Metallomics Research 2022 (3) #MR202206

Review https://metallomicsresearch.brte.org

Redox Chemistry of Selenols and Diselenides as Potential Manipulators for Structural Maturation of Peptides and Proteins

Kenta Arai [1, 2*], Rumi Mikami [1]

[1] Department of Chemistry, School of Science, Tokai University, Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan

[2] Institute of Advanced Biosciences, Tokai University, Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan

Summary

Oxidative folding is an essential process for polypeptide chains containing cysteine (Cys) residues to form a bioactive three-dimensional structure. During this process, the folded state with the correct disulfide (SS) combination, which is found in the native state, cannot be obtained at 100% yield because various byproducts, such as misfolded states with mispaired SS bonds, oligomers, and aggregates are simultaneously produced. The formation of misfolded states *in vivo* has been suggested to cause critical human diseases such as neurodegenerative disorders. Therefore, the development of methods to promote the correct structural maturation of polypeptides, including Cys residues, both *in vivo* and *in vitro*, is a challenging task in protein synthesis, medicine, and drug discovery. To rapidly form correctly folded proteins at high yields, two potential strategies are available. First, called the *outside strategy*, is to control oxidative folding from the *outside* of proteins using artificial small molecules as catalyst and a reagent that mimics the function of protein disulfide isomerase, which catalyzes SS-related reactions during oxidative folding in cells. Second, called the *inside strategy*, is to insert mimics of SS linkage(s) into the *inside* of polypeptides to form a rigid covalent bond chemoselectively, thereby avoiding the formation of a misfolded state having mispaired SS bonds. In this review, recent developments and trends based on the unique redox properties of selenols and diselenides, which are selenium analogs of thiols and disulfides, respectively, are outlined, and their future prospects are discussed.

Key words: protein folding, cysteine, selenocysteine, enzyme mimic, catalyst, misfolding

Statements about COI: There are no conflicts of interest to declare.

*Correspondence:
Kenta Arai
Department of Chemistry, School of Science, Tokai University,
Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan
Tel: +81 463 58 1211
E-mail: k-arai4470@tokai-u.jp

Received: October 06, 2022
Accepted: November 21, 2022
Released online: December 12, 2022

1. Introduction

Proteins are first synthesized as polypeptide chains through transcription from genetic information in DNA into mRNA and subsequent translation, during which amino acids corresponding to three specific bases (codons) in mRNA are sequentially elongated (i.e., central dogma, **Fig. 1**). For the synthesized polypeptide chains to exert their physiological functions as proteins, they must gain a unique three-dimensional structure through protein folding. In classical protein science, polypeptide chains spontaneously fold into their most stable conformation in an aqueous solution under properly controlled reaction

conditions (**Fig. 1**, *in vitro* folding) [1]. However, only a few proteins strictly follow the basic principle of biophysics, known as the Anfinsen's dogma. In cells with high concentrations of biomolecules, intermolecular interactions and the resulting irreversible aggregation are inevitable; thus, correct folding is inhibited. Therefore, in modern protein science, it is important to understand the structures and functions of enzymes that promote correct folding [2].

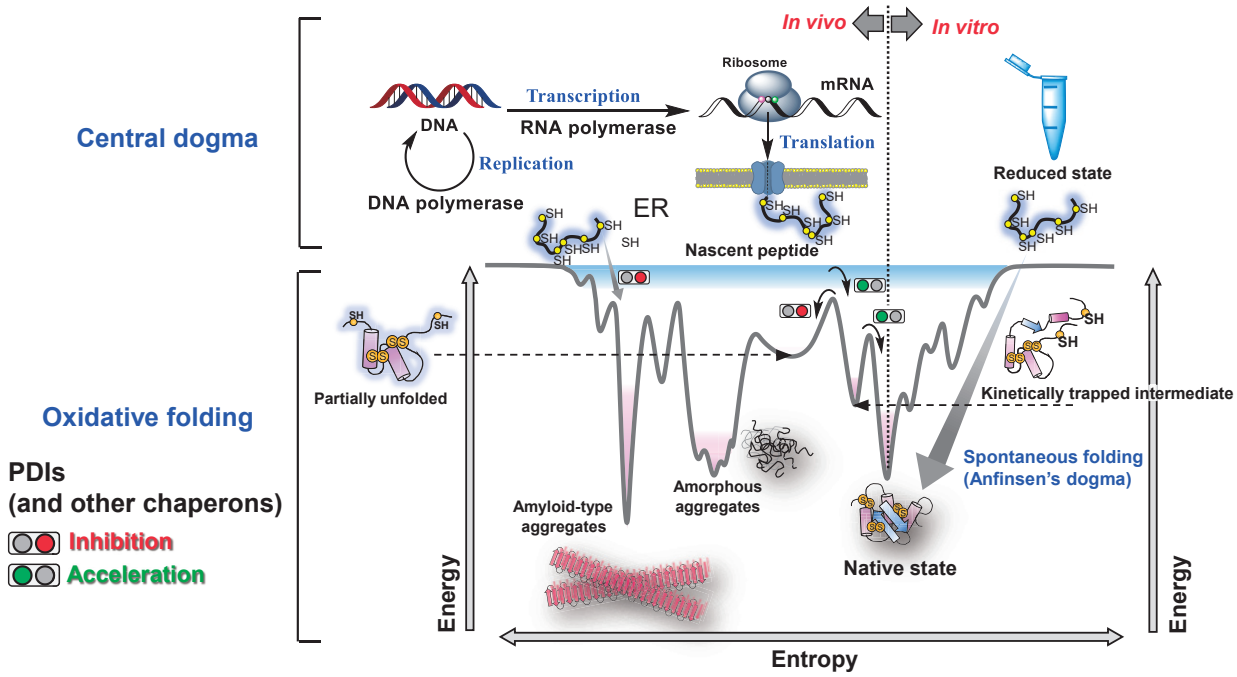


Fig. 1. Biosynthesis of polypeptides following the central dogma and subsequent oxidative protein folding controlled by protein disulfide isomerase family members (PDIs). For oxidative folding, two-dimensional energy landscapes (folding funnels) for *in vivo* and *in vitro* folding are shown, which represent the relationship of energy and entropy (i.e., diversity of peptidyl conformation).

Oxidative cross-linking between cysteinyl thiol (SH) groups to form disulfide (SS) bond(s) is a typical and important posttranslational modification, particularly for secreted proteins; folding coupled with SS formation is commonly termed oxidative folding [3]. Synthesized nascent polypeptides undergo oxidative folding in the endoplasmic reticulum (ER), where the oxidative environment is maintained. Protein disulfide isomerase (PDI), a typical ER-resident SS-based oxidoreductase, cooperatively controls proteostasis together with more than 20 PDI family enzymes (PDIs) in the ER (**Fig. 1**) [4]. PDI has four functional cysteine (Cys) residues that are important for its enzymatic activity (see below). The generation and accumulation of reactive nitrogen species (RNS) due to excessive oxidative stress in the ER leads to undesired oxidative modifications of Cys residues in PDI and significantly decreases enzymatic activity [5]. Dysfunction of PDI leads to disruption of proteostasis in the ER, resulting in misfolding, oligomerization, aggregation, and amyloidogenesis of proteins (**Fig. 1**), eventually triggering various fatal human diseases such as cancer, neurodegenerative disorders, diabetes, and atherothrombotic stroke [6–12].

Oxidative folding is also an essential process for chemically synthesized polypeptides to exert their physiological functions. The solid-phase peptide synthesis (SPPS) method pioneered by Merrifield enabled the synthesis of polypeptides with arbitrary amino acid sequences [13]. Consequently, rare proteins, non-natural proteins, and peptide-based formulations have been easily synthesized, and related fields such as structural biology, medicine, and drug discovery have progressed remarkably [14]. Generally, oxidative folding of synthetic polypeptides can be completed within a reasonable reaction time (hours to days) under mild oxidative conditions. However, considerable effort is often required to determine the optimal reaction conditions. While the use of PDI as a catalyst is effective for efficient folding, it is costly, and separation of the folded protein from PDI is usually difficult. In addition, peptide chains containing three or more Cys residues generally convert to isomeric species with non-native SS bonds (scrambled species) in addition to the native state during oxidative folding. Because the SS bonding pattern depends on the thermodynamic stability of the peptide loop, it is impossible to achieve 100% folding yield, even under precisely optimized

conditions. Therefore, purification of the target protein by high-performance liquid chromatography (HPLC) is usually required after oxidative folding, thus reducing cost and time efficiency of the overall protein synthesis.

Consequently, developing tools for efficient oxidative folding, which has troublesome aspects both *in vivo* and *in vitro*, should be valuable in medicinal science and drug discovery where protein misfolding is a concern. Currently, two chemical strategies are available for this purpose:

a) Indirect control of oxidative folding from the *outside* of protein molecules using an artificial organocatalyst with PDI-like functions (called the *outside strategy*; **Fig. 2a**).

b) Insertion of regioselective covalent bond(s), instead of SS bond(s), and promotion of structural maturation of proteins directly from the *inside* of the protein molecule (called the *inside strategy*; **Fig. 2b**).

In this review, the outside and inside strategies utilizing the redox properties of organoselenium molecules and peptides are discussed. First, the latest research trends and important achievements, in addition to some significant pioneering studies for each strategy, are outlined, and future perspectives of these strategies for artificial manipulation of *in vivo* and *in vitro* oxidative folding are discussed.

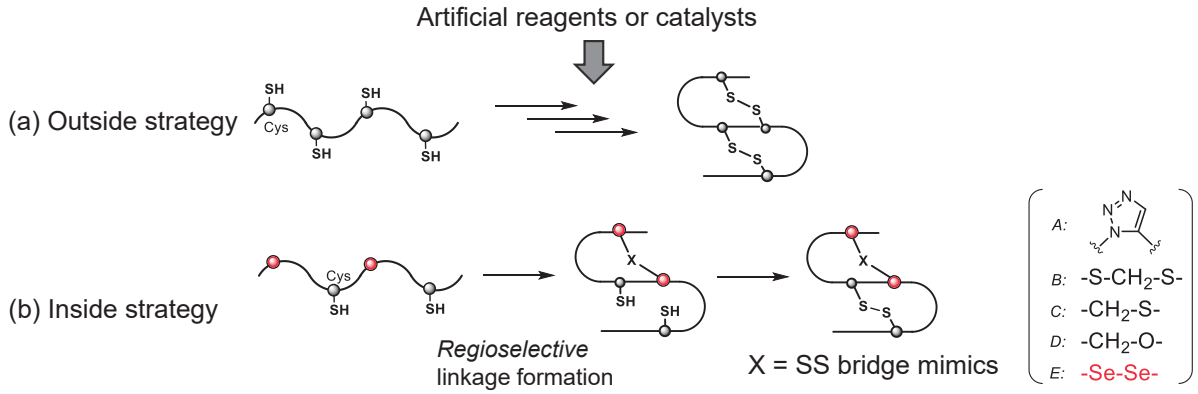


Fig. 2. Potential manipulation strategies for oxidative folding.
(a) Control of oxidative folding using artificial reagents and catalysts (*outside strategy*).
(b) Regioselective covariant bond formation in peptides and proteins by substituting native SS bonds with its representative mimics (*inside strategy*). Disulfide (SS) mimic E (diselenide bridge) was discussed in this review. For SS mimics A, B, C, and D, see the reports in Refs. [15], [16], [17], and [18], respectively.

2. Protein folding coupled with disulfide bond formation

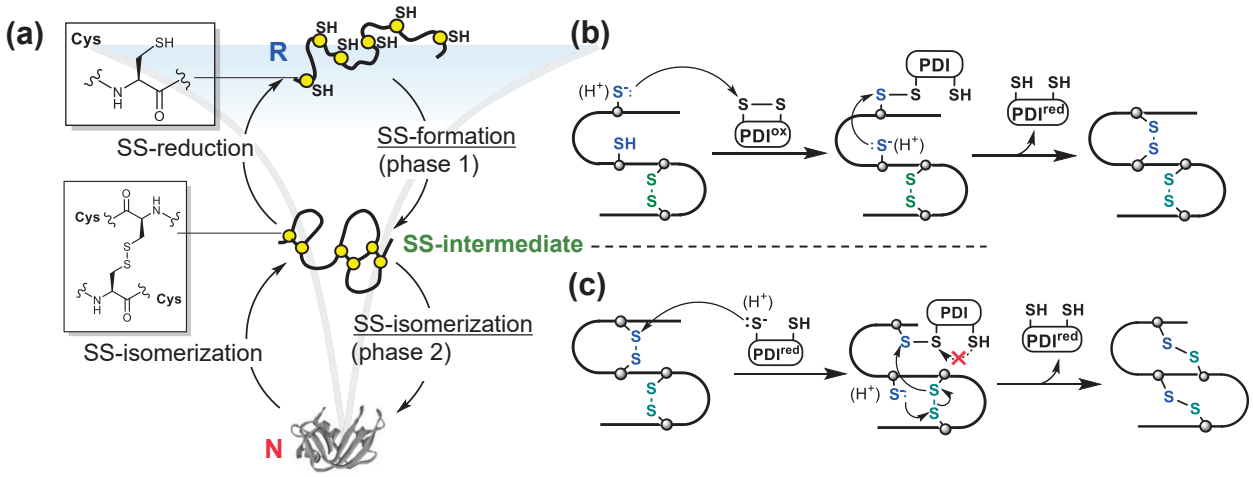


Fig. 3. Chemical perspective of oxidative folding.
(a) Structural maturation coupled with SS-formation (phase 1) and subsequent SS-isomerization (phase 2).
(b) SS-formation promoted by PDI^{ox} via an intermolecular bond exchange reaction.
(c) SS-isomerization catalyzed by PDI^{red} via a temporal SS-cleavage in substrate proteins.

Generally, during *in vitro* oxidative folding, two chemical reaction phases, SS-formation (phase 1) and SS-isomerization (phase 2), are observed (**Fig. 3a**). In phase 1, a reduced protein (R) loses its chain entropy with the hydrophobic collapse of the polypeptide, accompanied by the formation of intramolecular non-native SS bonds. In phase 2, the SS intermediates (scrambled species) generated in the first phase undergo conformational folding accompanied by rearrangement of SS bonds to search for correctly paired SS bonding patterns found in the native state. PDI accelerates the overall rate of oxidative folding by exhaustively catalyzing SS-related reactions in these phases (**Fig. 3b, c**) [19–22].

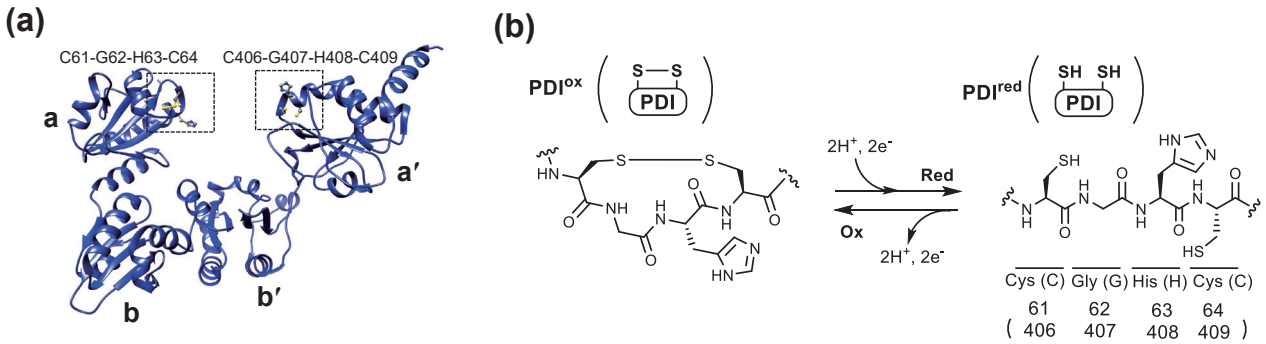


Fig. 4. Protein disulfide isomerase (PDI).
(a) Molecular structure of PDI composed of four tandem thioredoxin (Trx)-like domains.
(b) Reversible redox state of CGHC motif as the redox active sequence of PDI.

PDI is composed of four tandem thioredoxin (Trx)-like domains, in which the N- and C-terminal domains (a and a′, respectively) contain a redox active sequence, namely Cys-Gly-His-Cys (CGHC) (**Fig. 4a**). Under physiological conditions, CGHC sequences are in equilibrium between the two redox states, disulfide (PDI^{ox}) and dithiol (PDI^{red}) states (**Fig. 4b**). During phase 1, PDI^{ox} rapidly introduces SS bonds to substrate proteins via an intermolecular bond exchange reaction (**Fig. 3b**). On the other hand, PDI^{red} catalyzes SS-isomerization during phase 2 by temporarily cleaving the miscrossed SS bonds due to the superior nucleophilic capability of the SH groups in CGHC (**Fig. 3c**). CGHC in PDI^{ox} and PDI^{red} have two crucial physicochemical properties so that PDI effectively catalyzes these reactions. First, the SS reduction potential ($E^{o\prime}$) of PDI^{ox} (–180 mV) is considerably higher than that of the CXXC motifs in other PDI family members except for ERdj5 [23]. This indicates that PDI^{ox} possesses a strong oxidizing capability, which is an ideal property for promoting phase 1. Furthermore, the pK_a values (ca. 6.7) of the SH groups in CGHC are substantially lower than those of general cysteinyl SH groups in proteins (ca. 8.3) due to the effect of the neighboring basic His residue. Thus, for phase 2, which is initiated by nucleophilic attack from the SH group in CGHC, PDI also has ideal reactivity. Moreover, the high $E^{o\prime}$ indicates that PDI^{red} is less likely to be ring-closed (oxidized) to PDI^{ox}, indicating that PDI^{red} promotes SS-isomerization rather than SS-reduction (**Fig. 3c**).

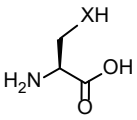
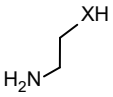
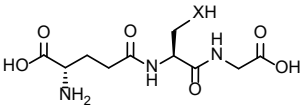
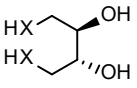
Since organothiols (RSH) exist in redox equilibrium states with coexisting disulfide (R′SSR′) compounds in an aqueous solution (Eq. 1), they can promote SS-related reactions via a PDI-like catalytic activity during oxidative folding.

$$2RSH + R'SSR' \rightleftharpoons RSSR + 2R'SH \quad \cdots (1)$$

In addition to monothiol glutathione (GSH) and its oxidized form (GSSG), which have been conventionally used as oxidative folding reagents, a number of sulfur-based compounds that mimic the physicochemical properties and catalytic functions of PDI active centers have been reported [24–29]. However, their catalytic ability is remarkably lower than that of PDI, and compounds must be added at a molar ratio of 10 to 100-fold with respect to the substrate proteins to achieve reasonable folding yields and rates.

3. Redox chemistry of diselenides and selenols

Table 1. | Physicochemical properties of conventional thiols and selenols.

Compound	Analog	pK_a	Diselenide reduction potential $E^{\circ\prime}$ (mV)
	X = S (Cys)	8.22 [a]	–238 [b]
	X = Se (Sec)	ca. 5.2 [c]	–383 [b]
	X = S (CA)	8.37 [d]	–236 [b]
	X = Se (SeCA)	ND [a]	–352 [b]
	X = S (GSH)	9.42 [a]	–256 [b]
	X = Se (GSeH)	ND [b]	–407 [b]
	X = S (DTT^{red})	9.21/10.1 [e]	–327 [f]
	X = Se (DST^{red})	ND [b]	–396 [g]

Values are from Refs. [a] [30], [b] [31], [c] [32], [d] [33], [e] [34], [f] [35], and [g] [36]. [h] No data.

Selenium, an essential micronutrient in living organisms, is incorporated into selenocysteine (Sec). Sec is an analog of Cys, in which the SH group in cysteine is replaced by a selenol (SeH) group. Sec is a building block in proteins that functions as an active center in various reductases to reduce various biological substrates, due to the excellent two-electron-donating ability of the SeH groups. Various organoselenols (RSeH), including Sec, can also be in redox equilibrium with coexisting disulfide compounds (R'SS R'), as RSH (Eq. 2).

$$RSeSeR + 2R'SH \rightleftharpoons 2RSeH + R'SSR' \quad \bullet\bullet\bullet (2)$$

In general, the pK_a value of RSeH is considerably lower than that of RSH. Although there are few reports on the pK_a of a SeH group because the isolation and purification of organoselenols are generally difficult due to their enormously high reactivity, the pK_a values of Cys and Sec residues have been reported to be 8.22 and ca. 5.2, respectively (**Table 1**). Thus, the SeH group almost exists as a deprotonated state (selenolate; Se^-) under physiological conditions and functions as a superior nucleophile. The acidity of the SeH group exceeds that of Cys residues in the active centers of PDI, indicating that organoselenols have more ideal chemical properties as reagents for promoting SS-isomerization (phase 2, **Fig. 3c**). On the other hand, the reduction potential of the diselenide (SeSe) bond ($E^{\circ\prime}_{SeSe}$) is generally lower than that of the SS bond (**Table 1**), and the oxidation of SH groups to SS bonds with SeSe compounds (eq. 2, forward reaction) is thermodynamically unfavorable [37]. Therefore, molecular design to increase the $E^{\circ\prime}_{SeSe}$ values is important for improving the function of organodiselenides as PDI mimics.

4. Outside strategy

4.1. Selenoglutathione: The first selenocompound as an oxidative folding catalyst

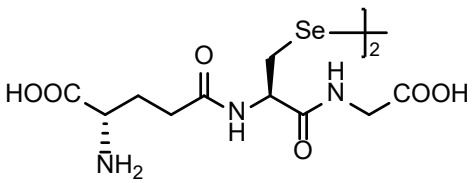


Fig. 5. | Oxidized selenoglutathione (GSeSeG) as the first diselenide-based folding catalyst.

Selenoglutathione (GSeSeG, **Fig. 5**), in which Cys residues in glutathione disulfide (GSSG) are replaced by Sec residues, was first reported as a water-soluble diselenide-based reagent used in oxidative folding [31]. Hilvert *et al*. used GSeSeG as an oxidant to the oxidative folding of the reduced state of ribonuclease A (RNase A) with no SS bond, and found that GSeSeG promoted oxidative folding at a higher rate than GSSG. They further demonstrated that in the oxidative folding of the reduced state of bovine pancreatic trypsin inhibitor (BPTI), GSeSeG promoted early SS-formation events (phase 1, **Fig. 3b**) more effectively and provided a higher final folding yield than GSSG. GSeSeG, despite having a considerably lower reduction potential ($E^{\circ\prime}_{SeSe} = -407$ mV) than GSSG ($E^{\circ\prime}_{SS} = -256$ mV), exerts a higher oxidizing ability for SS-formation than GSSG. The nucleophilic attack of an SH group on the SeSe bond was previously shown to be up to four orders of magnitude faster than the attack on the SS bond, indicating that the kinetics of organodiselenides make them more favorable SS-forming reagents [38,39]. In addition, monoselenol GSeH, which is produced as a byproduct during SS-formation (GSeSeG + Protein[SH,SH] → $2GSe^- + 2H^+$ + Protein[S-S]), promotes SS-isomerization reactions (phase 2, **Fig. 3c**) more effectively than GSH because of its higher nucleophilicity, which accelerates the overall folding rate.

In the oxidative folding of BPTI, the accumulation of two kinetically trapped SS-intermediates decelerates the overall folding rate and reduces the yield of the folded state (see section 5-2). Metanis and Hirvert *et al*. previously showed that GSeH promotes SS-isomerization of SS-intermediates on unproductive pathways, thus improving folding efficiency [40]. Furthermore, the addition of catalytic amounts of GSeSeG can substantially promote oxidative folding under aerobic conditions because the GSeH generated during the reaction is readily reoxidized by molecular oxygen to GSeSeG (i.e., $4GSeH + O_2 \rightarrow 2GSeSeG + 2H_2O$) as an SS-forming agent [41]. GSeSeG was applied to the oxidative folding of various reduced proteins, and its wide substrate scope has been demonstrated [42].

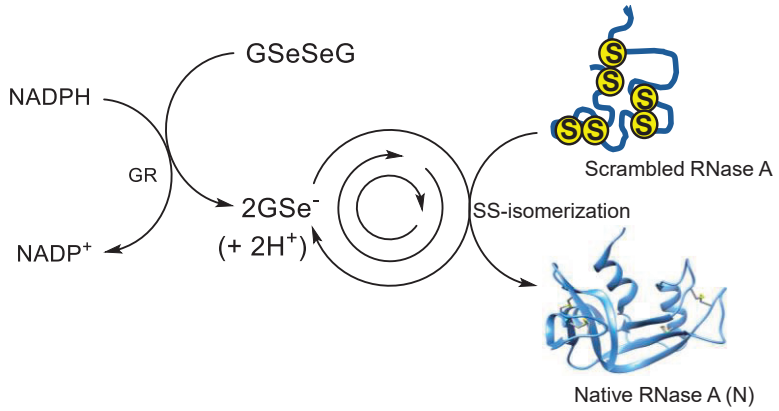


Fig. 6. | Refolding of scrambled RNase A coupled with GSeSeG reduction by glutathione reductase (GR).

Recently, Iwaoka *et al*. reported an effective synthetic route for preparing GSeSeG using a liquid-phase peptide synthesis method [43]. Furthermore, they showed that scrambled RNase A with four non-native SS bonds can be quickly repaired to the native state by treatment with NADPH and glutathione reductase (GR) in the presence of catalytic amounts of GSeSeG (1 mol%) (**Fig. 6**). This is because GSeSeG is reduced to GSeH, which catalyzes SS-isomerization, with NADPH in the presence GR that can recognize both GSSG and GSeSeG as substrates [44].

4.2. Modification of chemical structures

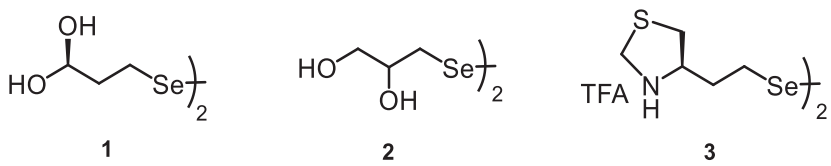


Fig. 7. | Structures of small molecule diselenides **1–3** as oxidative folding catalysts.

Metanis *et al*. synthesized small-molecule diselenides **1–3** as second-generation dimeric-type diselenide compounds to replace GSeSeG (**Fig. 7**) [45]. These compounds can be synthesized more easily and at a higher yield than peptide-based compounds through a short synthetic route using inexpensive starting materials. Furthermore, the compound is smaller than GSeSeG, improving the probability of contact with free cysteinyl SH groups buried in kinetically trapped SS-intermediates, and consequently accelerating the overall velocity of oxidative folding. Indeed, in the oxidative folding of BPTI, selenol states generated from corresponding diselenide compounds smoothly mediated SS-isomerization of the kinetically trapped SS-intermediates generated during the folding, and remarkably improved the oxidative folding rate and yield more than GSSG. Notably, the capability of compounds **2** and **3** as folding promoters was comparable to or slightly higher than that of GSeSeG.

4.3. Cyclic diselenides

Not only dimeric diselenides as shown above but also cyclic diselenide compounds are frequently used to manipulate the structural maturation of denatured and reduced proteins. Raines *et al*. reported that the diselenolate form (**4a**) produced by the reduction of (*S*)-1,2-diselenan-4-amine (**4**) can rapidly reduce protein SS bonds [46] (**Fig. 8**). Arai *et al*. extended the potential application of compound **4** to oxidative folding [36]. In addition to **4**, analogs **5** and **6**, which differ in ring size and functional groups, respectively, were also synthesized, and the ability of the oxidative folding catalyst was compared by folding experiments with reduced RNase A. Five-membered ring diselenide **5** was found to have the highest SS-formation and SS-isomerization abilities [36]. Among the six-membered ring diselenides (**4** and **6**) (**Fig. 8**), the $E^{\circ\prime}_{SeSe}$ value of **4**, which has an amino group, is higher than that of **6**, which has two hydroxy groups. This may be due to the ammonium ($-NH_3^+$) group in the aqueous solution that stabilizes the chain-opened (diselenol) form (**4a**) by electrostatic interactions with the Se^- group in the molecule. Moreover, compound **5**, which has a stronger ring strain, had a significantly higher $E^{\circ\prime}_{SeSe}$ value than compound **4**. These results suggest that the $E^{\circ\prime}_{SeSe}$ value, and thus the folding catalytic activity of the cyclic diselenides, can be modulated by changing the ring size and functional groups.

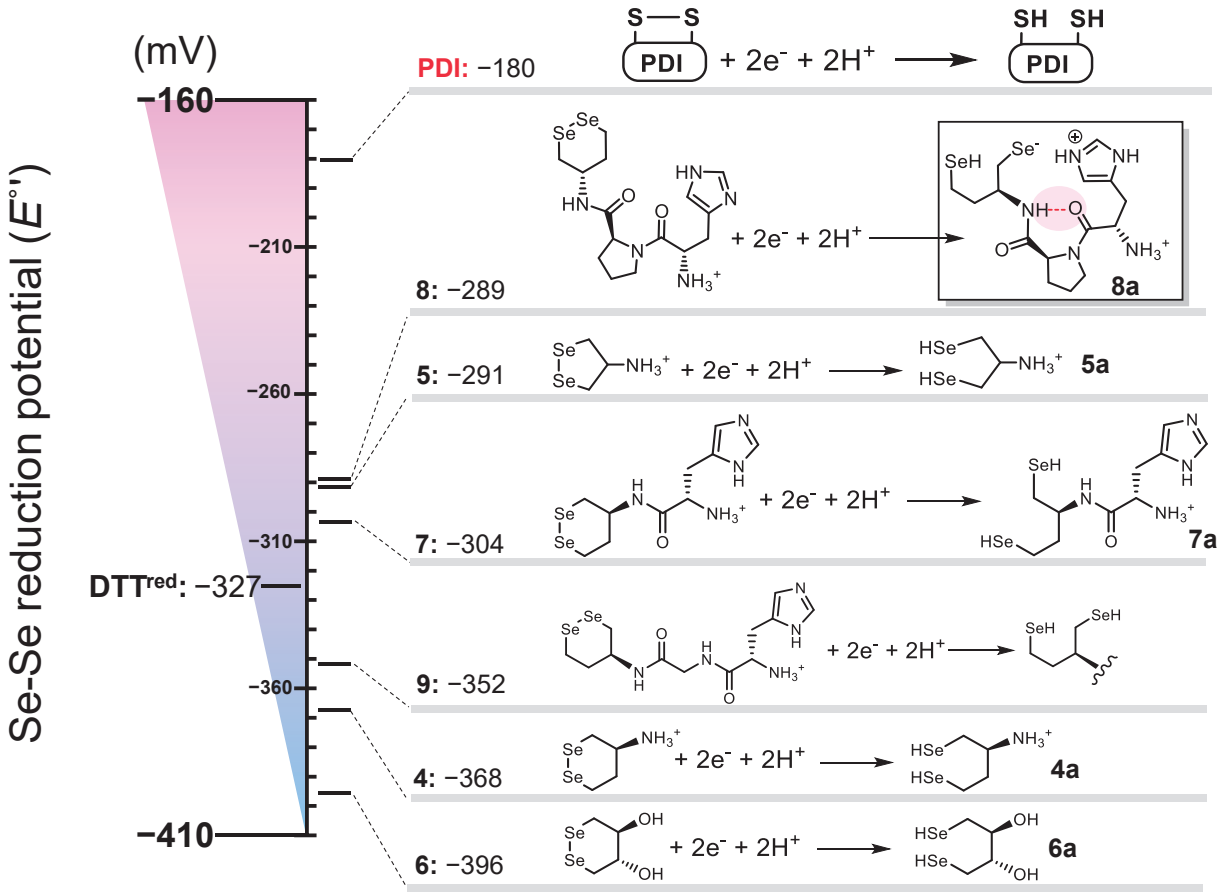


Fig. 8. | Comparison of reduction potentials of cyclic diselenide compounds.

However, such structural modifications of simple Se-containing aliphatic compounds have not been readily achieved in practical organic syntheses [47]. Meanwhile, we also reported that the $E^{\circ\prime}_{SeSe}$ values could be easily increased by conjugating His via an amide bond with compound **4** [48] (**Fig. 8**). This result may be attributed to the stabilization of the reduced form due to the formation of NH•••Se hydrogen bonds between the selenium atom in the diselenol state and the imidazole ring of His in **7a** (**Fig. 8**) [49]. Consequently, in the oxidative folding of hen egg-white lysozyme (HEL), compound **7** showed higher catalytic activity than the parent compound (**4**) for both oxidative folding of the reduced state without SS bonds and repair of the misfolded state (scrambled species) with non-native SS bonds. Importantly, the addition of compound **7** (0.3–0.5 mM) also efficiently inhibited the aggregation of HEL (1.4 mg/mL) induced by thermal denaturation. In the oxidative folding of high concentrations of reduced HEL (0.7 mg/mL), correct folding of the monomeric protein was also accompanied with undesired aggregation and oligomerization in a redox buffer solution containing common glutathione (GSH/GSSG, 1.0 mM/0.2 mM), finally recovering only 25% yield of biologically active state. In contrast, the coexistence of compound **7** (0.2 mM) instead of GSSG inhibited intermolecular contacts of proteins, accelerated the oxidative folding rate, and improved the folding yield to 55% [48]. Thus, these results strongly suggest that the conjugation of His with compound **4** simultaneously enhances its redox reactivity as a PDI-like catalyst and chaperone-like capability to suppress protein aggregation.

More recently, compound **8**, in which Pro is inserted as a spacer amino acid between compound **4** and His, had a significantly higher $E^{\circ\prime}_{SeSe}$ value than **7** and **9** without or with Gly spacer instead of Pro residue, respectively (**Fig. 8**) [50,51]. Detailed structural analyses of the compounds by circular dichroism (CD) and 2-dimensional nuclear magnetic resonance (NMR) spectroscopy clearly showed that reductive cleavage of the SeSe bond in **8** induces transformation of the peptide backbone into a rigid γ-turn structure. Therefore, the SeH and imidazole groups are spatially close to each other in the reduced state, forming NH•••Se hydrogen bonds (or salt bridges) that thermodynamically stabilize the diselenol moiety. Thus, compound **8** functions as an excellent oxidative folding catalyst. Moreover, aliphatic cyclic diselenols are promising regulators of intracellular redox balance in cells because they function as good two-electron donors and are effective not only in SS-related reactions during folding, but also in the reduction of reactive oxygen/nitrogen species, such as peroxides and nitrosothiols, which cause protein misfolding [51,52].

4.4. Enhancement of oxidative folding in cells by selenocystamine

Hilvert *et al*. previously reported that selenocystamine ($[SeCA]_2$), an oxidized state of SeCA (**Table 1**) and a smaller diselenide compound than GSeSeG, also significantly accelerates the oxidative folding of RNase A. However, its catalytic ability is slightly lower than that of GSeSeG. [41]. They further reported that low concentrations (1–10 μM) of $[SeCA]_2$ administered to *Escherichia coli* lacking disulfide bond isomerase A (DsbA), a typical CXXC motif-containing folding enzyme in the periplasm of gram-negative bacteria, promoted oxidative protein folding instead of DsbA [53].

Recently, eukaryotic green algae have attracted attention as platforms for the production of recombinant proteins. The rate-limiting step in the accumulation of recombinant proteins is the structural maturation of proteins coupled with SS-formation and SS-isomerization in chloroplasts, which contain protein-folding enzymes, such as chaperones, peptidyl prolyl isomerases, and PDIs. Interestingly, the addition of $[SeCA]_2$ was found to improve the accumulation of SS-containing proteins in the chloroplasts, suggesting that the selenocompound would be promising for effective protein production by genetic engineering [54].

5. Inside strategy

5.1. Folding pathway kinetically controlled by insertion of Sec residues

Because SS-boding patterns in proteins with multiple Cys residues are essentially governed by the thermodynamics of polypeptide chains, it is impossible to form SS bonds at arbitrary positions at 100% yield without direct modification of peptide chains in the synthetic process. Moloder *et al*. pioneered a method to kinetically govern oxidative protein folding by internalizing the physicochemical properties of the SeH group into the polypeptide itself [55]. Endothelin-1 (ET1; 21 amino acids), a vasoconstrictive peptide, is a mini protein stabilized by two intramolecular SS bonds (Cys1–Cys15 and Cys3–Cys11). Under optimal conditions, oxidation of reduced ET1 without SS bond by O_2 provides a regioisomer with two non-native SS bonds (isomer A) and native ET1 (isomer B) in a 1:3 ratio, depending on the relative thermodynamic stability of the peptide loops (**Fig. 9a**) [56]. Moroder *et al*. synthesized an ET1 analog ([C3U,C11U]-ET1), in which the two Cys residues (Cys3 and Cys11) that form SS bonds in the native state were substituted by Sec residues to selectively yield correctly folded ET1. Because the pK_a

value of the SeH groups in the reduced state is significantly lower than that of the cysteinyl SH groups (**Table 1**), SeSe bond formation proceeds preferentially even when multiple Cys residues coexist in the peptide chain. In addition, the SeSe bond in the peptide chain is more thermodynamically stable than the SS bond [37], and thus bond isomerization via SH-SeSe exchange is thermodynamically less likely to proceed. Consequently, the generation of a misfolded state with incorrect bonding pairs was inhibited during the oxidative folding of [C3U,C11U]-ET1 (**Fig. 9a**). Notably, replacing SS with SeSe did not affect the biological activity and folded structure of ET1.

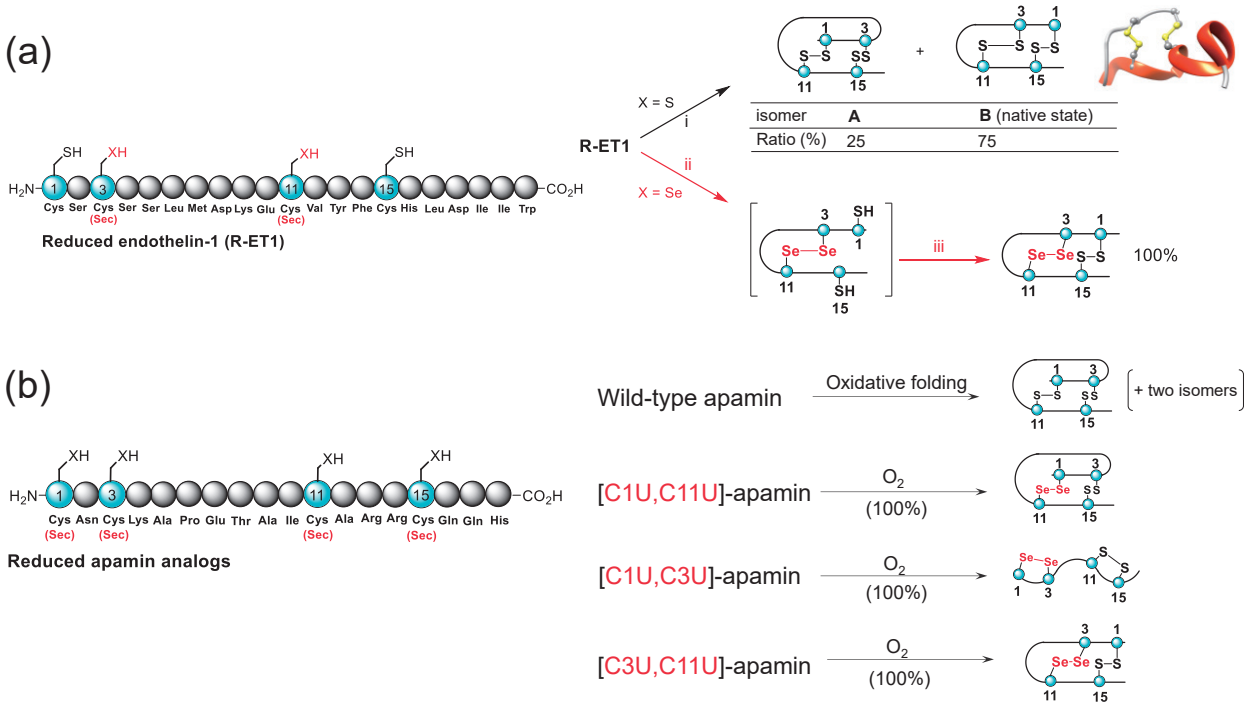


Fig. 9. Oxidative folding of mini proteins kinetically controlled by highly reactive selenol (SeH) groups in selenocysteine (Sec) residues.
(a) Predominant oxidation of Sec residues and selective production of folded endothelin-1 (ET1) analog ([C3U,C11U]-ET1).
(b) Regioselective diselenide- (SeSe) and SS-formation to produce possible apamin isomers.

Apamin, a bee toxin protein, has the same SS bond topology as ET1. Moroder *et al*. selectively synthesized three possible isomers, including the native state, by utilizing the Sec-substituting strategy (**Fig. 9b**) [57,58]. The results strongly suggest that beyond the structural information encoded in the primary sequence, the kinetic and thermodynamic properties of the SeH groups and SeSe bonds, respectively, contribute more strongly to the folding pathway and SS-bonding pattern in the final product. Consequently, Sec was also utilized to generate misfolded forms in a purposeful manner [55]. Since then, this method has been applied to structural analysis of protein folding intermediates, elucidation of the folding pathway, and effective production of peptide formulations, as described below.

5.2. Enhancement of the foldability of conotoxins

SS-rich bioactive peptides, such as neurotoxins, plant cyclotides, antimicrobial peptides, and protease inhibitors, are potential therapeutic agents for analgesics, antihypertensives, antiarrhythmics, antitumor medicines, antivirals, and antibiotics. SS-rich conotoxins, known as neurotoxins from *Conus*, are peptide drug candidates that have been explored to enhance the foldability and structural stability of their native states by the Sec-substituting strategy. Multiple SS bonds in conotoxins are readily reduced or isomerized in the presence of an SH-based reductant, such as GSH, thioredoxin, and albumin, thus easily degrading them in the blood. Alewood *et al*. synthesized α-selenoconotoxins, in which one or two SS bonds in wild-type α-conotoxin were replaced by SeSe bonds [59]. Due to regioselective SeSe bonding, the folded state of α-selenoconotoxins is effectively produced at high yields, and all analogs exhibited remarkable stability against SH-based biomolecules [59,60]. Similarly, for pharmacologically

relevant conotoxins containing three SS bonds, such as μ-conotoxin [61], ω-selenoconotoxin GVIA [62], μ-conotoxin KIIIA [63], and μO-conotoxin MrVIB [64], the folding yield was improved by replacing one SS bond with a SeSe bond. In both cases, the substitution of SeSe bonds did not alter the 3D structure and reduce the biological activity. Furthermore, Bulaj *et al*. compared the overall folding rates of seleno-analogs of ω-conotoxin GVIA and μ-conotoxin SIIIA with wild types. Both selenopeptides, in which one of the three SS bonds was replaced by SeSe bond, promoted SS-formation and SS-isomerization during oxidative folding at a higher rate than the wild-type peptides, even in the absence of chemical redox reagents. This is due to the autocatalytic functions of inserted Sec residues in the peptides (**Fig. 10**). [65].

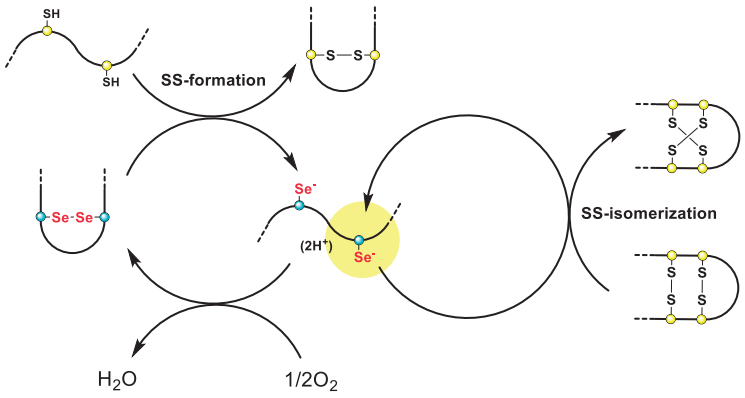


Fig. 10. | Redox reagent-free oxidative folding of conotoxins catalyzed by intramolecular Sec residues.

5.3. Steering of the folding pathway by inserting Sec residues

A detailed oxidative folding pathway has been reported for wild-type BPTI (58 amino acids), which has long been employed as a benchmark protein in folding studies (**Fig. 11a**) [66–68]. In the key intermediates, N* and N′, which have native-like structures, free SH groups are buried inside the molecules. Generation of these kinetically trapped intermediates consequently decelerates the overall folding rate of BPTI. Conversely, if the formation of N* and N′ could be avoided, reduced BPTI (R) could lead to rapid and high-yield formation of the native state (N).

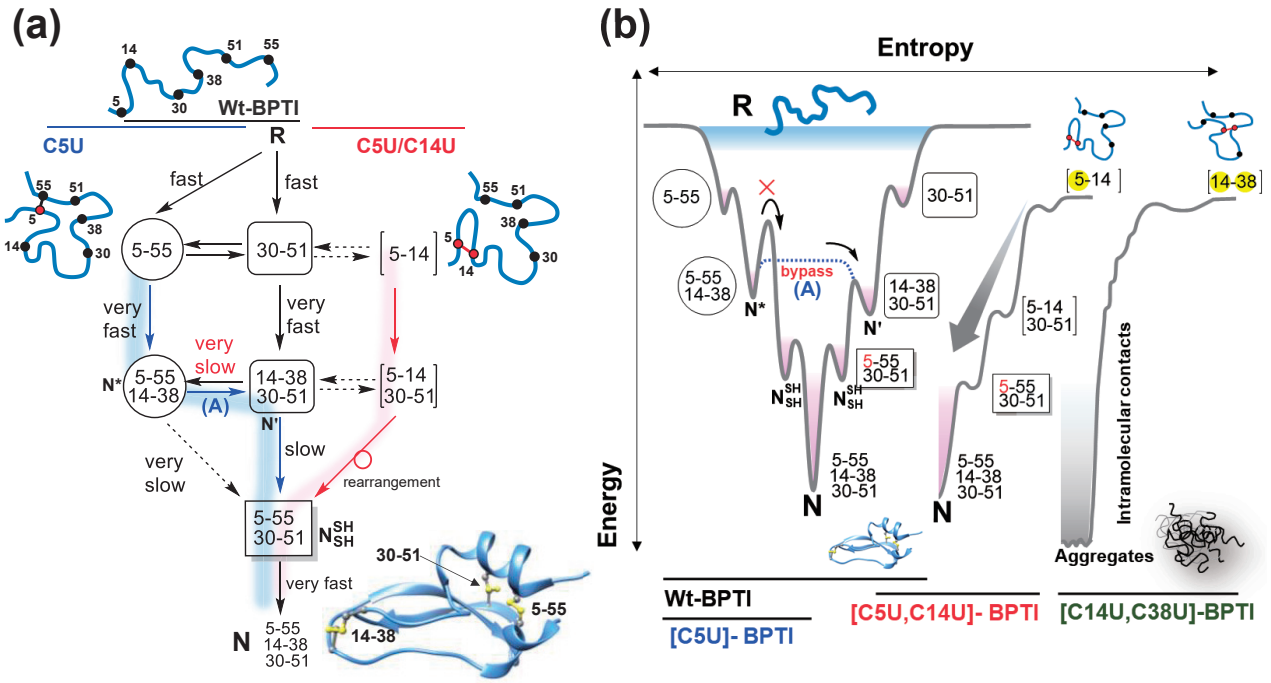


Fig. 11. | Oxidative folding of bovine pancreatic trypsin inhibitor (BPTI).
(a) Major oxidative folding pathways of BPTI and its Sec-substituted analogs.
(b) Modification of energy landscape for oxidative folding of BPTI by insertion of Sec, instead of cysteine (Cys) residues.

Based on this concept, Hilvert *et al*. proposed a new strategy to efficiently yield N by replacing Cys5 and Cys14 of BPTI with Sec residues, preferentially undergoing a pathway in which N* and N′ are not involved (**Fig. 11a**, pathways highlighted in pink) [69]. Indeed, the seleno-analog, [C5U,C14U]-BPTI, folded into N at 70–80% yield at a higher rate than wild-type BPTI under weakly basic and aerobic conditions, whereas oxidative folding of wild-type BPTI produced N* and N′ as the major products (80%) with N at a low yield. This result indicates that [C5U,C14U]-BPTI can selectively undergo a reaction pathway with smaller activation barriers than those in wild-type BPTI by preferentially forming the non-native crosslink via the SeSe bond at the early folding phase (**Fig. 11b**, middle funnel). On the other hand, for [C14U,C38U]-BPTI, intermolecular contacts preferentially form aggregates because of the formation of an unfoldable intermediate with a SeSe bond at the non-native position (**Fig. 11b**, right funnel) [70]. In the case of [C5U]-BPIT, in which only one Cys residue (Cys5) is replaced by Sec, the folding pathway is essentially unaltered (**Fig. 11a**, pathways highlighted in blue), but N* can be quickly converted to N′, which is accessible to the precursor (N^{SH}_{SH}) through a bypass with a small activation barrier (**Fig. 11a**, path A), resulting in an increased overall folding rate (**Fig. 11b**, left funnel) [70]. Since a single Sec residue can be artificially introduced into a protein by genetic engineering, the result that the single Sec insertion instead of Cys reasonably enhances folding efficiency would be advantageous for the biological production of peptide-based formulations containing SS bonds [70–74].

5.4. Simplification of the oxidative folding pathway involving diverse SS-intermediates

A detailed oxidative folding pathway of hirudin (65 amino acid residues), a thrombin inhibitor with three SS bonds (Cys6–Cys14, Cys16–Cys28, and Cys22–Cys39), was reported in the 1990s by Chang *et al*. [75–77]. While hirudin has almost the same number of amino acid residues as BPTI with three SS bonds, their oxidative folding phenomena are completely different [78]. BPTI achieves oxidative folding through a limited number of SS-intermediates with native-like structures (**Fig. 11a**). In contrast, the oxidative folding of hirudin involves SS-intermediates (scrambled species), 1SS, 2SS, and 3SS, which have one, two, and three non-native SS bonds in the molecule, respectively. These intermediates are sequentially generated from the reduced state (R) (i.e., R→1SS→2SS→3SS), accompanied by the hydrophobic collapse of the polypeptide. 3SS subsequently undergoes SS-isomerization with conformational folding to obtain the native SS-bonding pattern and folded structure (**Fig. 12**).

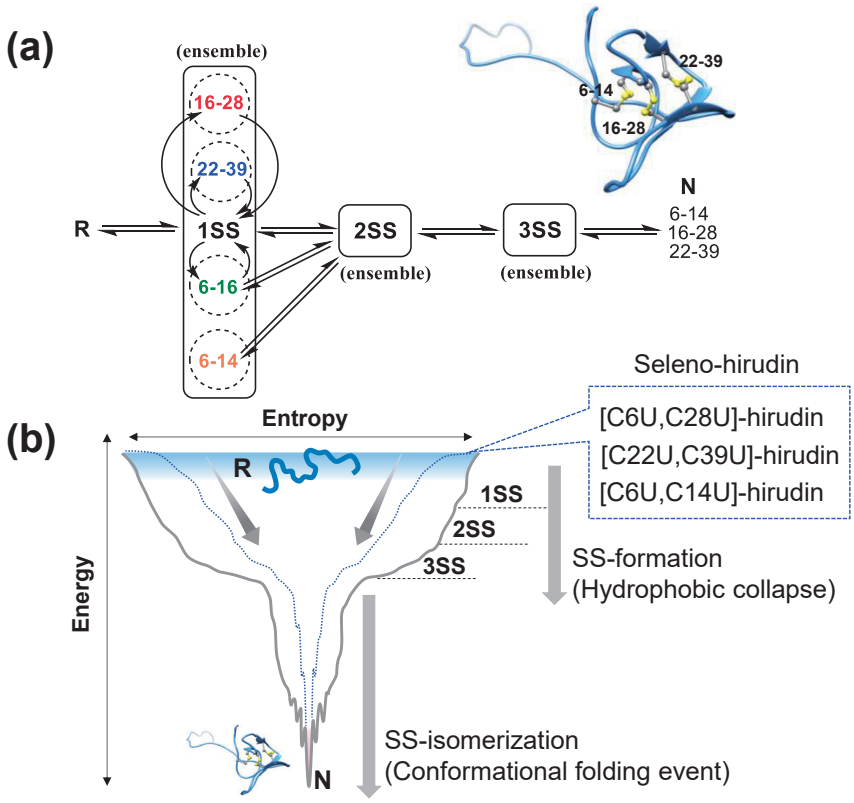


Fig. 12. Oxidative folding of hirudin with three SS bonds.
(a) Oxidative folding pathway of hirudin and seleno-hirudin via scrambled SS-intermediates. Heterogeneous ensembles with one (1SS), two (2SS), and three (3SS) SS bonds in the molecule are shown.
(b) Energy landscape for oxidative folding of wild-type hirudin (gray) and seleno-hirudin (blue).

Metanis *et al*. prepared seleno-hirudins ([C6U,C14U], [C16U,C28U] and [C22U,C39U]), in which one of the three native SS bonds was replaced by SeSe bond, and investigated their folding behavior [79]. All analogs folded into the native state at a higher rate than the wild type. During the early oxidative folding event (SS-formation) of wild-type hirudin, heterogeneous 1SS ensembles, including various isomers, are generated as scrambled species, whereas for seleno-hirudin, the components in the scrambled species are biased, decreasing the heterogeneity. Particularly, [C6U,C14U]-hirudin with the SeSe bond directly gained an additional SS bond without conversion into the scrambled species via the SH-SeSe exchange reaction (**Fig. 12a**). In contrast, [C6U,C16U]-hirudin with the SeSe bond at the non-native position rapidly isomerized to a heterogeneous ensemble. These results indicate that the introduction of the SeSe bond in the appropriate position significantly reduces the diversity (i.e., peptidyl entropy) of the folding intermediates (**Fig. 12b**) and that the folding rate is accelerated by undergoing a simpler pathway.

5.5. Potent tool for interchain cross-coupling

Insulin, an important peptide-based drug, is widely known as a hypoglycemic agent for diabetic patients. Its native structure is stabilized by two interchain SS bonds (Cys7^{A}–Cys7^{B} and Cys20^{A}–Cys19^{B}) between the A-chain (21 amino acid residues) and the B-chain (30 amino acid residues), in addition to the intrachain SS bond (Cys6^{A}–Cys11^{A}) in the A-chain. Direct coupling of the unprotected A-chain and B-chain via SS linkages in an oxidative environment to obtain the native form is the simplest and most rational synthetic method. However, intramolecular SS cross-linking in each peptide chain is usually preferred, producing a folding yield of less than 5% [80–82]. Although several efforts have been directed toward developing innovative synthetic methodologies, a technology that involves a simple synthetic process and exhibits high product yield is yet to be developed [83].

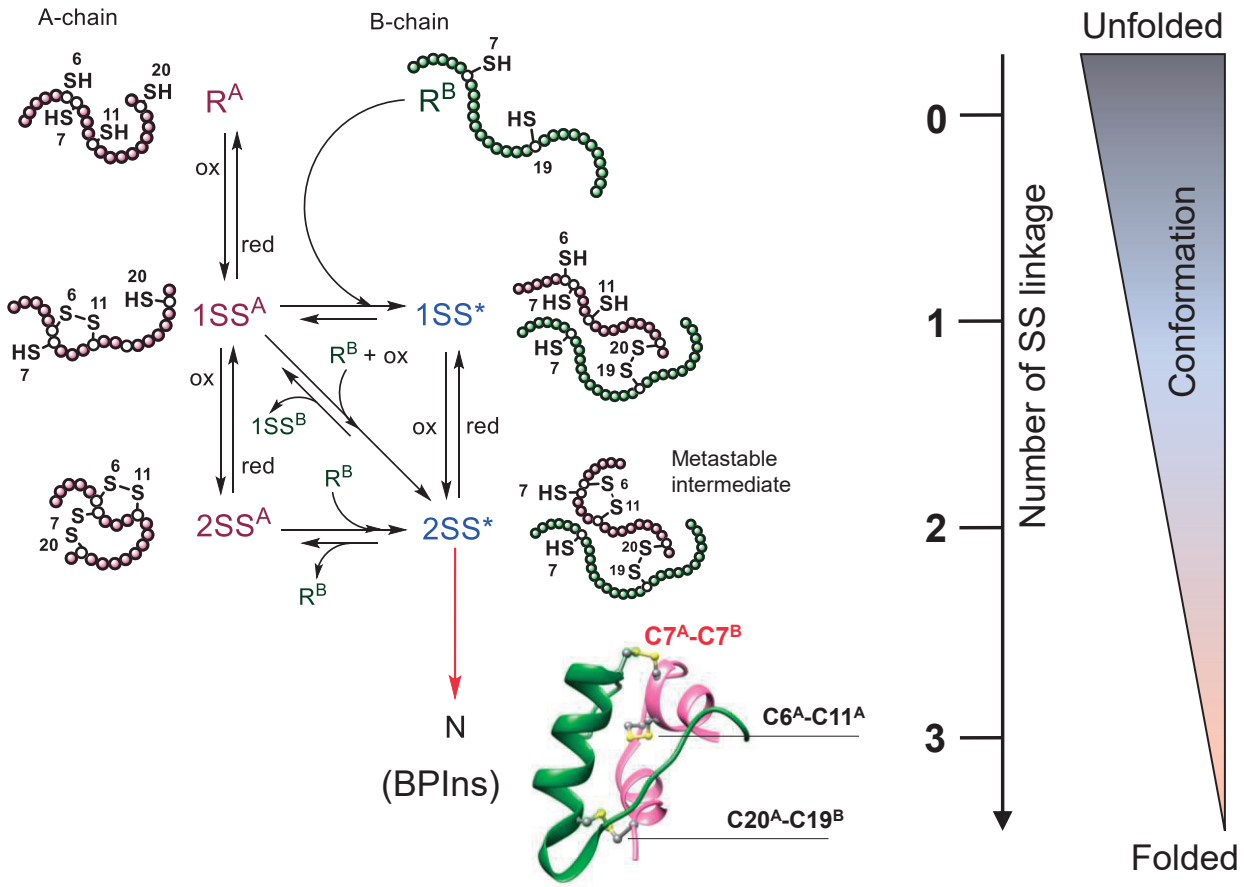


Fig. 13. Chain combination pathways of unprotected bovine pancreatic insulin (BPIns) A-chain and B-chain (native chain assembly; NCA).

Arai *et al*. predicted that the A-chain and B-chain could be coupled directly and effectively by utilizing the Sec-substituting strategy, which has been applied to single-chain polypeptide folding (see above). Prior to the trial of this strategy, an exhaustive mechanistic investigation on oxidative folding of the unprotected A-chain and B-chain, namely native chain assembly (NCA), was performed to determine the appropriate positions for inserting Sec residues [84]. The results revealed that the NCA pathway of insulin includes several pathways, all of which involve a common metastable precursor (2SS*) that lacks one interchain SS bond ($Cys7^A$–$Cys7^B$) (**Fig. 13**). Furthermore, $Cys7^A$–$Cys7^B$, which is solvent-exposed in the native structure, was found to be the most kinetically and thermodynamically unstable among the three native SS bonds.

Consequently, Arai *et al*. synthesized the [C7U]-A-chain and [C7U]-B-chain, which are seleno-analogs of the component peptides of bovine pancreatic insulin (BPIns) [85]. The A-chain and B-chain were isolated as 2-pyridylsulfanyl- (Pys) protected derivatives with one SeS bond and an oxidized form including one SeS bond, respectively (**Fig. 14a**). The peptide chains were activated to reactive species containing SH and SeH groups under weakly reductive conditions, and the chains gradually coupled with each other by O_2 as an oxidant to form the [$C7U^A$,$C7U^B$] variant of BPIns, namely selenoinsulin (SeIns), at up to 27% isolated yield. SeIns has almost the same biological activity and 3D structure as the wild type. More notably, SeIns exhibited much higher resistance against insulin-degrading enzyme (IDE), which is found in the liver and pancreas in mammals, than wild-type insulin (i.e., $\tau_{1/2} \approx 8$ h vs. ≈ 1 h for BPIns), and was found to have potential as a long-acting formulation that could circulate in the body for a long time. X-ray crystallographic analysis of SeIns suggests that the SeSe bond ($Sec7^A$–$Sec7^B$) has enhanced native interactions in the N-terminal region of the B-chain, resulting in increased stability of the monomeric state, which may explain the high IDE resistances.

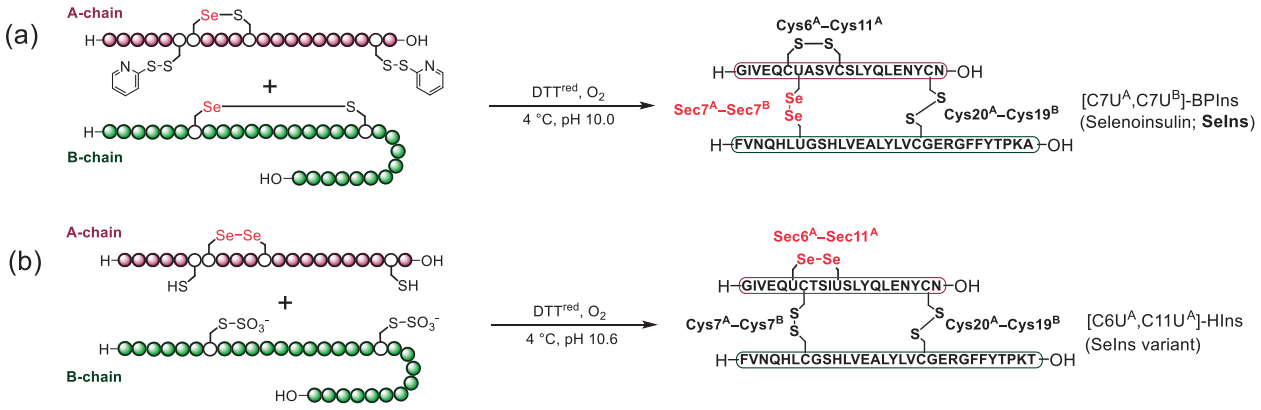


Fig. 14. Preparation of selenoinsulins. Components and primary sequences of selenoinsulin series are shown.
(a) [$C7U^A$,$C7U^B$]-bovine pancreatic insulin (SeIns) with external SeSe bond reported by Arai *et al*.
(b) Human selenoinsulin with internal SeSe bond reported by Metanis *et al*.

The second selenoinsulin [$C6U^A$,$C11U^A$] variant of human insulin (HIns) (**Fig. 14b**) was synthesized by Metanis *et al*. [86]. They focused on $Cys6^A$–$Cys11^A$, which is formed as a major SS component in $1SS^A$ during the early NCA event (**Fig. 13**), and prepared [$C6U^A$,$C11U^A$]-A-chain through SPPS. Interestingly, the A-chain analog, which has an intrachain SeSe bond, coupled effectively with the wild-type B-chain to produce [$C6U^A$,$C11U^A$]-HIns with a correctly folded structure at 31% isolation yield. Importantly, the replacement of internal SS bond, which is buried in the hydrophobic core of the molecule, enhanced thermodynamic stability against guanidinium chloride (GdmCl) and improved resistance to peptide hydrolysis by Glu-V8 protease and reductive unfolding by GSH. These results suggest that replacement of SS bonds with SeSe bonds in proteins enable effective oxidative folding and improve the pharmacological effects of peptide formulations.

6. Future prospective and concluding remark

In vivo folding studies over the past decades have revealed that the oxidative folding pathway of proteins can be flexibly changed to non-native pathways that are not encoded in the primary amino acid sequence by regulating the environment, such as the additives, pH, temperature, and peptide modifications [87]. As described above, the unique redox properties of the SeSe bond and SeH groups can also modify the energy landscape of oxidative folding and accelerate folding velocity by avoiding a pathway

involving a kinetically trapped intermediate, or by altering the unproductive pathway to a productive pathway with lower energetic barriers. The addition of a catalytic amount of small molecule diselenide to the oxidative folding of wild-type peptides greatly improves both the rate and yield despite its ease of use; however, its catalytic activity is still lower than that of PDI. One reason for the enormously high catalytic activity of PDI is that it aggressively incorporates structurally immature proteins, which have exposed hydrophobic regions in the molecules, into its own hydrophobic cavity [88]. To further enhance the catalytic activity of SeSe-based catalysts, it is necessary to improve their redox properties and design novel molecules to selectively recognize undesired species that prohibit oxidative folding, such as misfolded states, kinetically trapped intermediates, and oligomers, and finally lead them to productive pathways.

In addition, although diselenide compounds may be promising in regulating protein quality control in cells, there are only a few reports on their biological applications (see Section 4.4). This is probably because SeSe-based compounds are highly reactive, and therefore, often highly toxic to cells. To develop diselenide-based therapeutics for protein misfolding diseases, it is also necessary to design molecules that can exert appropriate catalytic activity at the target organelles. Furthermore, disulfides/thiols redox reactions are involved in a variety of biological phenomena related to protein quality control as well as oxidative folding in the ER. For example, inositol-requiring enzyme 1 (IRE1), which is an ER transmembrane protein, can detect misfolded proteins, and subsequently assembles with other IRE1 molecules via intermolecular SS bonds, eventually activating the unfolded protein response (UPR) mechanisms [89]. To avoid excessive accumulation and prolonged retention of IRE1-oligomers that cause undesired UPR-associated apoptosis, PDI family A member 6 (PDIA6 or P5) cleaves the intermolecular SS bonds in the oligomers to reproduce monomeric IRE1 [90,91]. Organoselenol compounds are also well known to function as a potent reductant for protein SS bonds due to their high nucleophilic potency [36,46,50], and thus could also be expected to possess the capability to chemically mimic the SS-reductase activity of PDIA6, which attenuates the IRE1 activity.

Meanwhile, the replacement of SS bonds with SeSe bonds in peptides provides various advantages, such as the promotion of oxidative folding, enhancement of structural stability, improvement of intrinsic bioactivity, and introduction of a novel biological function. However, although many SeSe-containing peptides have been reported as potential candidates for practical formulations, their clinical application has not yet been achieved. For drug discovery, further knowledge of the toxicity and pharmacokinetics, as well as the chemical phenomena of the SeSe bond *in vivo*, is necessary.

Acknowledgements

This work was supported by the Promotion and Mutual Aid Corporation (PMAC) for Private Schools of Japan [The Science Research Promotion Fund] and the Research and Study Project of Tokai University, Educational System General Research Organization.

References

[1] Anfinsen CB: Principles that govern the folding of protein chains. Science 181: 223-230, 1973.

[2] Kanemura S, Matsusaki M, Inaba K, Okumura M: PDI family members as guides for client folding and assembly. Int J Mol Sci 21: 9351, 2020.

[3] Matthias JF, Ineke B, Linda MH. Disulfide bonds in protein folding and stability. Matthias JF (ed): Oxidative Folding of Proteins. Royal Society of Chemistry, United Kingdom, 2018, 1-33.

[4] Matsusaki M, Kanemura S, Kinoshita M, Lee YH, Inaba K, Okumura M: The protein disulfide isomerase family: from proteostasis to pathogenesis. Biochim Biophys Acta Gen Subj 1864: 129338, 2020.

[5] Uehara T, Nakamura T, Yao D, Shi ZQ, Gu Z, Ma Y, Masliah E, Nomura Y, Lipton SA: S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. Nature 441: 513-517, 2006.

[6] Gaut JR, Hendershot LM: The modification and assembly of proteins in the endoplasmic reticulum. Curr Opin Cell Bio 5: 589-595, 1993.

[7] Ellgaard L, Helenius A: Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol 4: 181-191, 2003.

[8] Meusse B, Hirsch C, Jarosch E, Sommer T: ERAD: The long road to destruction. Nat Cell Biol 7: 766-772, 2005.

[9] Chaudhuri TK, Paul S: Protein-misfolding diseases and chaperone-based therapeutic approaches. FEBS J 273: 1331-1349, 2006.

[10] Wu J, Kaufman RJ: From Acute ER stress to physiological roles of the unfolded protein response. Cell Death Differ 13: 374-384, 2006.

[11] Kim I, Xu W, Reed JC: Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug

Discov 7: 1013-1030, 2008.

[12] Verfaillie T, Garg AD, Agostinis P: Targeting ER stress induced apoptosis and inflammation in cancer. Cancer Lett 332: 249-264, 2013.

[13] Merrifield RB: Solid phase peptide synthesis. I. the synthesis of a tetrapeptide. J Am Chem Soc. 85: 2149-2154, 1963.

[14] Jaradat DMM: Thirteen decades of peptide synthesis: key developments in solid phase peptide synthesis and amide bond formation utilized in peptide ligation. Amino Acids 50: 39-68, 2018.

[15] Empting M, Avrutina O, Meusinger R, Fabritz S, Reinwarth M, Biesalski M, Voigt S, Buntkowsky G, Kolmar H: "Triazole bridge": disulfide-bond replacement by ruthenium-catalyzed formation of 1,5-disubstituted 1,2,3-triazoles. Angew Chem Int Ed Engl 50: 5207-5211, 2011.

[16] Kourra CMBK, Cramer N: Converting disulfide bridges in native peptides to stable methylene thioacetals. Chem Sci. 7: 7007-7012, 2016.

[17] Wang T, Fan J, Chen XX, Zhao R, Xu Y, Bierer D, Liu L, Li YM, Shi J, Fang GM: Synthesis of peptide disulfide-bond mimics by using fully orthogonally protected diaminodiacids. Org Lett 20: 6074-6078, 2018.

[18] Zhao R, Shi P, Chen J, Sun S, Chen J, Cui J, Wu F, Fang G, Tian C, Shi J, Bierer D, Liu L, Li YM: Chemical synthesis and biological activity of peptides incorporating an ether bridge as a surrogate for a disulfide bond. Chem Sci 11: 7927-7932, 2020.

[19] Wilkinson B, Gilbert HF: Protein disulfide isomerase. Biochim Biophys Acta - Proteins Proteom 1699: 35-44, 2004.

[20] Gruber CW, Čemažar M, Heras B, Martin JL, Craik DJ: Protein disulfide isomerase: the structure of oxidative folding. Trends Biochem Sci 31: 455-464, 2006.

[21] Oka OBV, Bulleid NJ: Forming disulfides in the endoplasmic reticulum. Biochim Biophys Acta - Mol Cell Res 1833: 2425-2429, 2013.

[22] Wang L, Wang X, Wang CC: Protein disulfide–isomerase, a folding catalyst and a redox-regulated chaperone. Free Radic Biol Med 83: 305-313, 2015.

[23] Hagiwara M, Maegawa K, Suzuki M, Ushioda R, Araki K, Matsumoto Y, Hoseki J, Nagata K, Inaba K: Structural basis of an ERAD pathway mediated by the ER-resident protein disulfide reductase ERdj5. Mol Cell 41: 432-444, 2011.

[24] Lees WJ: Small-molecule catalysts of oxidative protein folding. Curr Opin Chem Biol 12: 740-745, 2008.

[25] Madar DJ, Patel AS, Lees WJ: Comparison of the oxidative folding of lysozyme at a high protein concentration using aromatic thiols versus glutathione. J Biotechnol 142: 214-219, 2009.

[26] Potempa M, Hafner M, Frech C: Mechanism of gemini disulfide detergent mediated oxidative refolding of lysozyme in a new artificial chaperone system. Protein J 29: 457-465, 2010.

[27] Lukesh III JC, Andersen KA, Wallin KK, Raines RT: Organocatalysts of oxidative protein folding inspired by protein disulfide isomerase. Org Biomol Chem 12: 8598-8602, 2014.

[28] Okada S, Matsusaki M, Arai K, Hidaka Y, Inaba K, Okumura M, Muraoka T: Coupling effects of thiol and urea-type groups for promotion of oxidative protein folding. Chem Commun 55: 759-762, 2019.

[29] Okada S, Matsusaki M, Okumura M, Muraoka T: Conjugate of thiol and guanidyl units with oligoethylene glycol linkage for manipulation of oxidative protein folding. Molecules 26: 879, 2021.

[30] Tajc SG, Tolbert BS, Basavappa R, Miller BL: Direct determination of thiol pK_a by isothermal titration microcalorimetry. J Am Chem Soc 126: 10508-10509, 2004.

[31] Beld J, Woycechowsky KJ, Hilvert D: Selenoglutathione: efficient oxidative protein folding by a diselenide. Biochemistry 46: 5382-5390, 2007.

[32] Huber RE, Criddle RS: Comparison of the chemical properties of selenocysteine and selenocystine with their sulfur analogs. Arch Biochem Biophys 122: 164-173, 1967.

[33] Connett PH, Wetterhahn KE: Reaction of chromium (VI) with thiols: pH dependence of chromium(VI) thio ester formation. J Am Chem Soc 108: 1842-1847, 1986.

[34] Whitesides GM, Lilburn JE, Szajewski RP: Rates of thiol-disulfide interchange reactions between mono- and dithiols and Ellman's reagent. J Org Chem 42: 332-338, 1977.

[35] Lees WJ, Whitesides GM: Equilibrium constants for thiol-disulfide interchange reactions: a coherent, corrected set. J Org Chem 58: 642-647, 1993.

[36] Arai K, Ueno H, Asano Y, Chakrabarty G, Shimodaira S, Mugesh G, Iwaoka M: Protein folding in the presence of water-soluble cyclic diselenides with novel oxidoreductase and isomerase activities. ChemBioChem 19: 207-211, 2018.

[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 36: 883-885, 1997.

[38] Metanis N, Keinan E, Dawson PE: Synthetic seleno-glutaredoxin 3 analogues are highly reducing oxidoreductases with enhanced catalytic efficiency. J Am Chem Soc 128: 16684-16691, 2006.

[39] Steinmann D, Nauser T, Koppenol WH: Selenium and sulfur in exchange reactions: a comparative study. J Org Chem 75: 6696-6699, 2010.

[40] Metanis N, Foletti C, Beld J, Hilvert D: Selenoglutathione-mediated rescue of kinetically trapped intermediates in oxidative protein

folding. Isr J Chem 51: 953-959, 2011.

[41] Beld J, Woycechowsky KJ, Hilvert D: Catalysis of oxidative protein folding by small-molecule diselenides. Biochemistry 47: 6985-6987, 2008.

[42] Beld J, Woycechowsky KJ, Hilvert D: Diselenides as universal oxidative folding catalysts of diverse proteins. J Biotechnol 150: 481-489, 2010.

[43] Shimodaira S, Asano Y, Arai K, Iwaoka M: Selenoglutathione diselenide: unique redox reactions in the GPx-like catalytic cycle and repairing of disulfide bonds in scrambled protein. Biochemistry 56: 5644-5653, 2017.

[44] Yoshida S, Kumakura F, Komatsu I, Arai K, Onuma Y, Hojo H, Singh BG, Priyadarsini KI, Iwaoka M: Antioxidative glutathione peroxidase activity of selenoglutathione. Angew Chem Int Ed Engl 50: 2125-2128, 2011.

[45] Reddy PS, Metanis N: Small Molecule Diselenide Additives for in Vitro Oxidative Protein Folding. Chem Commun 52: 3336-3339, 2016.

[46] Lukesh JC, VanVeller B, Raines RT: Thiols and selenols as electron-relay catalysts for disulfide-bond reduction. Angew Chem Int Ed 52: 12901-12904, 2013.

[47] Arai K, Kumakura F, Takahira M, Sekiyama N, Kuroda N, Suzuki T, Iwaoka M: Effects of ring size and polar functional groups on the glutathione peroxidase-like antioxidant activity of water-soluble cyclic selenides. J Org Chem 80: 5633-5642, 2015.

[48] Tsukagoshi S, Mikami R, Arai K: Basic amino acid conjugates of 1,2-diselenan-4-amine with protein disulfide isomerase-like functions as a manipulator of protein quality control. Chem Asian J 15: 2646-2652, 2020.

[49] Arai K, Matsunaga T, Ueno H, Akahoshi N, Sato Y, Chakrabarty G, Mugesh G, Iwaoka M: Modeling thioredoxin reductase-like activity with cyclic selenenyl sulfides: participation of an NH•••Se hydrogen bond through stabilization of the mixed Se–S intermediate. Chem Eur J 25: 12751-12760, 2019.

[50] Mikami R, Tsukagoshi S, Arai K: Abnormal enhancement of protein disulfide isomerase-like activity of a cyclic diselenide conjugated with a basic amino acid by inserting a glycine spacer. Biology 10: 1090, 2021.

[51] Mikami R, Tsukagoshi S, Oda Y, Arai K: S-denitrosylase-like activity of cyclic diselenides conjugated with Xaa-His dipeptide: role of proline spacer as a key activity booster. ChemBioChem 23: e202100394, 2022.

[52] Arai K, Sato Y, Nakajima I, Saito M, Sasaki M, Kanamori A, Iwaoka M: Glutathione peroxidase-like functions of 1,2-diselenane-4,5-diol and its amphiphilic derivatives: switchable catalytic cycles depending on peroxide substrates. Bioorg Med Chem 29: 115866, 2021.

[53] Beld J, Woycechowsky KJ, Hilvert D: Small-molecule diselenides catalyze oxidative protein folding in vivo. ACS Chem Biol 5: 177-182, 2010.

[54] Ferreira-Camargo LS, Tran M, Beld J, Burkart MD, Mayfield SP: Selenocystamine improves protein accumulation in chloroplasts of eukaryotic green algae. AMB Expr 5: 39, 2015.

[55] Moroder L, Musiol HJ, Götz M, Renner C: Synthesis of single- and multiple-stranded cystine-rich peptides. Biopolymers 80: 85-97, 2005.

[56] Pegoraro S, Fiori, S, Rudolph-Böhner 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 284: 779-792, 1998.

[57] Pegoraro S, Fiori S, Cramer J, Rudolph-Böhner S, Moroder L: The disulfide-coupled folding pathway of apamin as derived from diselenide-quenched analogs and intermediates. Protein Sci 8: 1605-1613, 1999.

[58] Fiori S, Pegoraro S, Rudolph-Böhner S, Cramer J, Moroder L: Synthesis and conformational analysis of apamin analogues with natural and non-natural cystine/selenocystine connectivities. Biopolymers 53: 550-564, 2000.

[59] Armishaw CJ, Daly NL, Nevin ST, Adams DJ, Craik DJ, Alewood PF: α-selenoconotoxins, a new class of potent A7 neuronal nicotinic receptor antagonists. J Biol Chem 281: 14136-14143, 2006.

[60] Muttenthaler M, Nevin ST, Grishin AA, Ngo ST, Choy PT, Daly NL, Hu SH, Armishaw CJ, Wang CIA, Lewis RJ, Martin JL, Noakes PG, Craik DJ, Adams DJ, Alewood PF: Solving the α-conotoxin folding problem: efficient selenium-directed on-resin generation of more potent and stable nicotinic acetylcholine receptor antagonists. J Am Chem Soc 132: 3514-3522, 2010.

[61] 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 48: 2221-2224, 2009.

[62] Gowd KH, Yarotskyy V, Elmslie KS, Skalicky JJ, Olivera BM, Bulaj G: Site-specific effects of diselenide bridges on the oxidative folding of a cystine knot peptide, ω-selenoconotoxin GVIA. Biochemistry 49: 2741-2752, 2010.

[63] Han TS, Zhang MM, Gowd KH, Walewska A, Yoshikami D, Olivera BM, Bulaj G: Disulfide-depleted selenoconopeptides: simplified oxidative folding of cysteine-rich peptides. ACS Med Chem Lett 1: 140-144, 2010.

[64] 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 50: 6527-6529, 2011.

[65] Steiner AM, Woycechowsky KJ, Olivera BM, Bulaj G: Reagentless oxidative folding of disulfide-rich peptides catalyzed by an intramolecular diselenide. Angew Chem Int Ed 51: 5580-5584, 2012.

[66] Creighton TE: Experimental studies of protein folding and unfolding. Prog Biophys Mol Biol 33: 231-297, 1978.

[67] Creighton TE, Goldenberg DP: Kinetic role of a meta-stable native-like two-disulphide species in the folding transition of bovine pancreatic

trypsin inhibitor. J Mol Biol 179: 497-526, 1984.

[68] Weissman JS, Kim PS: Reexamination of the folding of BPTI: Predominance of native intermediates. Science 253: 1386-1393, 1991.

[69] Metanis N, Hilvert D: Strategic use of non-native diselenide bridges to steer oxidative protein folding. Angew Chem Int Ed Engl 51: 5585-5588, 2012.

[70] Metanis N, Hilvert D: Harnessing selenocysteine reactivity for oxidative protein folding. Chem Sci 6: 322-325, 2014.

[71] Jiang Z, Arnér ESJ, Mu Y, Johansson L, Shi J, Zhao S, Liu S, Wang R, Zhang T, Yan G, Liu J, Shen J, Luo G: Expression of selenocysteine-containing glutathione s-transferase in *Escherichia coli*. Biochem Biophys Res Commun 321: 94-101, 2004.

[72] Aldag C, Gromov IA, García-Rubio I, von Koenig K, Schlichting I, Jaun B, Hilvert D: Probing the Role of the Proximal Heme Ligand in Cytochrome P450cam by recombinant incorporation of selenocysteine. Proc Natl Acad Sci U S A 106: 5481-5486, 2009.

[73] Bröcker MJ, Ho JML, Church GM, Söll D, O'Donoghue P: Recoding the genetic code with selenocysteine. Angew Chem Int Ed Engl 53: 319-323, 2014.

[74] Liu J, Chen Q, Rozovsky S: Utilizing Selenocysteine for expressed protein ligation and bioconjugations. J Am Chem Soc 139: 3430-3437, 2017.

[75] Chatrenet B, Chang JY: The folding of hirudin adopts a mechanism of trial and error. J Biol Chem 267: 3038-3043, 1992.

[76] Chatrenet B, Chang JY: The disulfide folding pathway of hirudin elucidated by stop/go folding experiments. J Biol Chem 268: 20988-20996, 1993.

[77] Chang JY: The properties of scrambled hirudins. J Biol Chem 270: 25661-25666, 1995.

[78] Arolas JL, Aviles FX, Chang JY, Ventura S: Folding of small disulfide-rich proteins: clarifying the puzzle. Trends Biochem Sci 31: 292-301, 2006.

[79] Mousa R, Hidmi T, Pomyalov S, Lansky S, Khouri L, Shalev DE, Shoham G, Metanis N: Diselenide crosslinks for enhanced and simplified oxidative protein folding. Commun Chem 4: 1-9, 2021.

[80] Meienhofer J, Schnabel E, Bremer H, Brinkhoff O, Zabel R, Sroka W, Klostermayer H, Brandenburg D, Okuda T, Zahn H: Synthesis of insulin chains and their combination to insulin-active preparations. Z Naturforsch B 18: 1120-1121, 1963.

[81] Katsoyannis PG, Fukuda K, Tometsko A, Suzuki K, Tilak M: Insulin Peptides. X. The synthesis of the b-chain of insulin and its combination with natural or synthetic a-chain to generate insulin activity. J Am Chem Soc 86: 930-932, 1964.

[82] Kung YT, Du YC, Huang WT, Chen CC, Ke LT: Total Synthesis of crystalline bovine insulin. Sci Sin 14: 1710-1716, 1965.

[83] Karas JA, Wade JD, Hossain MA: The Chemical synthesis of insulin: an enduring challenge. Chem Rev 121: 4531-4560, 2021.

[84] Arai K, Takei T, Shinozaki R, Noguchi M, Fujisawa S, Katayama H, Moroder L, Ando S, Okumura M, Inaba K, Inaba K, Iwaoka M: Characterization and optimization of two-chain folding pathways of insulin via native chain assembly. Commun Chem 1: 1-11, 2018.

[85] Arai K, Takei T, Okumura M, Watanabe S, Amagai Y, Asahina Y, Moroder L, Hojo H, Inaba K, Iwaoka M: Preparation of selenoinsulin as a long-lasting insulin analogue. Angew Chem Int Ed 56: 5522-5526, 2017.

[86] Weil-Ktorza O, Rege N, Lansky S, Shalev DE, Shoham G, Weiss MA, Metanis N: Substitution of an internal disulfide bridge with a diselenide enhances both foldability and stability of human insulin. Chem Eur J 25: 8513-8521, 2019.

[87] Arai K, Iwaoka M: Flexible folding: disulfide-containing peptides and proteins choose the pathway depending on the environments. Molecules 26: 195, 2021.

[88] Okumura M, Noi K, Kanemura S, Kinoshita M, Saio T, Inoue Y, Hikima T, Akiyama S, Ogura T, Inaba K: Dynamic assembly of protein disulfide isomerase in catalysis of oxidative folding. Nat Chem Biol 15: 499-509, 2019.

[89] Chen Y, Brandizzi F: IRE1: ER stress sensor and cell fate executor. Trends Cell Biol 23: 547-555, 2013.

[90] Eletto D, Eletto D, Dersh D, Gidalevitz T, Argon Y: Protein disulfide isomerase A6 controls the decay of IRE1α signaling via disulfide-dependent association. Mol Cell 53: 562-576, 2014.

[91] Okumura M, Kanemura S, Matsusaki M, Kinoshita M, Saio T, Ito D, Hirayama C, Kumeta H, Watabe M, Amagai Y, Lee Y-H, Akiyama S, Inaba K: A unique leucine-valine adhesive motif supports structure and function of protein disulfide isomerase P5 via dimerization. Structure 29: 1357-1370.e6, 2021.