, such as native chemical ligation (NCL), has made it possible to analyze specifically labeled proteins using biophysical measurement techniques (13, 14). More recently, recombinant techniques in biotechnology, combined with NCL, have emerged, making much larger molecular-sized proteins available for folding analyses. Therefore, recent applications of the regioselective formation of native and non-native disulfide bonds in elucidating the role of disulfide bonds of peptides and proteins are also reviewed.

Diselenide bonds between selenocysteine residues have been also introduced in place of disulfide bonds, in attempts to regulate peptide and protein conformation (15–20). A diselenide bond is essentially stable in the presence of thiol reagents, such as mercaptoethanol, and, because of this, disulfide-diselenide exchange reactions are generally not significant. Therefore, several peptides have been chemically synthesized and their folding regulated via the use of diselenide bonds. This method can be also applied to protein folding to regulate, not only the native conformation, but also non-native folding intermediates (19). In addition, the introduction of diselenide bonds into proteins permits disulfide-coupled protein folding to be regulated by restricting the folding intermediates in the folding pathway (15, 21). In this review, we introduce recent applications of the use of diselenide bonds in studies of peptide and protein folding.

Determination of the position of disulfide bonds

To investigate the role of disulfide bonds or the mechanism of disulfide-coupled folding, it is important to determine the positions of the disulfide bonds in folding intermediates or intact peptides and proteins. Here, both chemical and biophysical methods have been used to accomplish this. Chemical methods for the regioselective formation of disulfide bonds are classically employed to characterize the disulfide pairings of peptides using a combination of two or three types of thiol protecting groups, such as acetamidomethyl (Acm) and 4-methylbenzyl (MeBzl) groups, and the stepwise formation of disulfide bonds (12, 22). The authentic chemically synthesized peptides with disulfide bonds at specific positions are then compared to folding intermediates or intact peptides by HPLC to determine the positions of the disulfide bonds. However, this method has some limitations when applied to general proteins, the major limitation being difficulties associated with the chemical synthesis of the materials. Therefore, disulfide bonds are partially reduced for determining disulfide pairings of intact proteins or folding intermediates. Gray established an elegant method using tris-(2-carboxyethyl)-phosphine (TCEP) for disulfide reduction under acidic conditions, which permits disulfide exchange reactions to be avoided (23). After the reduction of disulfide bonds, the free thiol groups are rapidly protected by reaction with monoiodoacetamide or monoiodoacetic acid. The amino acid sequence of the protected peptides and proteins can then be determined (usually after enzymatic digestions) by Edman degradation to estimate the positions of the disulfide bonds (23). This represents the most popular

method for determining the positions of disulfide bonds in peptides and proteins.

Mass spectrometry has recently emerged as a powerful tool for the determination of disulfide bonds. Fukuyama et al. developed the use of a novel matrix, 1,5-diaminonaphthalene (1,5-DAN), for use in MALDI QIT TOFMS to assign the positions of disulfide bonds in peptides (24). They applied the method to guanylin, a peptide that contains 15 amino acid residues with two intra-molecular disulfide bonds, and determined the disulfide pairings by MS/MS analyses. This method can also be used to assign the intermolecular disulfide pairings of peptides, such as insulin (24). However, the method is only suitable for small or mid-sized peptides.

More recently, electron captured dissociation (ECD) and electron transfer dissociation (ETD) methods have been introduced for use in mass spectrometric analysis to conventionally determine amino acid sequences (25). Searching extracted ion chromatography by ETD tandem mass spectroscopy has been applied to map the disulfide pairings of proteins, such as recombinant HIV-1 Env (25). The disulfide parings for 16 of the 18 cysteine residues were successfully assigned using mass spectral data.

Chemical or enzymatic additives used in the regulation of disulfide-coupled peptide and protein folding

Disulfide bond formation proceeds in a cooperative manner during the formation of the main chain structure of peptides and proteins. Therefore, several types of non-native and native disulfide bonded species play an important role in the folding pathway. However, non-native disulfide (mis-bridged) isomers (folding intermediates) cause several problems, such as insolubility by its hydrophobic character, which constitutes impediment to studies of the role of disulfide bonds in protein folding. To overcome this shortcoming, several chemical additives have been utilized in disulfide-coupled protein folding. Urea, Gu/HCl, and Arg, known as chaotropic reagents, stabilize and destabilize protein structures at low and high concentrations, respectively. Urea stabilizes a protein conformation under entropic control at low concentrations, while Gu/HCl contributes to charge shielding and the suppression of inter- and intra-molecular electrostatic interactions (26). In general, Gu/HCl appears to be much effective for the stabilization of a protein structure compared to urea. Low concentrations of Gu/HCl are usually employed in disulfide-coupled protein folding, resulting in the formation of the native conformation of a protein. Arg has also been used to eliminate the formation of non-specific aggregation in protein folding. Arg does not affect the thermodynamic stability of a protein, but suppresses the aggregation of folding intermediates or the precipitation of mis-bridged disulfide isomers (27). However, the use of Arg does not appear to avoid the formation of mis-bridged disulfide isomers and may decrease the velocity of the disulfide-coupled folding reaction of peptides and proteins.

Organic solvents, such as trifluoroethanol (TFE), induce the formation of α-helical structures in peptides and proteins to artificially enhance intra-molecular hydrogen bonds (28, 29). Therefore, TFE is generally employed to regulate the disulfide-coupled folding of α-helical peptides. For example, the native disulfide pairings of endothelin were successfully achieved by oxidation with dimethyl sulfoxide in the presence of 30%–60% TFE under strongly alkaline conditions, whereas several disulfide isomers are produced under typical folding conditions (30). This suggests that the formation of an α-helical structure in an intermediate plays an important role in the disulfide-coupled folding of endothelin. Therefore, the disulfide bonds of endothelin may play a pivotal role in trapping the correct folding intermediates leading to the formation of the native conformation during folding. Thus, TFE has been used to examine the role of disulfide bonds in peptide folding. However, applications to protein folding are few. In this context, the use of TFE in protein folding promises to provide a new insight into the role of disulfide bonds in proteins, especially α-helical proteins.

Generally, disulfide-coupled folding reactions are carried out in the presence of redox reagents, such as the reduced and oxidized forms of glutathione. The addition of redox agents can accelerate disulfide-coupled peptide and protein folding and play an important role in achieving the native conformation under thermodynamic control. Several mis-bridged disulfide isomers and cross disulfide linked species are also observed during folding and the rate-determining step is thought to be the disulfide-exchange reaction from the mis-bridged disulfide isomer to the native disulfide bonded peptides and proteins. However, little is known regarding the disulfide exchange mechanism or the role of the cross-disulfide-linked intermediates between glutathione and peptides/proteins, because it is difficult to separate and characterize the cross disulfide linked species. To investigate the role of cross disulfide linked species during disulfide-coupled peptide and protein folding, Arg-Cys-Gly was recently applied to this process, using lysozyme and prouroguanylin as model proteins. The folding velocity using the Arg-Cys-Gly redox

system was three times higher than that using a typical glutathione redox system. Okumura et al. proposed that the positive net charge of the redox molecule in a mixed disulfide species is a factor in accelerating disulfide-coupled folding (31). More recently, a series of thiol reagents carrying alkyl amino groups were examined, in attempts to estimate the role of cross disulfide linked molecules in the acceleration of disulfide-coupled protein folding. The use of diisopropylaminoethane thiol (DPAET) results in a dramatic enhancement in folding efficiency to produce the correct conformation by accelerating the disulfide shuffling reaction (32). Local electrostatic environments around a Cys residue in a protein molecule have been predicted to affect the disulfide exchange reaction during protein folding (33). Among the thiol reagents examined in the study, DPAET has the largest ASA^+ (solvent accessible surface area with positive partial charges), indicating that DPAET effectively provides a positively charged environment for stabilizing the thiolate anion of a Cys residue of the cross disulfide linked protein molecule, as shown in Figure 1, although steric hindrance is also an important factor (32). Thus, a reducing agent with the larger ASA^+ is preferred for accelerating disulfide-coupled protein folding because it stabilizes (induces the formation of) the thiolate anion of the Cys residue of a protein in the cross disulfide linked folding intermediates. Such recent developments have resulted in considerable progress being made toward understanding the role of cross disulfide bonds in the folding pathway of disulfide-containing peptides and proteins.

Recently, several enzymes that catalyze disulfide bond formation/rearrangement have also been utilized in investigating the role of disulfide bonds. Disulfide bond formation in proteins is regulated by several types of oxidative folding systems *in vivo*. The protein disulfide isomerase (PDI)-endoplasmic reticulum oxidoreduclin 1 (Ero1) system in mammalian cells plays an important role in the quality control of proteins through disulfide bond formation, as does the DsbA-DsbB system in *Escherichia coli* cells (34). PDI is classified as a disulfide-donating enzyme to nascent proteins, while Ero1 specifically generates a disulfide bond in PDI. This oxidative folding system takes advantage of the reversibility of disulfide bond formation in PDI and Ero1 under physiological conditions. Thus, disulfide bonds play a central role in the quality control of proteins and redox signaling in cells. Therefore, PDI is also utilized as an enzymatic additive for the *in vitro* folding of disulfide-containing peptides and proteins to produce the native conformation (10, 11). For example, disulfide bond formation of conotoxins, α-GI and α-ImI, has been estimated in both the presence and absence of PDI (9). The native disulfide formation (corresponding to a globular form) of α-GI was significantly accelerated by the assistance of PDI among disulfide isomers although the folding yield of the correct conformation of α-GI was not affected by PDI. In addition, PDI did not affect the folding velocity of the globular form (native disulfide bonds) of α-ImI but significantly increased that of a ribbon form of α-ImI. This suggests that PDI does not discriminate between native and non-native disulfide bonded structures as well as that of RNase A, as has been reported previously (35). PDI may accelerate disulfide-coupled protein folding by specifically recognizing partially folded intermediates that are formed in the pathway.

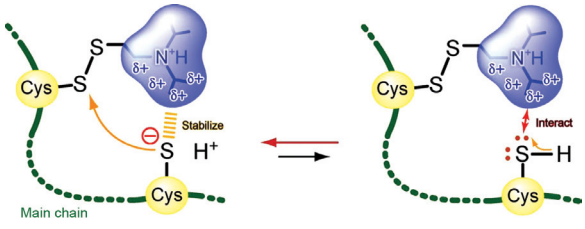

Figure 1 Mechanism for the acceleration of disulfide-coupled protein folding by a positively charged diisopropylaminoethyl group.
The positively charged alkylamino group of the cross disulfide linked species stabilizes the thiolate anion of the Cys residue intramolecularly, inducing the conversion of the thiol to the thiolate form of the Cys residue by decreasing the pK_a value of the thiol group. Therefore, the combined effects, namely, charge interactions between an alkylamino cation and a thiolate anion and the induction/stabilization of the thiolate form, accelerate disulfide-coupled peptide and protein folding.

Selenocysteine as the tool for estimating the role of disulfide bonds in peptide and protein folding

Several types of non-native and native disulfide bonded folding intermediates are produced during disulfide-coupled protein folding *in vitro*. To estimate the role of each disulfide bond of multi disulfide-containing peptides and proteins, a kinetic analysis can be carried out, which permits the folding mechanism of peptides and proteins to be predicted. However, in general, the lives of folding intermediates are quite short and it is difficult to trap and estimate their conformations. In the latter half of the 1990s, selenocysteine (SeCys, Sec), the 21st amino acid, has been applied for folding studies of cysteine-rich peptides (19, 20). SeCys and Cys share many chemical properties and

SeCys residues can form diselenide bonds, analogous to a disulfide bond by Cys residues. The replacement of disulfide with diselenide bonds in peptides usually has minimal effect on the structure and biological activity of the product (18). However, there are some differences between SeCys and Cys, such as pK_a (5.2 for SeCys vs. 8.3 for Cys) and redox potential (-383 mV for SeCys vs. -238 mV for Cys) (36, 37). This means that diselenide bonds (Se-Se) are preferred to either disulfide bonds (S-S) or selenylsulfide bonds (Se-S) and diselenide-disulfide exchange reactions are generally not significant (38). Therefore, many types of disulfide isomers of peptides can be prepared, including unstable disulfide isomers, which can be useful in studies of the conformation of folding intermediates or mature peptides. More recently, the chemical replacement of a disulfide with a diselenide bond, including non-native disulfide bonds, has been applied to a variety of peptides, including oxytocin, endothelin, apamin, a trypsin inhibitor, and various conotoxins, in attempts to regulate peptide conformation and estimate the role of each disulfide bond (16–20). All possible diselenide peptides were successfully produced and had stable conformations.

Importantly, diselenide formation can be performed in an aqueous buffer even under mildly acidic and/or reducing conditions and the diselenide bond is essentially resistant to the disulfide exchange reaction by a thiolate anion, as described above. These properties provide a strong benefit for peptide and protein folding, such as the regioselective formation of diselenide and disulfide bonds and represent a unique strategy for trapping (preparing) folding intermediates, including (unstable) disulfide isomers, under aqueous conditions (Figure 2). For example, each disulfide bond of apamin, which contains two disulfide bonds, was chemically replaced by a diselenide bond (19). Thus, three possible disulfide/diselenide isomers of a seleno-apamin analogue can be produced unless the conformational preferences of this peptide would prevail over the highly differentiated redox potentials of the diselenide and disulfide bonds. Importantly, no disulfide-diselenide exchange reactions were observed. Therefore, this approach makes it possible to selectively prepare stable non-native disulfide intermediates in aqueous buffers at near neutral pH's and to facilitate studies of folding pathway in small peptides (Figure 2C).

In addition, techniques such as replacing disulfide bonds with isosteric diselenide bonds can be used to regulate a peptide conformation, including folding intermediates. Disulfide-rich peptides generally produce several types of kinetically trapped folding intermediates, including partially formed disulfide bonds, during *in vitro* folding. The overall yields for the native conformation of disulfide-rich peptides are generally low and the rate-determining step is the disulfide-exchange reaction between non-native and native disulfide bonds. To improve folding efficiency, Steiner et al. incorporated a diselenide bond into peptides and successfully accelerated the disulfide-coupled folding

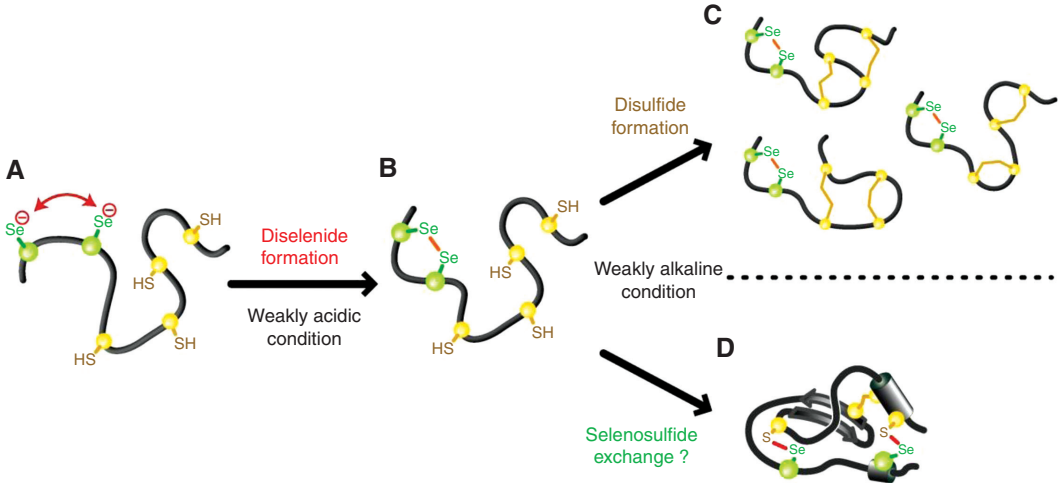

Figure 2 Regulation of disulfide-coupled peptide and protein folding using a diselenide bond.
(A) A diselenide bond can be regioselectively formed in SeCys-containing peptides or proteins in an aqueous buffer under weakly acidic conditions. (B) The protein with a diselenide bond and free thiol groups can be trapped and purified because the diselenide bond is generally resistant for thiol groups. (C) Disulfide bond formation occurs between Cys residues under weakly alkaline conditions without the diselenide-disulfide exchange reaction, providing the native or non-native disulfide proteins. (D) The net stability of proteins may overcome the partial stability of diselenide bonds. SeCys-containing BPTI was able to fold into the native conformation via the mis-bridged $SeCys^{5}$-$SeCys^{14}$ folding intermediate.

of conotoxins by defining the folding intermediates (39). Three disulfide-containing peptides, such as in conotoxins, theoretically produce 15 disulfide isomers with three disulfide bonds. As described above, the more stable diselenide bond would be initially formed at the initial stage of the folding reaction, especially under weakly acidic conditions, and so would provide a constraint to producing the initial structure in oxidative folding, resulting in the regulation of the folding pathway. Thus, the introduction of a diselenide bond to the native disulfide position of ω-conotoxin GVIA and μ-conotoxin SIIIA significantly increased the folding-velocities. These results indicate that the diselenide bond possesses the ability to regulate folding intermediates and improve folding efficiency and can be used to advantage in investigations of the role of disulfide bond in peptide folding.

More recently, diselenide bonds have been utilized to investigate the folding pathway of the protein, BPTI (15). The folding pathway of BPTI was extensively studied by Creighton, as well as Weissman and Kim, and predominantly consists of two kinetically trapped intermediates (2, 5, 40). In the early stage of folding, folding intermediates with a native disulfide bond (Cys30-Cys51 or Cys5-Cys55) are rapidly produced, followed by the formation of another disulfide bond (Cys14-Cys38). Non-native disulfide species (Cys5-Cys14 or Cys30-Cys51) are also observed during the folding of BPTI but the pathway is thought to be kinetically excluded from the folding pathway of BPTI. To validate the folding pathway for BPTI and the role of non-native disulfide bonds in its folding, Metanis and Hilvert replaced the non-native disulfide bond (Cys5-Cys14) with a diselenide bond (15). BPTI, which contains a diselenide bond between the SeCys5 and SeCys14 residues, was able to fold into the native conformation carrying selenylsulfide bonds (SeCys5-Cys55 and SeCys14-Cys38) under the condition used. As described above, the diselenide-disulfide exchange reaction does not generally occur in peptide folding. Surprisingly, the net stability of a BPTI molecule overcomes the partial stability of the diselenide bond in the case of SeCys-containing BPTI, driving the diselenide-disulfide exchange reaction (15). However, it should still be noted that the target folding intermediates can be specifically trapped during folding using the diselenide bond.

Outlook

Transiently trapped folding intermediates play a key role in the folding pathway of disulfide containing peptides and proteins. Therefore, it is important to regulate/investigate the conformation of the intermediates to determine the role of each disulfide bond of a peptide and protein. However, little is known concerning the folding intermediates that are produced in cases of disulfide-coupled folding of peptides and proteins on a molecular level. In this review, we introduced some of the recent developments in investigations of the role of disulfide bonds in the area of disulfide-coupled peptide and protein folding using diselenide bonds instead of disulfide bonds. A diselenide bond can be used for an advantage in investigating a folding pathway, and to regulate folding intermediates that are produced in disulfide-coupled peptide and protein folding. The strategies described herein can provide critical clues or new insights in the formation of disulfide-coupled peptides and protein folding. However, to utilize the method, some technical problems should be cleared for the preparation of SeCys-labeled peptides and proteins. Expressed protein ligation (EPL) is known as a combined method of chemical synthesis and the use of recombinant techniques for the preparation of specifically labeled proteins. Therefore, it appears likely that EPL will be further developed to permit the more convenient preparation of SeCys-labeled proteins.

In addition, analytical methods for investigating transiently produced disulfide species (folding intermediates) are required for the investigation of disulfide-coupled peptide and protein folding. Mass spectrometry has been utilized to analyze, not only the positions of disulfide bonds of peptides and proteins but also the complex structure of proteins. The method possesses the potential for the analysis of transiently produced folding intermediates by real-time detection. This supporting technology is also important in terms of investigating the role of disulfide bonds and the regulation of disulfide-coupled peptide and protein folding.

Highlights

- Chemical and biophysical methods to determine the positions of disulfide bonds are introduced.
- The reactivity of the cross disulfide linked moiety of folding intermediates is discussed and explained based on the solvent accessible surface area with positive partial charges.
- PDI does not discriminate between native and non-native disulfide bonded structures. PDI may accelerate disulfide-coupled protein folding by specifically recognizing partially folded intermediates that are formed in the pathway.

- A diselenide bond can be used for an advantage in investigating the folding pathway and regulate folding intermediates of disulfide-coupled peptide and protein folding by replacing a disulfide bond.
- Diselenide bonds have been utilized to trap folding intermediates in disulfide-coupled peptide and protein folding processes.

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References

1. Anfinsen CB. Principles that govern the folding of protein chains. Science 1973; 181: 223–30.
2. Creighton TE. Folding pathways determined using disulfide bonds. In: Creighton TE, editor. Protein folding. New York: W.H. Freeman, 1992: 301–52.
3. Goldenberg DP, Creighton TE. Energetics of protein structure and folding. Biopolymers 1985; 24: 167–82.
4. Moroder L, Besse D, Musiol HJ, Rudolph-Bohner S, Siedler F. Oxidative folding of cystine-rich peptides vs. regioselective cysteine pairing strategies. Biopolymers 1996; 40: 207–34.
5. Weissman JS, Kim PS. Reexamination of the folding of BPTI: predominance of native intermediates. Science 1991; 253: 1386–93.
6. Arolas JL, Aviles FX, Chang JY, Ventura S. Folding of small disulfide-rich proteins: clarifying the puzzle. Trends Biochem Sci 2006; 31: 292–301.
7. Levinthal C. How to fold graciously. Mossbauer spectroscopy in biological systems: proceedings of a meeting held at Allerton House, Monticello, Illinois: University of Illinois Press, 1969: 22–4.
8. Konishi Y, Ooi T, Scheraga HA. Regeneration of ribonuclease A from the reduced protein. Rate-limiting steps. Biochemistry 1982; 21: 4734–40.
9. Safavi-Hemami H, Gorasia DG, Steiner AM, Williamson NA, Karas JA, Gajewiak J, Olivera BM, Bulaj G, Purcell AW. Modulation of conotoxin structure and function is achieved through a multienzyme complex in the venom glands of cone snails. J Biol Chem 2012; 287: 34288–303.
10. Narayan M. Disulfide bonds: protein folding and subcellular protein trafficking. FEBS J 2012; 279: 2272–82.
11. Lyles MM, Gilbert HF. Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. Biochemistry 1991; 30: 613–9.
12. Okumura M, Shimamoto S, Hidaka Y. A chemical method for investigating disulfide-coupled peptide and protein folding. FEBS J 2012; 279: 2283–95.
13. Evans TC Jr, Benner J, Xu MQ. Semisynthesis of cytotoxic proteins using a modified protein splicing element. Protein Sci 1998; 7: 2256–64.
14. Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. Science 1994; 266: 776–9.
15. Metanis N, Hilvert D. Strategic use of non-native diselenide bridges to steer oxidative protein folding. Angew Chem Int Ed Engl 2012; 51: 5585–8.
16. de Araujo AD, Callaghan B, Nevin ST, Daly NL, Craik DJ, Moretta M, Hopping G, Christie MJ, Adams DJ, Alewood PF. Total synthesis of the analgesic conotoxin MrVIB through selenocysteine-assisted folding. Angew Chem Int Ed Engl 2011; 50: 6527–9.
17. Walewska A, Zhang MM, Skalicky JJ, Yoshikami D, Olivera BM, Bulaj G. Integrated oxidative folding of cysteine/selenocysteine containing peptides: improving chemical synthesis of conotoxins. Angew Chem Int Ed Engl 2009; 48: 2221–4.
18. Armishaw CJ, Daly NL, Nevin ST, Adams DJ, Craik DJ, Alewood PF. α-Selenoconotoxins, a new class of potent α_7 neuronal nicotinic receptor antagonists. J Biol Chem 2006; 281: 14136–43.
19. Pegoraro S, Fiori S, Cramer J, Rudolph-Bohner S, Moroder L. The disulfide-coupled folding pathway of apamin as derived from diselenide-quenched analogs and intermediates. Protein Sci 1999; 8: 1605–13.
20. Pegoraro S, Fiori S, Rudolph-Bohner S, Watanabe TX, Moroder L. Isomorphous replacement of cystine with selenocystine in endothelin: oxidative refolding, biological and conformational properties of [Sec3,Sec11,Nle7]-endothelin-1. J Mol Biol 1998; 284: 779–92.
21. Craik DJ. Protein folding: turbo-charged crosslinking. Nat Chem 2012; 4: 600–2.
22. Kamber B, Hartmann A, Eisler K, Riniker B, Rink H, Sieber P, Rittel W. The synthesis of cystine peptides by iodine oxidation of S-trityl-cysteine and S-acetamidomethyl-cysteine peptides. Helv Chim Acta 1980; 63: 899–915.
23. Gray WR. Disulfide structures of highly bridged peptides: a new strategy for analysis. Protein Sci 1993; 2: 1732–48.
24. Fukuyama Y, Iwamoto S, Tanaka K. Rapid sequencing and disulfide mapping of peptides containing disulfide bonds by using 1,5-diaminonaphthalene as a reductive matrix. J Mass Spectrom 2006; 41: 191–201.
25. Clark DF, Go EP, Desaire H. Simple approach to assign disulfide connectivity using extracted ion chromatograms of electron transfer dissociation spectra. Anal Chem 2013; 85: 1192–9.
26. Bhuyan AK. Protein stabilization by urea and guanidine hydrochloride. Biochemistry 2002; 41: 13386–94.

27. Reddy KR, Lilie H, Rudolph R, Lange C. L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. Protein Sci 2005; 14: 929–35.
28. Kentsis A, Sosnick TR. Trifluoroethanol promotes helix formation by destabilizing backbone exposure: desolvation rather than native hydrogen bonding defines the kinetic pathway of dimeric coiled coil folding. Biochemistry 1998; 37: 14613–22.
29. Luo P, Baldwin RL. Mechanism of helix induction by trifluoroethanol: a framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water. Biochemistry 1997; 36: 8413–21.
30. Tam JP, Deng XC, Wang RJ. Solvent assistance in regiospecific disulfide formation in dimethylsulfoxide. Lett Pept Sci 1999; 6: 265–73.
31. Okumura M, Saiki M, Yamaguchi H, Hidaka Y. Acceleration of disulfide-coupled protein folding using glutathione derivatives. FEBS J 2011; 278: 1137–44.
32. Okumura M, Shimamoto S, Nakanishi T, Yoshida Y, Konogami T, Maeda S, Hidaka Y. Effects of positively charged redox molecules on disulfide-coupled protein folding. FEBS Lett 2012; 586: 3926–30.
33. Snyder GH, Cennerazzo MJ, Karalis AJ, Field D. Electrostatic influence of local cysteine environments on disulfide exchange kinetics. Biochemistry 1981; 20: 6509–19.
34. Sato Y, Inaba K. Disulfide bond formation network in the three biological kingdoms, bacteria, fungi and mammals. FEBS J 2012; 279: 2262–71.
35. Gonzalez V, Pal R, Narayan M. The oxidoreductase behavior of protein disulfide isomerase impedes fold maturation of endoplasmic reticulum-processed proteins in the pivotal structure-coupled step of oxidative folding: implications for subcellular protein trafficking. Biochemistry 2010; 49: 6282–9.
36. Huber RE, Criddle RS. Comparison of the chemical properties of selenocysteine and selenocystine with their sulfur analogs. Arch Biochem Biophys 1967; 122: 164–73.
37. Besse D, Siedler F, Diercks T, Kessler H, Moroder L. The redox potential of selenocystine in unconstrained cyclic peptides. Angew Chem Int Ed Engl 1997; 36: 883–5.
38. Steinmann D, Nauser T, Koppenol WH. Selenium and sulfur in exchange reactions: a comparative study. J Org Chem 2010; 75: 6696–9.
39. Steiner AM, Woycechowsky KJ, Olivera BM, Bulaj G. Reagentless oxidative folding of disulfide-rich peptides catalyzed by an intramolecular diselenide. Angew Chem Int Ed Engl 2012; 51: 5580–4.
40. Creighton TE. Protein folding pathways determined using disulphide bonds. Bioessays 1992; 14: 195–9.

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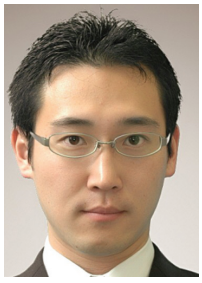

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