Journal of Peptide Science
J. Peptide Sci. **11**: 187–214 (2005)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.654

JOURNAL OF PEPTIDE SCIENCE

Review

Isosteric replacement of sulfur with other chalcogens in peptides and proteins‡

LUIS MORODER*
Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany

Received 20 December 2004; Accepted 6 January 2005

Abstract: The review addresses the functional and structural properties of the two series of chalcogen analogues of amino acids in peptides and proteins, the methionine and the serine/cysteine series, and discusses the synthesis of the related selenium/tellurium analogues as well as their use in peptide synthesis and protein expression. Advances in synthetic methodologies and recombinant technologies and their combined applications in native and expressed protein ligation allows the isomorphous character of selenium- and tellurium-containing amino acids to be exploited for production of heavy metal mutants of proteins and thus to facilitate the phasing problem in x-ray crystallography. In addition, selenocysteine has been recognized as an ideal tool for the production of selenoenzymes with new catalytic activities. Moreover, the fully isomorphous character of disulfide replacement with diselenide is well suited to increase the robustness of cystine frameworks in cystine-rich peptides and proteins and for the *de novo* design of even non-native cystine frameworks by exploiting the highly negative redox potential of selenols.

Keywords: methionine and cysteine; selenium and tellurium analogues; synthesis; peptide synthesis; protein expression; structural and functional properties; selenocysteine; selenocystine; tellurocysteine; tellurocystine; methoxinine; selenomethionine; telluromethionine; ligation

INTRODUCTION

The chalcogen¶ elements oxygen, sulfur and selenium are essential constituents of functional groups of amino acids which in related biomolecules play a unique role in terms of chemistry and structure. In comparison, no similar structural and biological functions have yet been discovered in the case of the heavier tellurium. The underlying reasons can be sought in its more metallic properties which are expressed by a distinct weakening of covalent bonds with hydrogen and carbon atoms when compared with sulfur and selenium. The physicochemical properties of the chalcogen elements are listed in Table 1. From these it is evident that much of the difference between oxygen and sulfur is a consequence of the larger size of the sulfur atom in its various oxidation states. This accounts for its lower electronegativity and the greater polarizability; both properties result in greater reactivity and explain the extreme toxicity of hydrogen sulfide compared with water. The lower electronegativity of sulfur is also responsible for its concatenating ability which makes disulfides relatively stable, whereas peroxides are unstable. Conversely, the higher electronegativity of oxygen is responsible for the formation of hydrogen bonds, whilst neither reduced nor oxidized sulfur readily forms hydrogen bonds. These differences between oxygen and sulfur are even more pronounced when comparing oxygen with selenium.

Another major aspect that largely determines the biochemistry of sulfur compared with that of oxygen is the availability of *d* orbitals for bonding, which allows sulfur to occur in a number of oxidation states ranging from −2 to +6. Correspondingly, the sulfur containing amino acid residues methionine and cysteine in proteins can participate in a wide range of different redox reactions and thus endow proteins with a powerful and biologically fundamental redox chemistry [1–4]. In selenoproteins the constituent amino acids selenomethionine and selenocysteine were found to occur as selenoxide, selenol, selenylsulfide and selenenic acid, which does not imply that other selenium oxidation states are principally absent *in vivo*; these selenium species are similarly involved in diverse biologically relevant redox reactions [3,5].

Taking into account that the different oxidation states of sulfur and selenium, identified so far in biological systems, represent separate post-translational protein modifications with unique functional properties, chalcogen biochemistry in eukaryotes is clearly very complex. While this aspect has been comprehensively reviewed [2,3], the present article is focused

* Correspondence to: Professor Luis Moroder, Max-Planck-Institut für Biochemie, Am Klopferspitz 18, D-82152 Martinsried, Germany;
e-mail: moroder@biochem.mpg.de

‡ Parts of this review were presented by the author in the *Rudinger Lecture* at the 28th European Peptide Symposium, Prague, September 2004.

¶ Chalcogen: "ore former" from the greek *chalcos "ore"* and *-gen* "formation".

BIOGRAPHY

Prof. Dr Luis Moroder

Luis Moroder was born at St Ulrich in Italy in 1940. He had his early training in peptide chemistry with Professor E. Scoffone at Padua and later spent a year with Professor K. Hofmann in Pittsburgh. Since 1991, with distinguished visiting appointments elsewhere, he has been Head of the Laboratory of Bioorganic Chemistry, Max-Planck-Institute of Biochemistry, Martinsried. He has been awarded the Max Bergman Medal, and in 2004 was Josef Rudinger Lecturer of the European Peptide Society. The author or co-author of over 500 publications, he is not only prolific but also diverse in his interests, which currently include hormone receptors, collagen peptides, proteinase inhibitors and photomodulation of peptide properties as well as the area covered by the present review.

mainly on the structural properties of chalcogen amino acids in synthetic peptides and bioexpressed proteins. The first section deals with methionine and its isomorphous chalcogen analogues including the isosteric carba-analogue 2-aminohexanoic acid (norleucine, Nle) and addresses the perspectives of using these amino acids in structural biology. In the second part mainly the unique redox properties of selenocysteine as an isomorphous analogue of cysteine are discussed as these can be purposely exploited to increase the robustness of natural and non-natural cystine frameworks.

METHIONINE AND RELATED ANALOGUES

Methionine (Met) is one of the nine essential amino acids, which upon reaction with ATP produces *S*-(adenosyl)methionine as the methyl donor in biological methylation reactions. The resulting homocysteine then acts as a precursor in the biosynthesis of cysteine or can be reconverted into methionine by reaction with N^5-methyl tetrahydrofolate [6]. As a proteinogenic amino acid, methionine contributes to protein structures by hydrophobic interactions and to lesser extent by hydrogen bonding. It is relatively rare as it accounts for only about 1.5% of all residues in proteins of known structures, and usually it is inaccessible to solvent with only 15% of all Met residues exposed to the surface [7]. Its thioether group is susceptible to oxidation with formation of the two diastereomeric sulfoxides and the sulfone. As a consequence, methionine residues on protein surfaces can provide a high concentration of antioxidants, which may play a significant role during oxidative stress [3,8]. This redox activity of methionine is further supported by the discovery of the enzyme methionine sulfoxide-reductase which acts as a repair enzyme to prevent the accumulation of methionine sulfoxide residues in proteins [9].

In the synthesis of bioactive peptides the facile oxidation of methionine to the sulfoxides, which generally is accompanied by a significant loss of bioactivity, has always been a matter of great concern. Efficient methods have been developed over the years for the quantitative reduction of methionine sulfoxides [10], but more suitable has proved to be the replacement of Met residues with leucine [11–14] and particularly with the isosteric norleucine [15], since bioactivities are usually retained almost quantitatively by the related peptide analogues. Similar to the positive experiences gained by the replacement of Met with Nle in bioactive peptides, substitution of even single methionine residues in semisynthetic proteins with the carba-analogue norleucine was without detectable effects on protein structure and function, as shown, for example, by ribonuclease S′ [16].

From these early experiences with isosteric replacements of methionine by Nle it was expected that substitution of the sulfur atom in methionine with chalcogen elements such as oxygen, selenium and tellurium (Figure 1) should lead to structurally and functionally isomorphous peptides and proteins, despite their differences in van der Waals radii, electronegativity and bond lengths (Table 1).

Norleucine as Isosteric Methionine Analogue in Peptides and Proteins

The early studies of Cowie *et al.* [17] have shown that norleucine can substitute methionine in bacterial protein synthesis. In fact, the carba-analogue is charged onto $tRNA^{Met}$ to replace Met in proteins [18]. Finally, using a methionine-auxotrophic strain of *Staphylococcus aureus* and a limiting amount of methionine in the growth medium, but larger excesses of norleucine, Anfinsen and Corley [19] succeeded in producing a staphylococcal nuclease containing about 15% of Nle in place of Met residues. Upon CNBr-mediated cleavage of all residual methionine residues a *per*-norleucine-nuclease was obtained, which essentially retained complete enzymatic activity. The ability of norleucine to replace Met residues in proteins without substantial effects on structure and function was confirmed with other proteins as reported for example in references [20,21]. However, norleucine was found to be on the borderline for being edited by *E. coli* methionyl-tRNA synthetase [22] and at the same time to be toxic to cells since it severely interferes with metabolic pathways functionally dependent on methionine. Therefore, only by optimizing the expression procedures and by

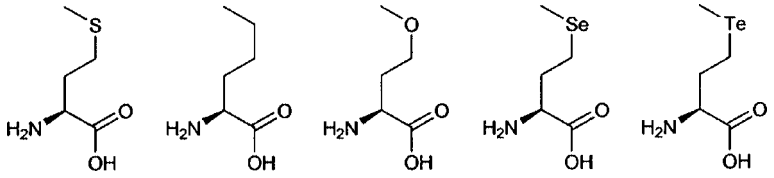


Figure 1 Methionine and the related isosteric analogues L-2-aminohexanoic acid (Nle), L-methoxinine (Mox), L-selenomethionine (Sem) and L-telluromethionine (Tem).

Table 1 Physicochemical Properties of the Chalcogen Elements

Property	X = oxygen	X = sulfur	X = selenium	X = tellurium
Electronegativity	3.44	2.58	2.55	2.1
Covalent radius (Å)	0.73	1.02	1.17	1.35
van der Waals radii (Å)	1.52	1.80	1.90	2.06
Bond length, C-X (Å)	1.43	1.82	1.95–1.99	2.4
Torsion angle χ^3 (°) $-C^{\beta}-C^{\gamma}-(X)^{\delta}-C^{\varepsilon}-$	180	179.7	174.4	180

applying the selective pressure incorporation method [23] did Budisa *et al.* [24] succeed in the quantitative replacement of all Met residues in model proteins. X-ray crystallographic analysis of a *per*-norleucine-annexin V variant fully confirmed the isomorphous character of these substitutions [24]. A comparison of wild-type annexin V with the *per*-norleucine- and the *per*-selenomethionine (Sem) variant (*vide infra*) in terms of thermodynamic stability clearly revealed a similarly enhanced stability of the two protein analogues as a result of the increased hydrophobicity of both Nle and Sem residues compared with Met [25]. The differences in the side chain geometries as a result of the different coordination spheres of CH_2, S and Se as well as of the C–C, C–S and C–Se bond lengths (Table 1) are well accepted by the plasticity of the protein structure. These findings are further supported by sequence comparisons of proteins, which usually assign similar hydrophobicities to methionine, leucine and isoleucine residues [7].

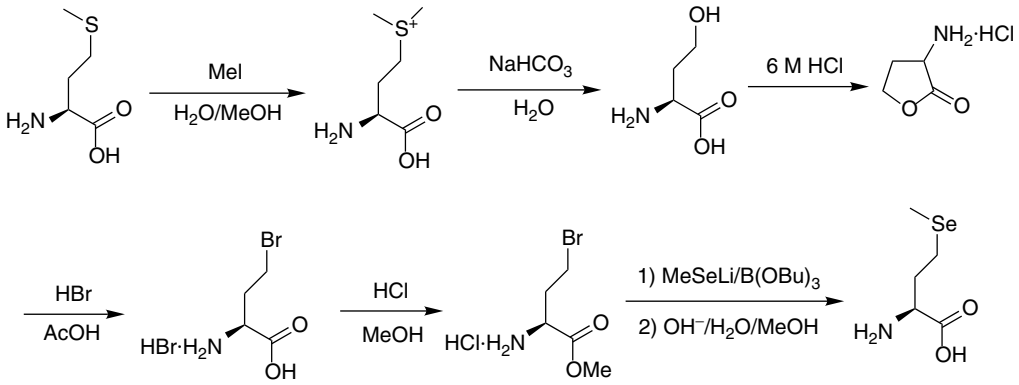
Methoxinine

Several syntheses have been reported for methoxinine (Mox) [26–28], among which the most efficient proved to be the conversion of β-methoxypropionaldehyde into the hydantoin by the Bücherer-Lieb procedure (modified Strecker synthesis) followed by hydrolysis and isolation of the racemic methoxinine as N^{α}-Z derivative [28]. Enantiomeric resolution was achieved by deacylation of Ac-D,L-Mox-OH with hog renal acylase I. With this oxa-analogue a bypass to the problem of methionine sulfoxide formation was obtained, but the effects of Met replacements with Mox on the bioactivities of peptide hormones was found to vary from lowered to equal or even enhanced potencies compared with the wild-type peptide [15,28–30]. These contrasting results may well be assigned to the reduced van der Waals radius of oxygen compared with sulfur, and to its higher electronegativity, and thus stronger tendency for hydrogen bonding.

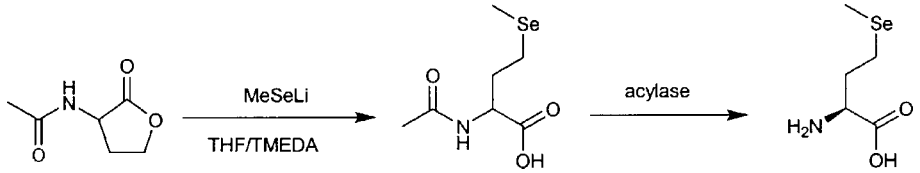
Although isolation of L-methoxinine from the culture fluid of *Corynebacterium* grown in a medium containing methanol was reported [31], to the best of our knowledge occurrence of this methionine analogue in proteins as a result of the tolerance in the editing range of methionyl-tRNA synthetase has not been observed so far. The absence of methoxinine residues in naturally occurring proteins fully agrees with the surprising observation from our laboratory that even by applying a highly efficient method for the specific and quantitative bioincorporation of isosteric Met analogues into proteins, expression of methoxinine-variants failed in the first instance with the model proteins examined [32]. However, by simultaneous overexpression of the methionyl-tRNA synthetase incorporation of methoxinine residues in the place of methionine does occur, but their effect on structure and function of the proteins has yet to be established (N. Budisa, unpublished results).

Selenomethionine

Selenomethionine is a naturally occurring amino acid and its abundance in living organisms strongly depends on the availability of dietary selenium as essential element in nutrition. Amino acid analysis of selenium-enriched yeast or Brazil nuts, as popular selenium supplements for cancer prevention [33,34], clearly revealed selenomethionine as the primary elemental Se-containing species in the methanesulfonic acid-derived protein hydrolysates [35]. Selenomethionine mimics



Scheme 1 Synthesis of L-selenomethionine from L-2-amino-4-bromobutanoic acid according to Koch and Buchardt [38].



Scheme 2 Synthesis of L-selenomethionine from *N*-acetyl-(*R*,*S*)-2-amino-4-butyrolactone according to Karnbrock *et al.* [39].

almost all the roles of methionine and therefore, it is randomly incorporated into proteins due to the editing tolerance of methionyl-tRNA synthetase [36,37]. In fact, it is the only known non-encoded amino acid that allows for uniform labelling of all cellular proteins, i.e. of the proteome, while all other non-encoded amino acids are more or less inhibitors of cellular growth. Correspondingly, in the latter case quantitative bioincorporation into proteins occurs only at the level of single proteins operating under special conditions [24].

For the synthesis of selenomethionine various procedures have been reported [38 and references therein], among which the most efficient methods are based on the reaction of lithium methyl selenolate with L-2-amino-4-bromo-butanoic acid hydrobromide, which is derived from L-Met as shown in Scheme 1 [38], or with *N*-acetyl-(*R*,*S*)-2-amino-4-butyrolactone, as outlined in Scheme 2. The latter procedure was applied conveniently in our laboratory for the synthesis of both seleno- and telluromethionine (*vide infra*) [39]. It is based on the ring opening of *N*-acetyl-(*R*,*S*)-2-amino-4-butyrolactone by the soft nucleophile methyl selenolate via an S_N2 ester cleavage reaction at the soft sp^3-centre. A small amount of byproduct is formed which was identified by mass spectrometry and NMR as *N*-acetyl-homoserine. This side product derives from the attack of the soft nucleophile at the sp^2-centre with formation of the methyl selenoester, which is then hydrolysed to *N*-acetyl-homoserine in the purification steps. Compared with other protecting groups, the *N*-acetyl derivative of the 2-amino-4-butyrolactone allowed for a smooth nucleophilic ring opening and, in addition, for the enantioselective enzymatic deacetylation by aminoacylase-based procedures to generate the desired L-selenomethionine. Attempts to bypass the enzymatic resolution of Ac-D,L-Sem-OH by the use of enantiomerically pure 2-amino-4-butyrolactone failed, since racemization was found to occur to significant extents during ring opening by the methyl selenolate (unpublished results). *Warning*: Selenium compounds are toxic and irritating to tissues. Procedures should be conducted in a hood and gloves should be worn to avoid contact with reagents.

Based on the early observation that selenomethionine is readily incorporated into bacterial proteins [36,37], the isomorphous replacement of sulfur by selenium has been proposed as an efficient new approach to provide proteins with an anomalous scattering nucleus for solving the phase problem in protein x-ray crystallography, as an alternative method to the classical multiple isomorphous replacement procedure [40]. Based on the availability of the synchroton radiation and on sophisticated computing methods, Hendrickson [41,42] has developed and proposed the multiwavelength anomalous diffraction (MAD) method that uses mainly selenium atoms as diffraction labels.

To ensure quantitative replacement of Met residues in proteins with the isomorphous selenomethionine analogue, a selective pressure method was developed by Budisa [23] which is based on (i) a strong methionine-auxotrophism of the host strain during the fermentation procedure, (ii) an efficient control of the cloned gene by the promoter, and (iii) a competitive expression system capable of using cytosolic enzymes to express mainly target DNA after induction of protein synthesis. Under these restricting conditions selenomethionine is accepted by the host translational machinery, activated by the methionyl-tRNA-synthetase, charged onto

$tRNA^{Met}$ and incorporated into the polypeptide chain at all the positions occupied by Met residues in the wild-type protein, thus producing *per*-selenomethionine-proteins [24,43–46].

In the meantime, the selenomethionine tool was shown to be very powerful in structure determination since a large part of all protein structures solved in recent years by MAD relied on this approach [47]. As a consequence of this success, selenomethionine has been defined as the 'magic bullet' in protein x-ray crystallography, although even this procedure bears its drawbacks. These are mainly caused by the readily occurring oxidation of the Sem residues during handling of the proteins. The heterogeneity resulting from spontaneous oxidation of the dialkyl selenide can be prevented or reversed by operating in the absence of air oxygen or by reducing the selenoxide, respectively. In fact, the selenoxide is more readily reduced than the sulfoxide analogue, and therefore, reductants such as glutathione or β-mercaptoethanol are sufficient for quantitative reversal of the selenoxide formation during handling of *per*-selenomethionine-proteins [47–49]. It has also been reported that an enhanced MAD phasing signal may result from quantitative chemical oxidation of the Sem-protein crystals [50–51], which is obtained with H_2O_2. The product of this type of oxidation may be predominantly the result of a four-electron oxidation to the selenone, and not the selenoxides [52], while selenoxides are expected to be the predominant products of a two-electron oxidation of selenomethionine by the milder oxidant peroxynitrite [53].

Possible structural differences observed between Sem- and Met-proteins are due to the slightly larger size of the selenium atom compared with sulfur (Figure 2) and to the differences in bond length between Se–C and S–C (Table 1). However, steric effects should not affect the properties of these protein analogues, since the protein structures generally retain sufficient plasticity and flexibility to accommodate the Sem residues within the geometries of the wild-type folds even in the densely populated cores where hydrophobic methionine residues are most commonly located. Because of the larger size of selenium compared with sulfur, the Sem side chain has a larger surface area and correspondingly it is more hydrophobic than that of Met. These differences in exposed hydrophobic surfaces may affect the protein solubility and thus crystallization. Moreover, since the hydrophobic Met residues are more often found in the core structures of proteins [54], their replacement with the more hydrophobic Sem residues is expected to increase the stability of folded proteins. In fact, a thermodynamic study of the *per*-selenomethionine-variant of annexin V clearly revealed an enhanced stability of the protein structure towards thermal and urea-induced denaturation [25]. Most interestingly, this annexin V variant was found to exhibit stability and unfolding properties very similar to those of the *per*-norleucine-variant [25]. A similar observation was reported for the multiple replacement of Met residues by Sem in the T4 lysozyme [55,56].

As a useful alternative to the *per*-selenomethionine-protein expression for x-ray crystallography, protein/Sem-peptide complexes can be used for such a purpose [57]. The synthesis of peptides containing selenomethionine is performed by standard Fmoc-based procedures [51,57], whereby particular care has to be taken to prevent oxidation to selenoxides, although as mentioned above, reversal of this side reaction is easier than in the case of sulfoxides. With the recent fast progress in ligation methodologies, semisynthetic Sem-containing proteins should be accessible with the great advantage of site-directed incorporation of the heavy selenium atom. In this context, the homoselenocysteine-mediated ligation of peptides followed by methylation of the resulting selenol under reducing conditions,

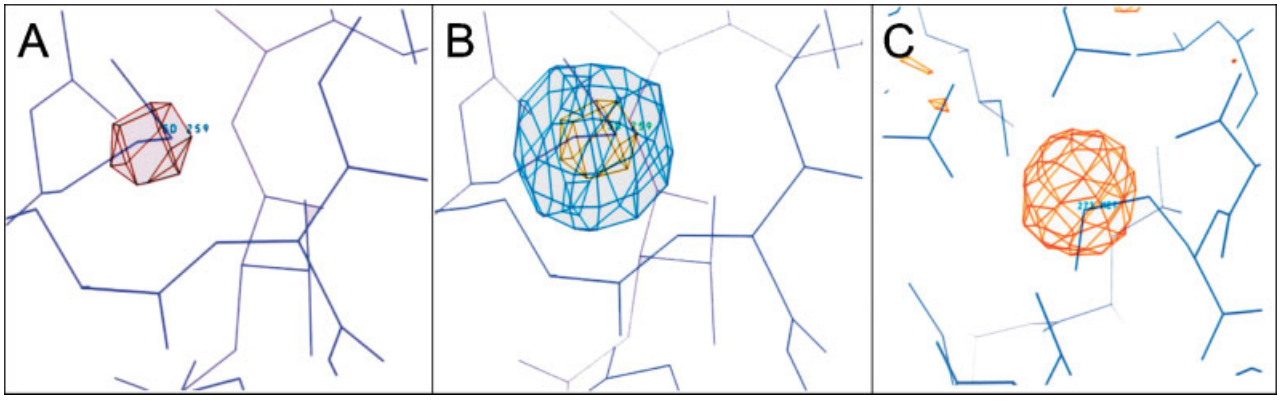
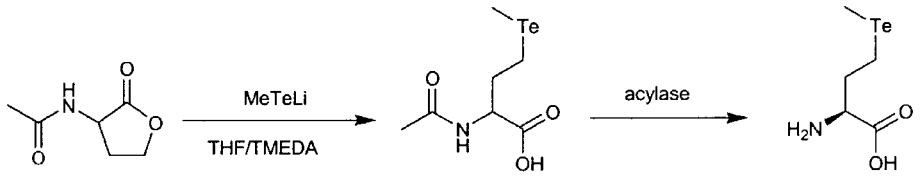


Figure 2 Crystallographic mapping of Nle (A), Sem (B) and Tem (C) residues in the spatial structure of recombinant human annexin V by the difference electron density maps (Fo-Fc) for the Met259 → Nle259 (A) and Met259 → Sem259 (B) replacements (all maps were contoured at 3.0 s), and by the Fo-Fc map (contouring level: 5.0 s) for the Met273 → Tem273 replacement (C) in the hydrophobic core of annexin V. Note that the overall fold and the structure of the whole protein as well as local environments upon substitutions are practically identical to those of the parent protein.



Scheme 3 Synthesis of L-telluromethionine from *N*-acetyl-(*R*,*S*)-2-amino-4-butyrolactone according to Karnbrock *et al.* [39].

i.e. in the presence of tris(2-carboxyethyl)phosphine (TCEP), with excesses of methyl 4-nitrobenzene sulfonate as methylating agent appears to be a highly promising procedure [58]. By this synthetic approach selenomethionine was incorporated site-selectively at position 17 of the bovine pancreatic polypeptide which allowed for detailed insights into unfolding processes of this polypeptide by ^{77}Se NMR spectroscopy. The L-homoselenocysteine required for this synthetic route is readily obtained from selenomethionine by treatment with sodium in liquid ammonia [59].

Besides the properties of selenomethionine as a heavy atom analogue in x-ray structural analysis, natural selenium contains 7.6% of the ^{77}Se isotope, which makes it a spin 1/2 nucleus suitable for ^{77}Se NMR spectroscopy [60]. Moreover, Sem is known to quench tryptophan fluorescence much more efficiently than methionine and thus it could well become a useful tool for studying protein folding as well as protein–protein and protein–peptide interactions [61]. Additional applications of selenomethionine and related peptides could derive from [^{73}Se]-selenomethionine as a short-lived positron emitter for *in vivo* positron-emission tomography (PET). Correspondingly, new synthetic approaches have been elaborated, which allow for fast access to the whole set of isotopomers [62].

Telluromethionine

First successful attempts to incorporate tellurium into proteins were made with Te-resistant fungi grown in the presence of tellurite on a sulfur-free medium, and the results were supportive for a biosynthesis of tellurium-containing amino acids [63]. More recently, telluromethionine (Tem) was found to replace Met residues in dihydrofolate reductase in high percentage (40%) using a methionine-auxotrophic *E. coli* strain and supplying synthetic telluromethionine to the medium [64]. Optimization of the selective pressure incorporation method finally allowed for quantitative Tem bioincorporation into a series of proteins, and the related x-ray crystallographic analysis confirmed the isomorphous character of these protein variants [24,65].

While for x-ray structural analysis of Sem-proteins by the MAD method intensity measurements with monochromatic synchroton-radiation at a minimum of three different wavelengths are required, the electron density of the tellurium atom is sufficient to generate clear signals in the isomorphous and anomalous difference Patterson maps at the commonly used CuK_α wavelength (Figure 2) [24,64,65].

Among the procedures reported for the synthesis of telluromethionine one is based on the reaction of 5-(β-bromoethyl)hydantoin with sodium methyl tellurolate followed by alkaline ring opening to generate the racemic Tem [66]. Since this method proved to be exceedingly difficult to reproduce, an alternative synthesis was proposed by Silks *et al.* [67], which relies on ring opening of (*S*)-2-amino-4-butyrolactone hydrochloride by lithium methyl tellurolate to directly afford L-telluromethionine. In our hands, reaction of lithium methyl tellurolate with *N*-acetyl-(*R*,*S*)-2-amino-4-butyrolactone in tetrahydrofuran and in the presence of tetramethylethylene diamine (TMEDA), as used for the synthesis of Sem, proved to be much more convenient and the desired Ac-D,L-Tem-OH lithium salt was obtained in very good yields (Scheme 3) [39]. The observation that even under identical reaction conditions, Ac-D,L-Tem-OH was obtained in all preparations in significantly higher yields (80%–90%) than the corresponding Sem derivative (60%–70%) can be attributed to the softer nucleophile character of the tellurolate compared with the selenolate. Finally, the desired L-telluromethionine was obtained by enantioselective enzymatic deacetylation of the racemic acetyl derivative. *Warning*: Tellurium compounds are toxic and irritating to tissues. Procedures should be conducted in a hood and gloves should be worn to avoid contact with reagents.

Dialkyl tellurium compounds are known to oxidize rapidly under various conditions [68,69], a fact that was confirmed by storing Ac-D,L-Tem-OH or L-Tem in aqueous solution (pH 7.0) [39,65]. The facile oxidation of telluromethionine was also found to present a serious drawback for the use of this methionine analogue for bioexpression of heavy atom variants of proteins, since oxidized forms were difficult to refold from inclusion bodies or to crystallize [65]. In particular, surface-located and, in a more pronounced manner, solvent-exposed Tem residues were found to oxidize readily and thus to generate electron densities of difficult assignment and local non-isomorphism. Nonetheless, telluromethionine represents a heavy-atom Met analogue well suited for isomorphous replacements in proteins. Although tellurium has a lower electronegativity and larger coordination-sphere radius than sulfur and selenium and the C–Te bonds are larger than Se–C and S–C (Table 1), the

plasticity of proteins allows for accommodation of the tellurium atoms without significantly affecting the native structures. In terms of structure stability in solution, the *per*-telluromethionine-variant of annexin V was less stable than the related wild-type protein and the selenomethionine-variant [25].

CYSTEINE AND RELATED ANALOGUES

The distinct physicochemical properties of the chalcogen elements impart to XH groups (X = O, S, Se and Te) significantly differentiated redox and nucleophilic properties. While for the chalcogen analogues of methionine the genetic code does not exploit functional or structural roles by coding specifically for the methoxinine or selenomethionine, the latter being randomly incorporated if sufficiently supplied to the organisms, the genetic code fully exploits the diversity in functional and structural properties of cysteine, serine and selenocysteine (Sem) (Figure 3) to translate these into the gene products. In fact, serine and cysteine are encoded by specific triplet units, and selenocysteine, which is often referred to as the 21st proteinogenic amino acid [70], is inserted co-translationally into proteins of archae, eubacteria and eukarya when intramolecularly and intermolecularly acting factors induce decoding of UGA for selenocysteine insertion rather than aborting translation [71,72]. Tellurium-tolerant fungi were shown to be able to grow on tellurite and to incorporate telluromethionine as well as tellurocystine/tellurocysteine (Tec) into several types of low and high molecular weight proteins [63]. However, the mechanism of this incorporation is yet unknown.

In contrast to what was observed by replacing methionine with the isosteric carba-analogue norleucine (*vide supra*), alanine can substitute cysteine as the carba-analogue only in terms of a quasi-isosteric space-filling residue, but without exhibiting all the redox and nucleophilic properties of the thiol group. Although Cys → Ala replacements were used in synthetic peptides and protein mutants to mimic and study protein folding intermediates [73–75], more appropriate for such a purpose proved to be the Cys → Ser substitutions which prevent the microenvironmental changes induced by the hydrophobic alanine side chain [76–80].

Cysteine

As mentioned in the introduction, sulfur has the ability to occur in many different oxidation states in biological systems [2,3], and the resulting post-translational modifications that have been identified so far are outlined in Figure 4. From these most diverse reactive sulfur species new concepts about cysteine-based redox signallings have emerged, and defined biochemical roles could be assigned to the different sulfur oxidation steps.

Cysteine-based sulfur reactive species. Some of the most common redox reactions in which sulfur reactive species such as thiol (**1**), thyil radical (**2**) and sulfenic acid (**3**) are involved, are listed in Table 2. Among these reactions the thiol/disulfide exchange occurs not only for breaking, i.e. reducing disulfide bonds, but most prominently to generate them. The enzymes that catalyse these reactions *in vivo* are the thiol-protein oxidoreductases whose active sites are formed by the Cys-Xaa-Yaa-Cys sequence motif, where a pair of cysteine residues is separated by two other residues [81,82]. This motif is employed by thioredoxins and glutaredoxins for reduction of inter- and intramolecular disulfide bonds and other forms of oxidized cysteines, and by protein disulfide isomerases for catalysing the oxidative folding of secretory proteins into their native structures [83]. The Cys-Xaa-Yaa-Cys motif has, therefore, been called a 'rheostat at the active centre', because changes in residues that separate the two cysteines influence the redox potentials, thus configuring the proteins for a particular redox function [84,85].

Responsible for the maintenance of the global intracellular redox balance is glutathione (γ-Glu-Cys-Gly, GSH) which is readily oxidized to GSSG, and the GSH/GSSG ratio adjusts the intracellular redox potential [3,86]. Most healthy cells have a GSH/GSSG ratio in the range of 100 : 1 [87,88], which decreases during oxidative stress affecting cellular proteins, DNA and membranes. GSH participates in electron-transfer, atom-transfer and thiol/disulfide exchange reactions by reacting with a wide spectrum of cellular oxidants and is regenerated in enzyme-mediated thiol/disulfide exchange reactions. Consequently, GSH/GSSG and its associated redox proteins represent the switchboard of cellular redox chemistry [3].

In the GSH-mediated reducing environment of the cells the enzymes PDI in eukaryotic cells [89,90] and DsbA in prokaryotes [91,92] provide the oxidizing equivalents to folding proteins and for this purpose are specifically oxidized by Ero1p and DsbB, respectively [92–94]. Thereby molecular oxygen can serve as the terminal electron acceptor for disulfide formation.

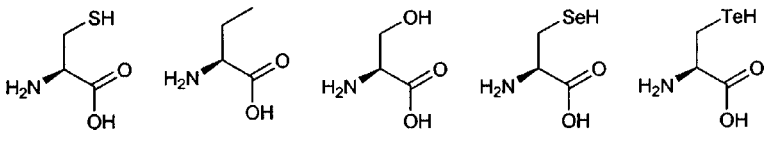


Figure 3 L-Cysteine and the isosteric analogues L-alanine, L-serine, L-selenocysteine (Sec) and L-tellurocysteine (Tec).

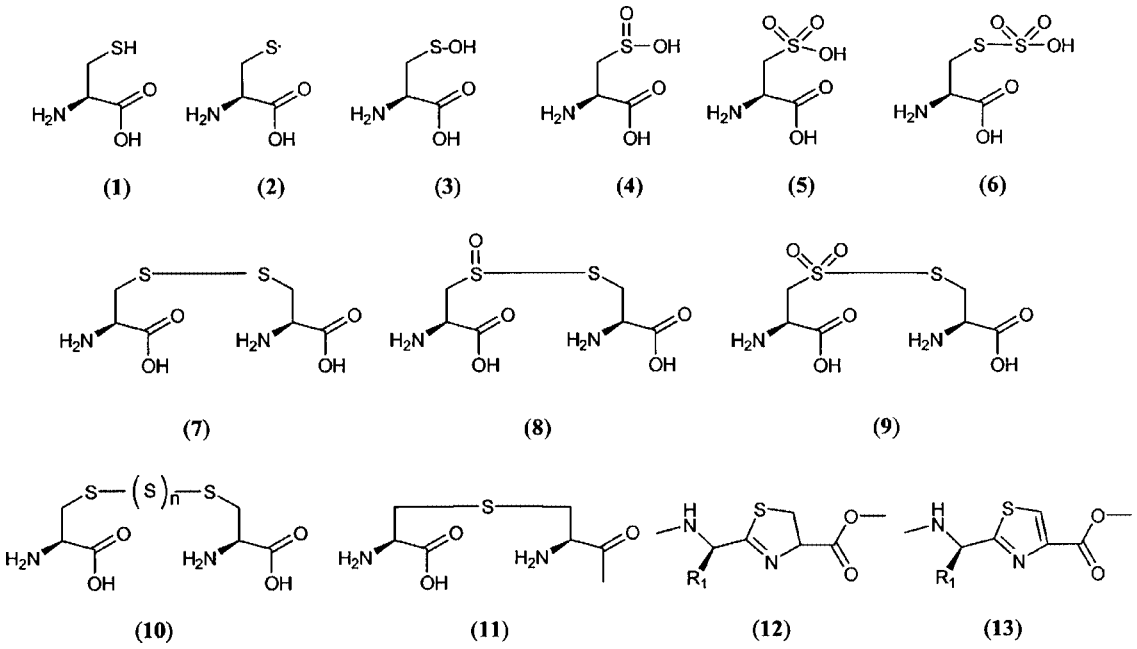


Figure 4 Post-translational modifications of L-cysteine residues that have been identified in proteins; the symmetrical *S*,*S*-dioxide has not been observed so far [3].

Table 2 Some Characteristic Reactions involving Various Cysteine Species in Enzymes

$R^1SH + R^2SSR^2 \leftrightarrow R^1SSR^2 + R^2SH$	thiol/disulfide exchange	e.g. glutathione reductase,
$2\ RSH \leftrightarrow RSSR + 2\ H^+ + 2\ e^-$	or two-electron transfer	thiol/disulfide oxidoreductases
$RSH \leftrightarrow RS^\bullet + H^+ + e^-$	single-electron transfer	ribonucleotide reductase,
$RSH \leftrightarrow RS^\bullet + H^\bullet$	or hydrogen-atom transfer	pyruvate formate lyase
$RSOH + H^- \leftrightarrow RSH + HO^-$	oxygen atom transfer	peroxiredoxins: peroxidases
$R^1SOH + R^2SH \leftrightarrow R^1SSR^2 + H_2O$	or hydride transfer	

The redox activity of these thiol/disulfide oxidoreductases is exerted by their typical Cys-Xaa-Yaa-Cys active centres whose redox potential reflects the equilibrium between the reduced state of the bis(cysteinyl)protein portion and its disulfide-linked oxidized state. This equilibrium is dictated both by the Xaa-Yaa intervening sequence, as well being supported by the redox potentials of synthetic fragments related to their active sites (Table 3) [98] and by extensive mutagenesis studies [97,104–108]. But it is also governed by the conformational restrictions imparted by the overall almost identical tyroxine-like fold of the enzymes [82,104,108]. These structural effects were further evidenced by the redox potentials of conformationally restricted cyclic active-site bis(cysteinyl)hexapeptides related to oxidoreductases [99], when compared with those of the linear unconstrained peptides (Table 3).

To possibly disclose the structural elements responsible for the observed changes in redox potentials upon cyclization of the active-site bis(cysteinyl)peptides, the solution structures of the cyclic peptides in the reduced and oxidized form were determined by NMR [109]. As shown in Figure 5 for the cyclic PDI hexapeptide with its strongly oxidizing redox potential, the ensemble of structures calculated from the NMR-derived distance constraints for the oxidized bicyclic peptide shows low convergence both in the backbone and side chain conformations, while in the reduced state the hexapeptide is characterized by a well-defined structural preference. This would suggest a significantly more relaxed state for the reduced PDI peptide compared with its oxidized form. In fact, by superimposing the structures of the cyclo- and the bicyclo-hexapeptide (Figure 5c) it can be seen that the backbone segment His^{38}-Cys^{39}-Trp^{40} is forced out of the plane in the bicyclic form, whereas in the reduced monocyclic form it lies on the ring plane. This structural change involving Cys^{39} is indicative of a higher conformational tension of the backbone in the oxidized state, which is then released upon disulfide reduction with consequent stabilization of the monocyclic over the bicyclic form. This could well explain the strongly oxidizing character of the PDI-cyclopeptide ($E'_0 = -130$ mV [99]).

Table 3 Redox Potentials (E'_0, [mV]) of Thiol/disulfide Oxidoreductases, their Linear Active-site fragments and Related Cyclic Hexapeptides. The redox potentials were determined using (a) $E'_{0,GSH/GSSG} = -240$ mV [95] and (b) $E'_{0,cysteine/cystine} = -223$ mV [96] as reference

Glutaredoxin (Grx)	−233 [97]
Ac-Gly-Cys-Pro-Tyr-Cys-Val-Arg-Ala-NH_2 (Grx-[10–17])	−215 [98][a)]
c[Gly-Cys-Pro-Tyr-Cys-Val] (Grx-[10–15])	−178 [99][b)]
Thioredoxin (Trx)	−270 [100]
Ac-Trp-Cys-Gly-Pro-Cys-Lys-His-Ile-NH_2 (Trx-His^{37}-[31–38])	−190 [98][a)]
c[Trp-Cys-Gly-Pro-Cys-Lys] (Trx-His^{37}-[31–36])	−152 [99][b)]
Protein disulfide isomerase (PDI)	−110 [101], −145 [102]
Ac-Trp-Cys-Gly-His-Cys-Lys-Ala-Leu-NH_2 (PDI-[35–42])	−205 [98][a)]
c[Trp-Cys-Gly-His-Cys-Lys] (PDI-[35–40]	−130 [99][b)]
Thioredoxin reductase (Trr)	−254/−271 [103]
Ac-Ala-Cys-Ala-Thr-Cys-Asp-Gly-Phe-NH2 (Trr-[134–141])	−210 [98][a)]
c[Ala-Cys-Ala-Thr-Cys-Asp] (Trr-134-139])	−204 [99][b)]

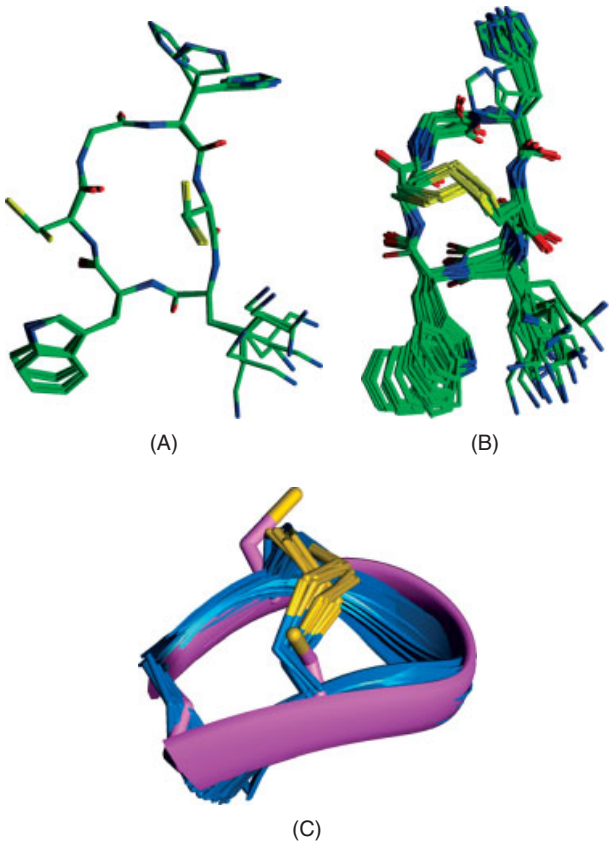


Figure 5 Solution structure of the cyclic PDI peptide in the reduced monocyclic form (**A**) and in the oxidized bicyclic form (**B**) in phosphate buffer (100 mM, pH 7) as determined by NMR [109]. The superposition of the backbones of the reduced and oxidized forms (**C**) clearly reveals that the sequence portion His-Cys-Trp is forced out of plane by the conformational restriction imposed by the disulfide bridge.

The function of the protein disulfide isomerases in oxidative folding of proteins is not only restricted to disulfide formation, but equally important in this context is the ability of these enzymes to reshuffle non-native disulfide bonds into the correct native fold. For this purpose a strongly nucleophilic thiol is required which is present in both PDI and DsbA. In fact, the *N*-terminal Cys residue of the bis(cysteinyl)active sites of both enzymes are extraordinarily nucleophilic with a pK_a of 3.3 for DsbA [104] and 4.5 for PDI [106] vs a pK_a of 7.1–7.4 for Trx [110], an enzyme not involved in protein folding, and a value of 8.4 for cysteine itself. This strong enhancement of the nucleophilicity of this active-site Cys residue has been attributed to its location at the *N*-terminus of an α-helix and to the stabilization of the thiolate by the α-helix dipole [111]. In this context it is interesting to note that the active-site thiol of cysteine proteases is also characterized by an extremely low pK_a value and thus by a strong nucleophilicity, which has been mainly assigned to its topochemical environment, but was additionally attributed to its location at the *N*-terminus of an α-helix (*vide infra*) [112].

While the radical-formation and -transfer mechanisms as well as the hydride-transfer reactions (Table 2) provide enzymes with additional cysteine-based redox pathways [3], the exact redox role of other cysteine derivatives is as yet not fully understood and may be formed as a result of oxidative stress. The sulfinic acid **4** (Figure 4) was found, e.g. in the active site of peroxiredoxins of human erythrocytes [113,114], despite the presence of a second cysteine residue in the active site, disulfide formation with the sulfinic acid is not possible, since unlike sulfenic acids, sulfinic acids cannot be easily reduced by other thiols [115]. Therefore, sulfinic acids would represent a dead end for these important human antioxidant enzymes; however, an enzyme named sulfiredoxin was recently discovered to be capable of reducing the sulfinic acid via intermediate phosphorylation and thus to rescue the peroxiredoxins [4,116].

The *S*-sulfate **6** has been found *in vivo* and it may act as a sulfate transfer intermediate in the detoxification of sulfite [3]. However, *S*-sulfation has not been observed so far as a post-translational modification of proteins, although *O*-sulfation represents a biologically relevant modification of hydroxy groups in peptides and proteins [117,118]. Other species such as the sulfonic acid **5** are effectively the result of oxidative stress since cysteine represents a prime target of many of the reactive oxygen species. As a consequence, even disulfides are oxidized to the highly reactive disulfide-*S*-monoxides (**8**) and disulfide-*S*-dioxides (**9**)

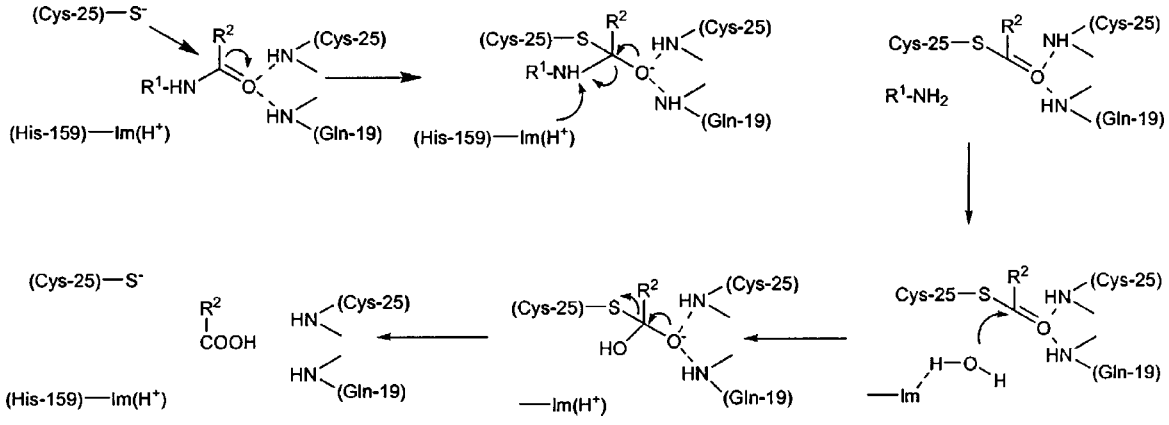
which react with thiols in proteins and enzymes and deactivate the enzymes in the process [3]. Such disulfide-*S*-monoxides could also form during oxidative refolding of cysteine-rich synthetic peptides, and because of their high reactivity could represent a serious source of scrambled oxidation products often encountered when oxidation is performed by air oxygen [119]. Indeed cysteine-*S*-sulfonate (**6**) and sulfinic acid thiol esters (**8**) are known to react with thiols forming unsymmetrical disulfides [296]. Although disulfide-S-monoxides are readily formed with H_2O_2, with air oxygen this reaction occurs only in the presence of heavy metals, particularly Ni^{2+} [120]. Additional transformations of cysteine residues are the recently discovered polysulfides (**10**) [121] as well as the well known enzyme-mediated transformations to lanthionine (**11**) [122,123], dihydrothiazole (**12**) and the related thiazole derivative (**13**) [124,125].

Because of the diversity of cysteine derivatives formed under oxidative conditions, the synthesis of cysteine- and cystine-rich peptides has been a challenge since the pioneering days of peptide chemistry. But the methodology has been significantly improved over the years with the development of orthogonal thiol-protection schemes [126] and efficient strategies for regioselective disulfide formation [127]. Similar advances were made in the synthesis of lanthionine- [123] as well as of dihydrothiazole- and thiazole-peptides [125].

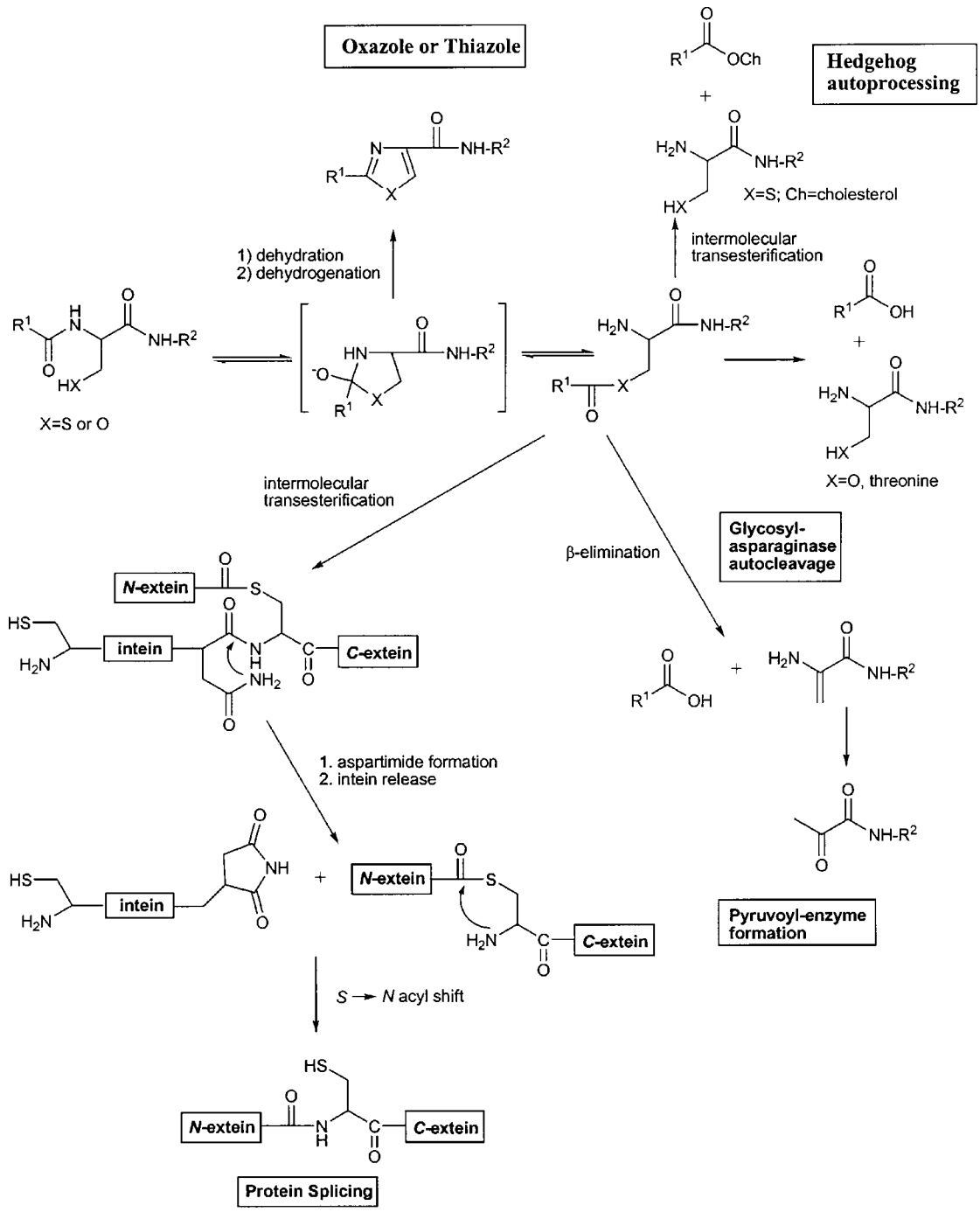
Cysteine as nucleophile in proteins. In addition to the involvement as a crucial structural element in redox active enzymes, the cysteine residue acts as a potent nucleophile in the proteolytically active sites of cysteine proteases. This family of enzymes takes advantage of the nucleophilicity of the thiolate to achieve peptide bond hydrolysis by a mechanism, which has to differ from that of the serine proteases (*vide infra*). The topochemical environment of the active site that stabilizes the imidazolium/thiolate ion pair between the active-site His and Cys residues leads, e.g. in papain, to a reduction of the pK_a value of the thiol group from the normal value of 8.5 for thiols to 4.0. Under neutral and even weakly acidic conditions the thiolate acts as nucleophile for an attack at the carbonyl of the scissile peptide bond. As outlined in Scheme 4, this first reaction step leads to the tetrahedral intermediate, which is stabilized in the oxyanion hole by hydrogen bonding to the backbone NH of Cys-25 and to the side-chain carboxamide of Gln-19. Thereby the imidazolium ion becomes sufficiently acidic to protonate the nitrogen of the leaving group and the acyl enzyme is formed. Formation of this intermediate is the rate limiting step [112,128]. The ion pair formation with the active-site His residue makes the thiol also a good leaving group and therefore, deacylation of the enzyme by water can take place even without acid catalysis as required in serine proteases since the C–S bond is weaker than the C–O bond.

The mechanistic difference between the cysteine and serine protease (*vide infra*) classes of enzymes is also supported by the observation that potent serine protease inhibitors, which take advantage of the enzyme's ability to stabilize a transition state, are not necessarily potent cysteine protease inhibitors. Moreover, early studies on chemoselective conversion of the bacterial serine protease, subtilisin, into thiol-subtilisin clearly revealed a dramatic loss of protease activity which confirmed that the serine-type active-site geometry of the newly generated thiol-subtilisin was responsible for this unexpected poor activity [129,130]. However, an increased aminolysis to hydrolysis ratio of tetrapeptide esters was observed for the thiol-subtilisin, converting this engineered cysteine protease to a promising enzyme for catalysing peptide bond formation [131]. An additional Pro225Ala mutation in the active site improved the catalytic activity [132], and the resulting thiol-subtilisin variant, termed subtiligase, proved to be well suited for enzymatic ligation of suitable peptide fragments into proteins [133,134].

Most of the post-translational modifications of proteins are catalysed by enzymes. Within the past years,



Scheme 4 Mechanism of proteolysis of peptide substrates by papain as an example for cysteine proteases.



Scheme 5 The role of acyl migration in initiating autocatalytic processing of proteins.

however, self-catalysed protein modifications were discovered, such as protein splicing [135,136], autoprocessing of amino-terminal nucleophilic hydrolases which include glycosylasparaginases, glutamine-5-ribosyl-1-pyrophosphate amidotransferases, penicillin acylase and the proteasome [137], as well as autoprocessing of pyruvoyl enzyme precursors [138] and *hedgehog* proteins [139,140]. All these processes are initiated by a self-catalysed $N \rightarrow S$ or $N \rightarrow O$ acyl rearrangement involving cysteine, serine and threonine residues, respectively, as the first step of activation of autoproteolysis in diverse biological systems. Because of the lower pK_a value of the thiol compared with the hydroxy group, in general cysteine can replace serine and threonine in these systems, albeit poorly, while the reverse situation is not observed. As a result, serine and threonine need assistance from surrounding residues in specific architectures for deprotonation of the side-chain hydroxy groups, whereas cysteine will be deprotonated at physiological pH. As shown in Scheme 5, the side-chain functions of cysteine, serine and threonine can attack the carbonyl carbon of the preceding

residue to generate the five-membered hydroxythiazolidine or hydroxyoxazolidine intermediates which upon dehydration and dehydrogenation yield the stable oxazole and thiazole ring, as present in certain bacterial antibiotics [141]. When the intermediates are protonated at the nitrogen, ester intermediates are formed whose fate depends upon the autocatalytic processes.

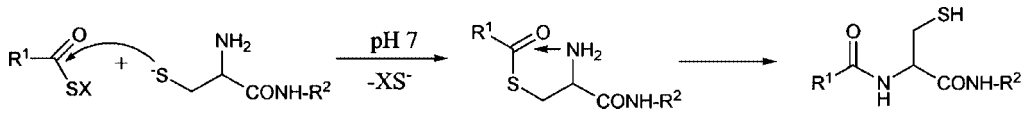
Among these processes, protein splicing involves the excision of an intervening protein domain (the intein) from a folded precursor protein with concomitant fusion of the two flanking portions, termed the *N*- and *C*-exteins, via a new peptide bond into the mature protein. The related mechanism has been extensively reviewed [142–144]. After the initial ester or thiol ester bond formation at the *N*-terminal splice site (step 1, acyl rearrangement), the ester carbonyl is attacked by the hydroxy/thiol group of the Ser/Thr/Cys residue at position 1 of the *C*-extein resulting in *N*-terminal cleavage of the precursor protein and formation of a branched intermediate (step 2, transesterification). The branched intermediate is resolved by cleavage of the peptide bond at the *C*-terminal splice site due to cyclization of the intein *C*-terminal asparagine residue to aspartimide (step 3, Asn cyclization). Finally, a spontaneous $S \rightarrow N$ or $O \rightarrow N$ acyl shift establishes a peptide bond between the exteins (step 4, acyl rearrangement).

While the initial $N \rightarrow S, O$ shifts require the assistance of a proton donor and acceptor such as the His residue in the intein [145], the aspartimide formation is assisted by the penultimate His residue in the intein segment [146]. A similar imidazole catalysis by a neighbouring His residue for ring closure of glutamine to pyroglutamine residue has been observed in synthetic peptides, too [147]. In general, cyclization of asparagine or aspartic acid β-esters, which is an acid-catalysed reaction known to take place particularly at Asn/Asp-Gly/Ser sequences as an undesired side reaction during peptide synthesis and storage, occurs by attack of the peptide bond nitrogen on the side chain carbonyl carbon to form the aspartimide-peptide. This intermediate undergoes rapid hydrolysis at either the α- or β-carbonyl group to regenerate the α-peptide bond or to produce the new β-aspartyl-peptide [147,148]. Less common is the attack of the side chain amide nitrogen on the peptide bond carbonyl with formation of a *C*-terminal succinimide and subsequent peptide bond cleavage [148,149]; however, such acid-catalysed peptide bond cleavage occurs readily at Asn-Pro sequences [150].

The reactions involved in these protein maturation processes are all well known in peptide chemistry. Regarding the spontaneous $X \rightarrow N$ acyl migration, Wieland showed in the early 1950s that *S*-aminoacyl-cysteine derivatives undergo an intramolecular transacylation to form N^{α}-aminoacyl-cysteine compounds as shown in Scheme 6 [151]. The concept of entropic activation, and its significance in the context of peptide bond formation via intramolecular acyl transfer, was introduced by Brenner [152,153] and then further developed by Kemp to the *Prior Thiol Capture Strategy* [154]. This principle was subsequently applied almost simultaneously by Kent [155] and Tam [156] for the development of a highly chemoselective ligation of unprotected peptide fragments via peptide amide bonds to produce larger polypeptide chains and proteins. This strategy, termed native chemical ligation (NCL), exploits the reversible intermolecular transesterification between a peptide thiol ester and the nucleophilic thiol group of an *N*-cysteinyl-peptide to produce the *S*-(peptidyl)-cysteinyl-peptide as intermediate. In the absence of structurally favoured proximity effects as well as the assistance of functionalities in the correct architecture, as present in the inteins for protein splicing, hydroxy groups and amines present in the unprotected peptide segments are too weak as nucleophiles to compete under neutral or slightly acidic media with the transesterification by the thiol groups. The thiol ester itself is generally relatively unreactive to aminolysis [157]; but the *S*-(peptidyl)-cysteinyl-peptide formed as an intermediate in the first step rapidly undergoes the $S \rightarrow N$ acyl shift, through the favourable five-membered ring intermediate, to produce the native peptide bond at the ligation site (Scheme 6). This entropically favoured aminolysis occurs at high rates and thus prevents competitive hydrolysis of the thiol ester at the slightly alkaline pH used in the reaction, despite the known higher rates of hydrolysis of thiol esters compared with carboxy esters [158].

Similar entropic proximity effects for aminolysis of thiol esters are exploited by nature in the modular peptide synthetases involved in nonribosomal peptide synthesis where amino acid phosphopantheteine thiol esters undergo aminolysis by the adjacent module-bound amino acid without enthalpic activation of the α-carboxy group [159,160].

The NCL strategy was subsequently extended to multiple repetitive peptide ligations taking advantage of *N*-terminal thioproline derivatives as intermediately



Scheme 6 Intermolecular trans-thiol-esterification and intramolecular trans-acylation as basic reactions of native chemical ligation of unprotected peptide segments [155,156].

masked cysteine residues [161]. The successful synthesis of various proteins by this procedure confirmed its considerable potential as a complementary approach to protein engineering based on ribosome-mediated protein synthesis [162–166]. It also fostered new developments to bypass the limitation of an *N*-terminal cysteine in one of the fragments, e.g. by the use of *N*-ethanemercapto- or *N*-oxyethanemercapto-derivatized glycine or alanine residues to provide the nucleophilic sulfur [167] or by the use of templates that allow for traceless ligations [168,169] in a manner similar to Kemp's thiol capture strategy [154].

The almost simultaneous discovery of protein splicing processes where trans-thiol-esterification reactions and $N \rightarrow S/S \rightarrow N$ acyl shifts are exploited for excision of the intein domains (*vide supra*) led to a manipulation of these biosystems for autoproteolysis at single splice junctions to produce protein thiol esters. These are as suitable reaction partners for ligation of recombinantly expressed larger protein portions with deliberately modified or labelled synthetic fragments to produce modified proteins [170,171]. This strategy, known as expressed protein ligation (EPL), enriches the arsenal of techniques available for the production of suitable protein variants and derivatives of particular interest in cell biology [166,172–174].

Disulfide bonds. Secretory proteins typically depend on disulfide bonds for their maturation and function. Formation of these bonds is thermodynamically coupled to folding and must be regarded as a key post-translational modification of secretory proteins, which are stabilized in their folded structures via the covalent crosslinks by 2 to 5 kcal/mol for each disulfide bond [175]. Although Anfinsen's classic *in vitro* experiments on the oxidative refolding of ribonuclease A [176,177] clearly established the central principle of self-assembly of proteins, *in vivo* the folding process is assisted by various chaperones and folding catalysts. These do not determine the final structure of the polypeptide chain, but rather accelerate slow chemical steps that accompany folding by inhibiting off-pathways such as aggregation phenomena [178,179] or by catalysing reshuffling of disulfides [180] and *cis-trans* isomerization of Xaa-Pro bonds [181] as rate-limiting steps.

In eukaryotes, oxidative folding of secreted proteins occurs in the endoplasmic reticulum, which provides an environment that is highly optimized for this purpose. It is more oxidizing than that of the cytosol with a GSH/GSSG ratio of approximately 1 : 1–3 : 1 [182]. But rather than relying on glutathione as an oxidant, disulfide formation is driven by a protein relay involving Ero1 and PDI; Ero1 is oxidized by molecular oxygen and in turn acts as a specific oxidant of PDI, which then directly oxidizes cysteines in folding proteins in addition to reshuffling incorrect disulfides [183]. *In vitro* and in the absence of such enzymatic assistance, oxidative protein-folding in the presence of glutathione [184] is a trial and error process with a stepwise conformational search in a funnel-like energy landscape (Figure 6) or along a redox-sensitive series of energy landscapes [185–188]. Indeed the redox potential of single disulfides can vary significantly in the folded cystine-rich proteins and peptides; e.g. for apamin, a relatively small peptide with two disulfide bonds, the one-disulfide folding intermediate exhibits a K_{ox} of 0.025 M, whereas the K_{ox} for formation of the second disulfide is approximately 17 M, thus making such folding processes highly cooperative [189,190].

Local structural preferences have been recognized as the nucleating sites of protein folding that form early in the process and direct subsequent folding events [191].

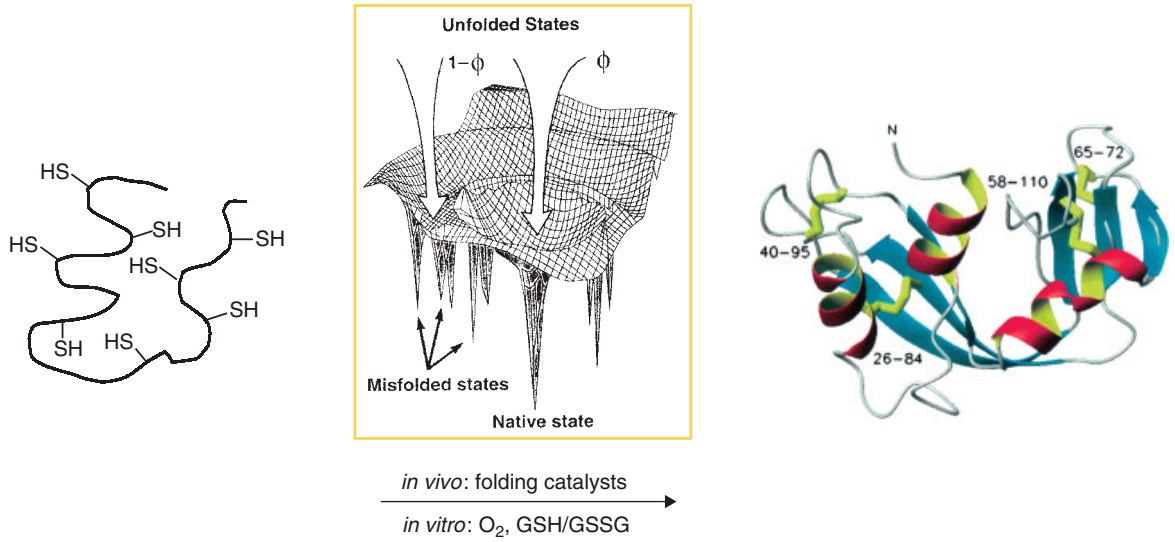


Figure 6 The general mechanism of oxidative protein-folding foresees a hierarchical conformational search in a funnel-like energy landscape as shown for example by ribonuclease A.

These may increase the effective concentrations for intermediate disulfide bonds [192,193], which are not necessarily retained in the final folded structure. Such effective concentrations which reflect the equilibrium constants for disulfide formation in the redox buffer applied, depend on the configurational entropy factor, i.e. on the probability of the cysteine residues colliding with each other. Studies on model peptides have clearly shown that in the absence of conformational constraints the readiness to form disulfide loops depends more on the number of residues m between the two cysteine residues than on the type of residues at least up to $m = 6$, and that disulfide loop formation is favoured by even m values and unfavoured by odd values [194,195]. This alternating odd-even pattern also appears in statistics of disulfides in naturally occurring proteins [195,196]. On the basis of this observation, disulfides between proximal cysteines should exhibit the highest rate of formation at least in folding intermediates. Via a decrease in entropy local constraints imparted by the disulfide could contribute to the formation of compact structures where both non-native and native contacts become important in directing the folding process since the compact intermediate could then rearrange to the more stable native state via disulfide reshuffling [197]. In fact, non-native interactions in protein folding have been suggested to play a potentially significant role even for small proteins with simple topologies [198].

In this context, disulfide bridging between two adjacent cysteine residues could increase the rigidity of local structures by inducing bends that help to bring various parts of the chain into closer proximity. Adjacent cysteine residues are often encountered in the sequence of cysteine-rich peptides and proteins. However, the eight-membered ring formed by the intramolecular Cys-Cys disulfide is very rarely found in native folds [199,200]. Theoretical calculations and first structural data on model peptides predicted that disulfide formation between adjacent residues requires a *cis*-like conformation of the Cys-Cys bond [201]. However, in the most recent examples even a *trans*-conformation was determined [199,200]. Such a vicinal disulfide was also detected as a folding intermediate of *Amaranthus* α-amylase inhibitor, a cysteine-rich peptide that in the native fold is characterized by a typical cystine knot motif [202]. This latter observation fully confirms the potential role of small intrachain disulfide loops, i.e. of bead-like structures, in the first steps of oxidative folding processes.

Despite the hierarchical protein folding pathways, formation of unproductive intermediates by incorrect disulfides or disulfides in the wrong temporal order can lead to off-pathway kinetic traps and cause the often encountered low yields of correctly folded proteins. Therefore, oxidative refolding of proteins still represents a considerable challenge particularly in terms of yields. With the advent of recombinant protein expression, the increased need for optimized folding procedures has fostered intense research in the field and continuous advances are reported including the use of artificial chaperone systems [203,204].

While cysteine-rich proteins contain sufficient sequence-encoded information for correct folding under optimized conditions, in oxidative refolding of cysteine-rich peptides often difficulties were encountered since these are products of post-translational processing of folded precursor molecules. Indeed, refolding of precursors generally leads to substantially increased folding yields, as well assessed by various propeptide forms [205,206].

Progress in the chemistry of regioselective disulfide formation in synthetic cysteine-rich peptides allows for the formation of up to three intra- or intermolecular disulfide bonds independently of the higher or lower propensity of the reduced peptide for correct oxidative refolding [127,207]. However, a continuously growing number of peptides containing even more disulfide bonds are being identified and isolated from the most diverse kingdoms of life such as fungi, plants, invertebrates and mammals, which function as hormones, neurotransmitters, growth factors, enzyme inhibitors, antimicrobial peptides including defensins and toxins [208–211]. Despite the impressive sequence diversity, very often the cysteine pattern in the sequence allows to one foresee the cystine frameworks. In fact, comparative analyses of the cysteine patterns and known 3D structures clearly revealed that identical cysteine patterns very often lead to identical cystine frameworks and thus to characteristic structural or substructural motifs such as the cystine-stabilized $\alpha\beta$ motif [212,213], the cystine knot motif [212,214,215] and the β-hairpin-like motif [199,216,217]. Although under optimized conditions oxidative folding of such cysteine-rich synthetic peptides was often successful [119], there is a continuous need for an improved methodology. Refolding chromatography represents one example of the most recent advances in the field where individual folding components are immobilized on solid supports [218,219]. Other procedures include immobilization of small molecule-dithiols [220], such as the Ellman's reagent [221], or the search for synthetic molecules capable of mimicking the disulfide reshuffling efficiency of PDI [99,222].

In addition to acting as an oxidant, PDI plays an important role in reshuffling incorrect disulfide bonds formed during oxidative folding of proteins. This property has been attributed mainly to the extraordinarily nucleophilic *N*-terminal Cys-36 residue of the active site sequence ($pK_a = 4.5$ [106]). The cyclic active site-peptide *c*[Trp-Cys-Gly-His-Cys-Lys] (PDI- [35–40]) exhibits a redox potential of −130 mV (Table 3) and very surprisingly differentiated pK_a values for the two Cys residues with the Cys-39 (the second

Cys in the sequence above) as the more acidic thiol ($pK_a = 7.3$) than generally observed for this amino acid ($pK_a \geq 8.4$) [99]. In fact, the cyclic bis(cysteinyl)peptide was found significantly to catalyse oxidative refolding of ribonuclease A both in the presence and absence of GSH/GSSG [99], although not with the efficiency of PDI [223]. This finding has to be attributed mainly to the obvious lack of chaperone activity by the small peptide which, however, is exerted by the large enzyme [224].

Serine

In terms of geometry and volume occupancy serine is a highly isosteric analogue of cysteine and is thus well suited for the design of protein mutants and peptide analogues for structure–function studies. But the two amino acids differ in basic chemical and physicochemical properties. The hydroxy group of serine lacks the redox properties of thiols at least in the range of redox potentials operating in biological systems, and serine residues do not form peroxide bridges because of the instability and highly oxidizing properties of peroxides. Therefore, the structural role of serine relies mainly on its hydrogen-bonding ability and the functional role on the nucleophilicity of the hydroxy group.

Serine as nucleophile in proteins. The hydroxy group is a weaker nucleophile than the cysteine thiol group; nonetheless, it constitutes the essential active-site nucleophile of serine proteases and it acts as a nucleophile in the autocatalytic protein splicing processes via intramolecular $N \rightarrow O$ and $O \rightarrow N$ transacylations.

Despite the weaker nucleophilicity of serine, the general mechanism of peptide bond hydrolysis by serine proteases is very similar to that shown in Scheme 4 for cysteine proteases. However, the topochemical environment of the active centres of serine proteases has evolved to stabilize optimally the highly charged tetrahedral transition state through oxyanion hole interactions. Correspondingly, both formation and breakdown of the tetrahedral intermediate are the rate limiting steps of peptide bond hydrolysis. This behaviour is in contrast to that of the cysteine proteases where the thio ester formation is the rate-limiting step (*vide supra*). Because of these fine mechanistic differences cysteine and serine residues are not functionally interchangeable in serine and cysteine proteases.

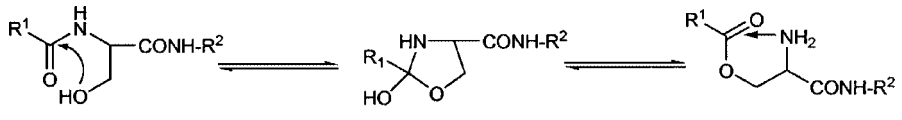
N → O/O → N *transacylations.* The mechanism of autocatalytic protein splicing at the *N*-terminal cysteine residues of the intein and the *C*-terminal extein (Scheme 5) has been well established by trapping the crucial intermediates [142–144]. Since protein splicing takes place even when the cysteine residues are replaced by the less nucleophilic serines, the structural environments have to provide the required acid/base catalysis particularly in the transesterification step involving the serine/threonine hydroxy groups and in the hydrolysis of the *O*-acyl intermediates in a manner similar to the mechanism of peptide hydrolysis by serine proteases.

From experiences in peptide synthesis it is known that even the $N \rightarrow O$ acyl shift is not a spontaneous reaction, but occurs in the absence of particular intramolecular assistance via the favourable 5-membered intermediate only under acidic conditions as required in the final deprotection steps (Scheme 7) [225–227]. Conversely, the opposite $O \rightarrow N$ transacylation takes place spontaneously and at high rates again via the hydroxyoxazolidine intermediate whenever the amino group is deprotonated [225–231] or protonated by weak acids [232]. However, this transacylation can also occur intra- and intermolecularly by the nucleophilic attack of an amino group when favoured by spatial proximity as induced in dimeric peptides [232]. Similar $O \rightarrow N$ transacylations via intermediate ring formation even of larger sizes have been successfully employed in the *Prior-Amine-Capture-Strategy* of Kemp [164,233] as well as in peptide-backbone protection strategies [234,235] entropically to facilitate coupling steps.

More recently the $O \rightarrow N$ acyl shift was applied for the design of prodrugs [236,237] and for the synthesis of 'difficult' peptide sequences by purposely incorporating depsipeptide structures to prevent aggregation phenomena directly correlated with the propensity for β-sheet formation [238–241]. Upon deprotection of the depsipeptides, their purification under acidic conditions and subsequent neutralization, these *O*-acyl compounds are readily converted into *all*-peptide amide compounds. This pH-dependent conversion of *O*-acyl peptides into *N*-acyl peptides was then ingeniously employed in the design of conformational switches that allow for studying the folding mechanisms in model peptides [242].

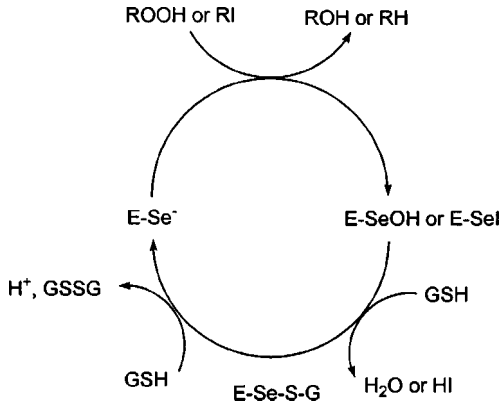
Selenocysteine

In vivo, selenocysteine is co-translationally inserted into the growing polypeptide chain via the normal biosynthetic machinery of the cell [70–72]. It was



Scheme 7 pH dependent $N \rightarrow O$ and $O \rightarrow N$ transacylations at serine residues in peptides and proteins.

recently established that the human selenoproteome consists of 25 selenoproteins [243], of which 18 have been described so far. The most studied are glutathione peroxidase, glycine reductase, thioredoxin reductase and iodothyronine 5′-deiodinase [70,244]. Most human selenoproteins have cysteine-homologues, in which selenocysteine is replaced by cysteine, but the sulfur proteins are generally weaker catalysts when compared with the selenoproteins [245]. The major difference between selenocysteine and cysteine residues is the significantly higher acidity of the selenol group ($pK_a = 5.2$) relative to that of the thiol group ($pK_a = 8.3$) [246,247]. As a consequence, selenols in proteins are normally present as selenolates at physiological pH and these are better nucleophiles than the thiolates. Selenocysteine in proteins occurs mainly in the reactive selenol and selenenic acid oxidation state. In fact, as exemplified by the redox cycle of glutathione peroxidase, selenocysteine is oxidized to selenenic acid, which in turn is reduced via an exchange reaction involving two equivalents of glutathione (Scheme 8) [3]. Unlike disulfides, reactive diselenides have not been found in proteins, but the mixed selenylsulfides are formed in glutathione peroxidase and thioredoxin reductase, which are then reduced in a first step by an exchange reaction with thiols to form the disulfide and in a second step by electron transfer as shown in Scheme 8 [3]. A similar reaction mechanism has been proposed for the deiodinases involved in the tyroxine metabolism [248].



Scheme 8 Reaction mechanisms of glutathione peroxidase and iodothyronine 5′-deiodinase.

Redox potential of selenocysteine. From cyclic voltammograms of cystine and selenocystine (pH 7.0; 25 °C) a difference in redox potential of about 250 mV between selenocysteine/selenocystine (−488 mV vs NHE) and cysteine/cystine (−233 vs NHE) was derived [3]. Since redox potentials of thiols and selenols are generally extracted from the equilibrium constants of exchange reactions with reference redox systems such as glutathione or DTT using the Nernst equation, the accuracy of these measurements strongly depends upon the values of the reference redox potentials used. Szajewski and Whitesides [249] have very precisely determined the redox potentials of DTT (−323 mV), β-mercaptoethanol (−207 mV) and glutathione (−205 mV) at pH 7.0 and 30 °C by equilibration against the lipoamide-lipoamide dehydrogenase couple and using the E'_0 value of −288 mV for lipoamide vs the standard hydrogen electrode as a reference. With these reference redox potentials an E'_0 value of −349 mV was derived for selenocystamine ($K_{ox} = 7.14$ M^{-1} [250]) (Table 4).

Since the redox potential of the glutaredoxin fragment grx-[10–17] was found to be similar to that of glutathione (Table 3), this bis(cysteinyl)peptide was chosen to determine the redox potential of a diselenide and mixed selenidesulfide bridge in unconstrained linear peptides [32,251,252]. As shown in Table 4, the redox potential of the bis(selenocysteine)peptide (−381 mV) proved to be remarkably lower than that of DTT (−323 mV), whereas the E'_0 value of the mixed (selenocysteine-cysteine)peptide (−326 mV) was found to be similar to DTT and thus significantly more reducing than glutathione and the parent bis(cysteinyl)peptide. The redox potential of diselenides clearly indicates that formation of a diselenide bridge is highly favoured over the formation of mixed selenidesulfide and even more of disulfide bridges in peptides and proteins.

Table 4 The Redox Potentials of Sec-containing Peptides in Comparison with the Cys-peptides and Selenocystamine at pH 7.0 as determined by the Nernst Equation ($E'_0 = E'_{0,DTT} - 0.03 \log K_{ox}$) from the K_{ox} Values of Equilibration with DTT as Reference Redox System ($E'_0 = -323$ mV [249])

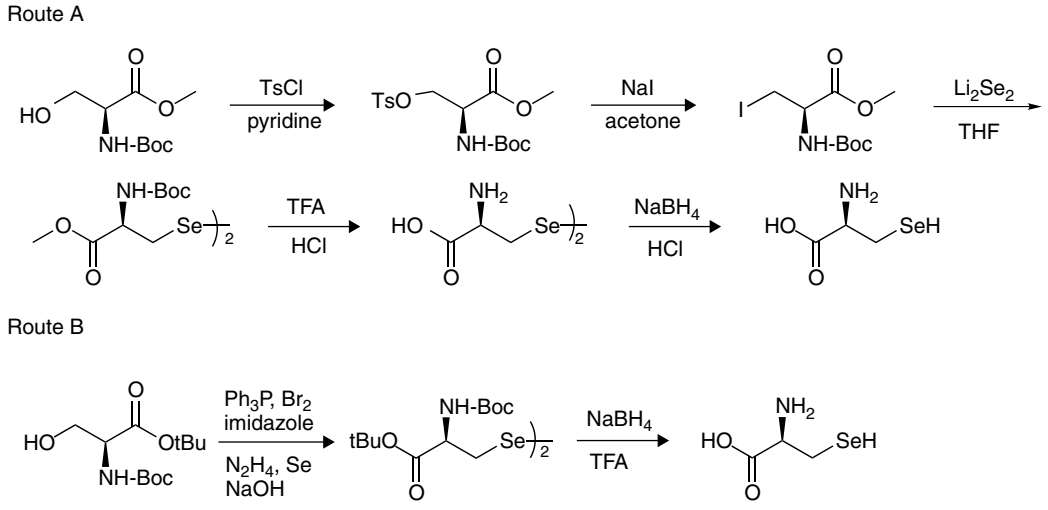
Compound	Reference redox system	E'_0 [mV]
Ac-Gly-Cys-Pro-Tyr-Cys-Val-Arg-Ala-NH_2	GSH/GSSG	−215 [98][a], −180[b]
Ac-Gly-Sec-Pro-Tyr-Sec-Val-Arg-Ala-NH_2	DTT	−381
Ac-Gly-Sec-Pro-Tyr-Cys-Val-Arg-Ala-NH_2	DTT	−326
$(H_2N\text{-}CH_2\text{-}CH_2\text{-}Se)_2$	DTT	−349

[a] Determined with $E'_0 = -240$ mV [95] for GSH/GSSG (see also Table 3).
[b] Determined with $E'_0 = -205$ mV [249] for GSH/GSSG.

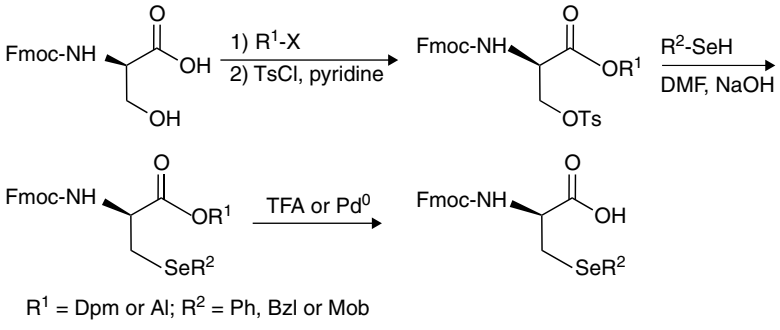
Synthesis of selenocysteine and related derivatives. Various syntheses of selenocysteine have been proposed, which all rely on the reaction of suitably protected β-haloalanine with lithium or sodium diselenide [62,253,254]. While Tanaka and Soda [253] used β-chloroalanine as the educt, the most recent synthetic routes are based on conversion of suitable serine derivatives into β-iodo- or β-bromoalanine compounds for reaction with diselenide anions to generate selenocystine upon removal of the protecting groups. The protected β-iodoalanine is conveniently prepared from Boc-Ser(Ts)-OMe with NaI (Scheme 9, route A) [254], whereas the bromo-derivative was obtained by direct reaction of Boc-Ser-OtBu with triphenylphosphine, bromine and imidazole (Scheme 9, route B) [62]. The β-haloalanine derivatives are then reacted with a diselenide anion, which is generated by reduction of selenium powder with either triethylborohydride or sodium borohydride [253–256], but can also be prepared *in situ* from selenium, hydrazine hydrate and NaOH [62].

The currently most used selenol protections in peptide synthesis are the 4-methoxybenzyl [256,257] and 4-methylbenzyl group [258,259]. These Sec derivatives are produced by reduction of selenocystine with $NaBH_4$ followed by *in situ* reaction with 4-methoxybenzyl chloride [257] or 4-methylbenzyl bromide [259]. A synthetic route more readily scalable is outlined in Scheme 10, where Fmoc-Ser-OAl or Fmoc-Ser-Dpm are activated as *O*-tosyl derivatives and reacted with aryl or aralkyl selenolates. These are obtained by treatment of selenium powder with 'super hydride' (1 M lithium triethylborohydride in THF) followed by the aryl or aralkyl chlorides and reduction of the resulting diselenides with hypophosphoric acid. Subsequent deprotection of the carboxy group yields the desired Sec derivatives by simple recrystallization of the crude reaction products [255].

Synthesis of selenocysteine/selenocystine peptides. Sec-peptides were synthesized both by the Fmoc and Boc strategy, and the procedures applied and difficulties encountered were comprehensively reviewed [207,260]. The main drawbacks encountered in these syntheses are the facile racemization of Sec derivatives during coupling steps and their high tendency to deselenate via β-elimination in the iterative piperidine-mediated Fmoc deprotection steps [207,251]. For deprotection of the *Se*-4-methylbenzyl group the hydrogen fluoride methods were applied



Scheme 9 Synthesis of L-selenocysteine by reaction of β-haloalanine derivatives with diselenide anions [62,254].

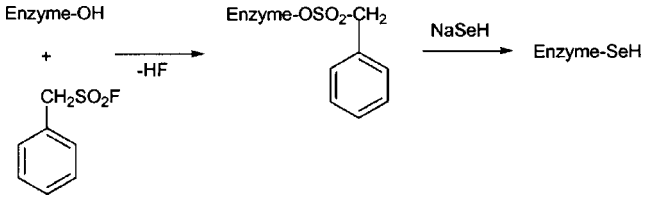


Scheme 10 Synthesis of protected L-selenocysteine derivatives for peptide synthesis [255].

apparently without particular difficulties [258,261], while cleavage of the *Se*-4-methoxybenzyl group can be performed with TFA in the presence of a strong Lewis acid such as trimethylsilyl-trifluoromethane sulfonate [257,262]. Alternatively, it can be removed oxidatively with iodine in acetic acid, a method which leads to complications in the presence of a second intramolecular Sec or Cys residue [32,207,255]. Better results were obtained in this case by deprotection with TFA in the presence of dimethylsulfoxide, which leads directly to the diselenide and mixed selenidesulfide bridge, respectively [32,207,251,255,262]. An additional useful method was found to be the displacement of the 4-methoxybenzyl group with 2-nitrophenylsulfenyl chloride [263] in analogy to the procedure applied for deprotection of related cysteine derivatives [126,264]. Unlike the unsymmetrical disulfide formed in the case of *S*-protected Cys-compounds, in addition to the 2-nitrophenylsulfide-selenide product the diselenide is formed, but the product mixture is readily converted to the Sec-peptides under reducing conditions. Interestingly, attempts to cleave the *Se*-4-methoxybenzyl groups in bis(selenocysteine)peptides with mercuric acetate led to the cyclic mercuric diselenolate peptides, from which regeneration of the free selenol groups failed even with large excess of mercaptanes. Possibly, by addition of HCl a displacement of the Hg^{2+} may occur, since these conditions were reported to destroy the complex of metalloselenonein with Cu^{+} [258].

Incorporation of selenocysteine into proteins. Selenocysteine has its own $tRNA^{Sec}$ that recognizes the opal stop codon UGA, but for co-translational insertion it needs intra- or intermolecularly acting factors in decoding UGA for Sec insertion [72]. In bacteria, a *stem*-loop structure in the mRNA immediately downstream of the UGA codon is recognized by a specialized Sec elongation factor, thus making site-specific insertion of selenocysteine into proteins possible only in a few *C*-terminal sequence positions [265]. This fact is very unfortunate as it prevents expression of proteins with selenocysteine placed into defined sequence positions for changing redox, and thus catalytic, properties of enzymes and for introducing unique sites suitable for chemoselective modification based on the peculiar properties of selenols.

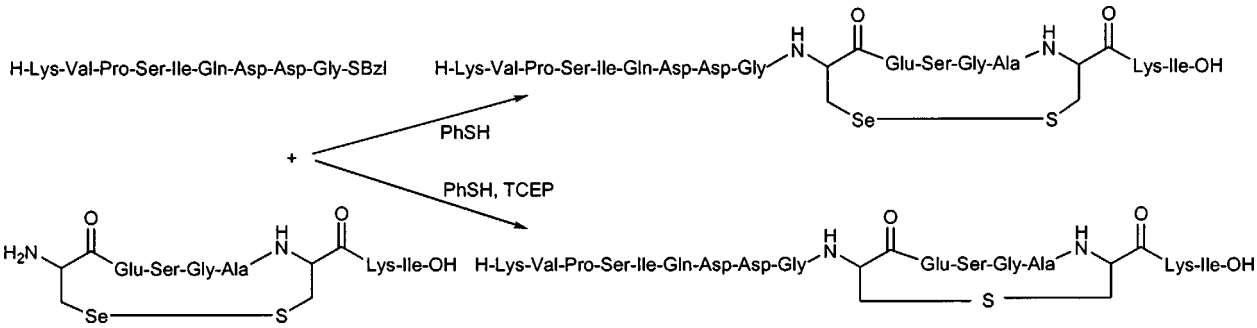
A first artificial selenoenzyme, selenosubtilisin, was synthesized by site-specific chemical modification of the active-site serine of subtilisin which reacts selectively with the inhibitor phenylmethanesulfonyl fluoride (Scheme 11). Treatment of the sulfonylated enzyme with excess hydrogen selenide resulted in the selenosubtilisin with novel hydrolytic and redox properties [266]. It proved to be an efficient acyl transferase in agreement with the faster aminolysis of selenol esters compared with esters and thiol esters [267,268], and to exhibit glutathione peroxidase activities.



Scheme 11 Site-specific selenation of subtilisin to produce selenosubtilisin [266].

An alternative approach for production of selenoproteins consists of *in vivo* charging of $tRNA^{Cys}$ with selenocysteine under cysteine-deprived growth conditions using cysteine-auxotrophic *E. coli* strains [269,270]. A possible disadvantage of this method, however, derives from the multiple replacement of Cys residues in the protein. For site-specific substitution of single Cys residues in peptides and proteins new methods based on native [155] and expressed chemical ligation [171] (*vide supra*) have been recently developed [255,263,271–273].

Native chemical ligation with selenocysteine. With the selenol being a stronger nucleophile than the thiol [246] and the selenoesters undergoing faster aminolysis than the thiol esters [267,268], native chemical ligation with *N*-terminal selenocysteine-peptides was expected to proceed faster and possibly even in better yields than with *N*-terminal cysteine-peptides. In fact, model studies on ligation of acetylglycine thiol esters with cystine and selenocystine in the presence of TCEP clearly confirmed significantly higher rates with the Sec derivatives in the pH range of 5 to 8 [271,274]. A complication was expected from the facile oxidation of the Sec residue. In the presence of a selenocystine dimer or of a mixed selenidesulfide peptide, thiophenol at high concentrations was sufficient to generate the selenol required for the transesterification step, although the ligation reaction itself was found to proceed at lower rates than with the cysteine-peptides [255]. Upon addition of TCEP as additional reductant, the ligation of two peptides related to ribonucleotide reductase was found to proceed very slowly with production of the lanthionine peptide shown in Scheme 12 as the only product and in low yields [255]. The very slow reaction can only be explained if the selenidesulfide bridge is deselenated at high rates leading to the lanthionine peptide which itself can than react with the thiol ester only via aminolysis. Such deselenation was attributed to the action of TCEP, since desulfurization of disulfides by tris(ethylamine)phosphine represents a well established reaction [275,276]. Conversely, Hilvert and coworkers [272] reported a smooth ligation in the presence of TCEP between the BPTI fragment 1–37 as ethylthiol ester and the fragment 38–58 containing the Cys^{51} and Cys^{54} residues in addition to the *N*-terminal Sec^{38} residue. Even in our hands deselenation has not been observed when reducing



Scheme 12 Side reaction in ligation of an oxidized [Sec,Cys]-peptide with a peptide thioester in the presence of TCEP [255].

diselenide-containing peptides with excess of TCEP or tributylphosphine [277,278]. However, we have repeatedly observed formation of dehydroalanine to high extents when treating Cys(StBu) compounds with some commercial charges of tributylphosphine [279], although desulfurization was reported to occur mainly with the basic tris(ethylamine)phosphine. Therefore, the question arises whether the side reaction reported by Gieselman *et al.* [255] derives from contaminants of unknown nature in the TCEP charge used.

Selenocystine as isomorphous replacement of cystine in peptides and proteins. The strongly reducing redox potential of selenocysteine clearly suggests a highly favoured diselenide over mixed selenidesulfide or disulfide formation. Correspondingly, selenocysteines when placed into appropriate positions of cysteine-rich peptides were expected to induce the correct diselenide/disulfide connectivities on oxidation. In fact, oxidative refolding of [Sec^{3,11},Cys^{1,15},Nle^{7}]-endothelin-1 [276] and [Sec^{1,11},Cys^{3,15}]-apamin [277,278] led to the wild-type diselenide/disulfide connectivities in quantitative yields. By placing the two Sec residues in different positions again, upon oxidation, the diselenide was formed quantitatively, thus producing the desired non-native topoisomers [278,280]. These results fully confirmed that suitable replacements of cysteine pairs with selenocysteine represents a highly efficient strategy for directing the oxidative folding of cysteine-rich peptides into the desired diselenide/disulfide framework.

Incorporation of a single selenocysteine residue in synthetic interleukin-8 [261] or by native chemical ligation into BPTI [272] and ribonuclease A [271] did not impede the correct oxidative refolding, but comparison of folding yields with the *all*-cysteine-polypeptides were not reported in these studies.

NMR conformational analysis of the diselenide-containing endothelin-1 and apamin analogues confirmed the fully isomorphous character of the disulfide/diselenide substitution as representatively shown in Figure 7 for wt-apamin and the [Sec^{1,11},Cys^{3,15}]-apamin analogue [277,278,280]. The identical results in terms of structure and function were reported for

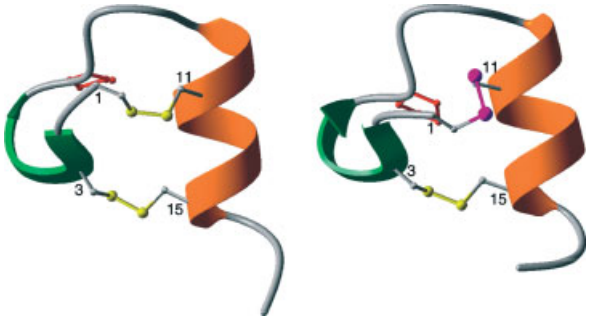


Figure 7 NMR solution structures of wt-apamin and its selenocystine analogue [Sec^{1,11},Cys^{3,15}]-apamin [278,280].

the interleukin-8 [263] and BPTI analogues [272] which contain a mixed selenidesulfide bridge. Correspondingly, such selenocystine-based heavy metal mutants of peptides and proteins were expected to serve well for solving the phase problem in protein crystallography [32] as an alternative to the selenomethionine [41,42], telluromethionine [24,39,64,65] and selenotryptophan mutants [281,282]. Indeed, success has been reported for the use of selenocystine in x-ray diffraction analysis of proteins [283,284]. These proteins were expressed in cysteine-auxotrophic *E. coli* by supplying the medium with D,L-selenocystine according to the procedure reported by Müller *et al.* [269]. By this expression method all cysteine residues are replaced by selenocysteine with an incorporation efficiency of about 70%–80%. Since the growth medium contains selenocystine, the reducing environment of *E. coli* has to be sufficiently strong to supply the translational machinery with selenocysteine and to allow for reshuffling of diselenides to the correct connectivities.

In addition to the usefulness of selenocysteine incorporation into peptides and proteins for MAD phasing in protein crystallography, uniform replacement of cystine residues by selenocystine was proposed for conformational analysis of unknown disulfide connectivities in cystine-rich peptides and proteins by ^{1}H–^{77}Se correlated NMR experiments [254]. However, ^{77}Se{^{1}H}, ^{1}H-HMBC experiments performed on the oxidized form of Ac-Gly-Sec-Pro-Tyr-Sec-Val-Arg-Ala-NH$_2$ at natural ^{77}Se abundance did not allow for assignment of the

diselenide connectivity [285]. Moreover, disulfides in peptides and proteins are known to be involved in tryptophan fluorescence quenching and charge transfer processes [286–288]. Taking into account the strong fluorescence quenching properties of selenomethionine [61,289], even selenocystines could provide interesting tools for studying peptide-protein or protein-protein interactions. Additionally, site-specific replacement of pairs of cysteine residues with selenocysteines was employed for selective reduction of the additional disulfides keeping the diselenides intact and thus to efficiently trap oxidative folding intermediates as well illustrated in the case of apamin [280].

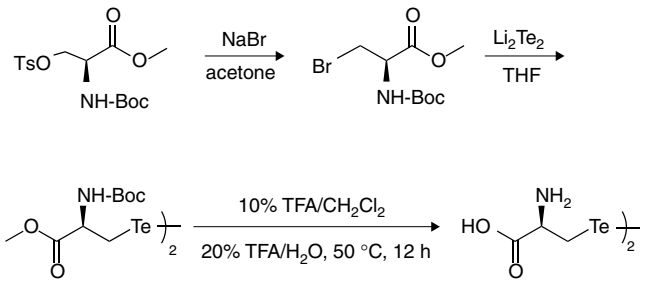
From a synthetic point of view, an additional application of selenocystein could derive from the mild and chemoselective oxidation of *Se*-phenylselenocysteine to dehydroalanine for site-specific intramolecular Michael additions to lanthionine or for site-specific introduction of external nucleophiles to produce peptide conjugates such as glycopeptides and lipopeptides [274].

Tellurocysteine

Unlike selenium, tellurium is not an essential micronutrient and, indeed, it induces both acute and chronic toxicity in a variety of species. But little is known about the molecular mechanism of tellurium toxicity. Tellurite is taken up by primary rat liver hepatocytes faster than selenite. It was shown that tellurite inhibits the ability of peroxidase to catalyse glutathione-dependent reduction of hydrogen peroxide and that inorganic tellurite delivers tellurium to the intracellular mileu in a form capable of binding to intracellular selenoproteins [290]. The nature of this binding seems not to be due to an insertion of tellurocysteine, although tellurium-tolerant fungi are capable of incorporating tellurocystine/tellurocysteine into proteins [63].

The synthesis of L-tellurocystine reported by Stocking *et al.* [254] differs from the related synthesis of L-selenocystine in the type of β-haloalanine used as educt (Scheme 13). Indeed, with the bromo-alanine derivative better yields were obtained than with the iodo compound. Moreover, the deprotection step was optimized and both cleavage of the Boc and hydrolysis of the methyl ester were performed with TFA to increase the yield in comparison with the cleavage with 6 M HCl used for selenocystine. The lithium ditelluride was prepared from elemental tellurium in dry tetrahydrofuran with 'super hydride' (1 M lithium triethylborohydride in THF) according to a known procedure [291].

In addition, the synthesis of *Te*-phenyl-L-tellurocysteine from β-chloroalanine and diphenyl ditelluride in the presence of $NaBH_4$ has been reported to proceed in satisfactory yields [292,293], suggesting that even *Te*-4-methylbenzyl- or *Te*-4-methoxybenzyl-tellurocysteine should be accessible to study the



Scheme 13 Synthesis of L-tellurocystine [254].

synthetic incorporation of this tellurium-containing amino acid into peptides and to analyse its stability versus demetallation via β-elimination as well as the properties of ditelluride bridges in model peptides.

Since the ^{125}Te nucleus is NMR active (spin 1/2) like ^{77}Se, tellurocystine could represent an interesting probe for NMR structural analysis of peptides and proteins. In addition, tellurocystine could provide heavy atom mutants of cystine-containing proteins with the advantage of a greater electron density than selenocystine. So far, attempts to express tellurocystine-containing thioredoxin failed [294] using cysteine-auxotrophic *E. coli* under the conditions successfully applied for the expression of the related selenocysteine mutant [269]. This failure may possibly be due to the insufficiently reducing the cytosolic environment of the host cells or to the lack of uptake of tellurocystine from the medium. Also the synthesis of tellurocystine-containing peptides has not been reported so far, although with the available ligation techniques telluro-proteins could possibly be accessible by this semisynthetic route. The ditelluride bridge is more space-filling than the disulfide and diselenide, but the plasticity of proteins is expected to accommodate such differences as was shown by replacing methione with telluromethionine. Moreover, analysis of disulfide bridges in a set of high resolution crystal structures has shown that the Cα distances can vary from 3.8 to 6.8 Å, as a result of the flexibility of the five different torsional angles of the bridged cystine residue [295].

Since *Te*-phenyl-L-tellurocysteine was found to be even more effective as a chemopreventive and antitumoural agent than the selenium and sulfur analogues [293], tellurium-containing peptides could well represent ideal delivery agents.

PERSPECTIVES

Nature exploits the distinct chemical and physicochemical properties of oxygen in hydroxy groups and of sulfur in thioether and thiol groups of peptides and proteins both structurally and functionally. Correspondingly, bioincorporation of the related amino acids serine, threonine and tyrosine as well as of methionine and

cysteine into polypeptide chains *in vivo* occurs under strict genetic control. Conversely, nature makes use of selenium, as is known to date, only with the amino acid analogues selenomethionine and selenocysteine, and genetically controlled bioincorporation occurs in the case of selenocysteine by a specific mechanism. Therefore, selenocysteine can be referred to as the 21st proteinogenic amino acid. Both selenomethionine and selenocysteine have attracted great interest in recent years as heavy metal, isomorphous analogues of methionine and cysteine, respectively, since their presence in peptides and proteins can significantly facilitate the phasing problem in x-ray crystallography. While a similar advantage derives from telluromethionine, the related tellurocysteine, so far, has not been used and studied as an isomorphous cysteine analogue.

Among the chalcogen-analogues of amino acids selenocysteine, in particular, was expected to offer additional interesting applications when incorporated synthetically or biosynthetically into peptides and proteins. Therefore, a new synthetic methodology has emerged recently to introduce selenocysteines into peptides, which concurrently allows for expansion of the cysteine-mediated native and expressed protein ligation to the selenocysteine approach and thus for application of this selenium-containing amino acid as a new promising tool in protein engineering. In fact, the characteristic physicochemical properties of selenocysteine such as the low pK_a, the low redox potential and high nucleophilicity allow the design of enzymes with a new reaction mechanism or changed redox properties to be addressed. Moreover, the highly preferential formation of diselenides and their stability towards reduction as observed in the oxidative refolding of selenocysteine/cysteine-rich peptides and proteins, should enhance the robustness of cystine frameworks and even allow the production of proteins with *de novo* designed diselenide/sulfide frameworks. In this context, extension of the peculiar properties of selenium to threonine and particularly to tyrosine could be highly promising. Indeed, tyrosine often plays decisive roles in enzyme catalysis and in natural products it is often structurally involved in side-chain crossbridges.

Acknowledgements

The author thanks the members of his research group for helpful discussions and Claudio Toniolo, University of Padova, for comments on the manuscript.

REFERENCES

1. Torchinsky YM. *Sulfur in Proteins*. Pergamon: Oxford, 1981; 1–277.
2. Giles NM, Watts AB, Giles GI, Fry FH, Littlechild JA, Jacob C. Metal and redox modulation of cysteine protein function. *Chem. Biol.* 2003; **10**: 677–693.
3. Jacob C, Giles GI, Giles NM, Sies H. Sulfur and selenium: the role of oxidation state in protein structure and function. *Angew. Chem. Int. Ed.* 2003; **42**: 4742–4758.
4. Jacob C, Holme AL, Fry FH. The sulfinic acid switch in proteins. *Org. Biol. Chem.* 2004; **2**: 1951–1956.
5. Mugesh G, du Mont WW, Sies H. Chemistry of biologically important synthetic organoselenium compounds. *Chem. Rev.* 2001; **101**: 2125–2179.
6. Stryer L. *Biochemistry*, 3rd edn. W. H. Freeman and Co: New York, USA, 1998.
7. Rose GD, Geselowitz AR, Lesser GJ, Lee RH, Zehfus MH. Hydrophobicity of amino acid residues in globular proteins. *Science* 1985; **229**: 834–838.
8. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reaction**s.** *Annu. Rev. Biochem.* 1993; **62**: 797–821.
9. Brot N, Weissbach H. Biochemistry of methionine sulfoxide residues in proteins. *Biofactors* 1991; **3**: 91–96.
10. Becker G, Musiol HJ. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22a), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 109–110.
11. Morley JS. Synthesis of human gastrin (I) and the biological properties of analogues. *Peptides 1966*, Beyermann H (ed.). North-Holland: Amsterdam, 1967; 226–234.
12. Kenner GW, Mendive JJ, Sheppard RC. Analogues of gastrin containing leucine in place of methionine. *J. Chem. Soc. (C)* 1968; 761–764.
13. Wünsch E, Jaeger E, Deffner M, Scharf R. Zur Synthese des [15-Leucin]Human-Gastrins I. III. Mitteilung: Zur Reindarstellung des synthetischen Heptadecapeptidamids. *Hoppe Seyler's Z. Physiol. Chem.* 1972; **353**: 1716–1720.
14. Wünsch E, Wendlberger G, Hallet A, Jaeger E, Knof S, Moroder L, Scharf R, Schmidt I, Thamm P, Wilschowitz L. Zur Totalsynthese des Human-Big-Gastrins I und seines 32-Leucin-Analogons. *Z. Naturforsch.* 1977; **32c**: 495–506.
15. Moroder L, Göhring W, Nyfeler R, Scharf R, Thamm P, Wendlberger G. Zur Synthese von Human-Little-Gastrin I und dessen Leucin-15-, Norleucin-25- und Methoxinin-15-Analoga. *Hoppe Seyler's Z. Physiol. Chem.* 1983; **364**: 157–171.
16. Rocchi R, Scatturin A, Moroder L, Marchiori M, Tamburro AM, Scoffone E. Synthesis of peptide analogues of the *N*-terminal eicosapeptide sequence of ribonuclease A. XI. Synthesis and conformational studies of [Orn^{10},Nle^{13}]-S-peptide. *J. Am. Chem. Soc.* 1969; **91**: 492–496.
17. Cowie DB, Cohen GN, Bolton ET, DeRobinchon-Szulmajster H. Amino acid analogue incorporation into bacterial proteins. *Biochim. Biophys. Acta* 1959; **34**: 39–46.
18. Barker DG, Bruton CJ. The fate of norleucine as a replacement for methionine in protein synthesis. *J. Mol. Biol.* 1979; **133**: 217–231.
19. Anfinsen CB, Corley LG. An active variant of staphylococcal nuclease containing norleucine in place of methionine. *J. Biol. Chem.* 1969; **244**: 5149–5152.
20. Gilles AM, Marliére P, Rose T, Sarfati R, Longin R, Meier A, Fermandjian S, Monnot M, Cohen GN, Bàrzu O. Conservative replacement of methionine by norleucine in *Escherichia coli* adenylate kinase. *J. Biol. Chem.* 1988; **263**: 8204–8209.
21. Koide H, Yokoyama S, Kawai G, Ha JM, Oka T, Kawai S, Miyake T, Fuwa T, Miyazawa T. Biosynthesis of a protein containing a nonprotein amino acid by *Escherichia coli*: L-2-aminohexanoic acid at position 21 in human epidermal growth factor. *Proc. Natl Acad. Sci. USA* 1988; **85**: 6237–6241.
22. Fersht AR, Dingwall C. An editing mechanism for the methionyl-tRNA synthetase in the selection of amino acids in protein synthesis. *Biochemistry* 1979; **18**: 1250–1255.
23. Budisa N. *Bioincorporation of Methionine Non-natural Amino Acid Analogues and Its Use for X-ray Crystallographic and Folding*

Studies of Proteins. Dissertation, Technical University of Munich: Munich, 1999.
24. Budisa N, Steipe B, Demange P, Eckerskorn C, Kellermann S, Huber R. High-level biosynthetic substitution of methionine in proteins by its analogues 2-aminohexanoic acid, selenomethionine, telluromethionine and ethionine in *Escherichia coli*. *Eur. J. Biochem.* 1995; **230**: 788–796.
25. Budisa N, Huber R, Golbik R, Minks C, Weyher E, Moroder L. Atomic mutations in annexin V. Thermodynamic studies of isomorphous protein variants. *Eur. J. Biochem.* 1998; **253**: 1–9.
26. Roblin RO, Lampen JO, English JP, Cole QP, Vaughan Jr. Studies in chemotherapy. VIII. Methionine and purine antagonists and their relation to the sulfonamides. *J. Am. Chem. Soc.* 1945; **67**: 290–294.
27. Christensen JA. On the synthesis of methoxinine. *Acta Chem. Scand.* 1954; **8**: 129.
28. Greven HM, Bruins AHNM, van der Vlugt FA. *Peptides 1971*, Nesvadba H (ed.). North-Holland: Amsterdam, 1973; 290–297.
29. Gillessen D, Trzeciak A, Müller RKM, Studer RO. Synthesis and biological activities of methoxinine-analogues of the *C*-terminal octapeptide of cholecystokinin-pancreozymin. *Int. J. Peptide Protein Res.* 1979; **13**: 130–136.
30. Wünsch E, Moroder L, Gillessen D, Soerensen UB, Bali JP. Biological and immunological properties of human gastrin-I analogues. *Hoppe Seyler's Z. Physiol. Chem.* 1982; **363**: 665–669.
31. Harada T, Seto K, Murooka Y. *O*-Alkylhomoserine and methionine biosynthesis in *Corynebacterium*. *J. Biochem.* 1969; **65**: 493–496.
32. Besse D, Budisa N, Karnbrock W, Minks C, Musiol HJ, Pegoraro S, Siedler F, Weyher E, Moroder L. Chalcogen-analogues of amino acids. Their use in x-ray crystallographic and folding studies of peptides and proteins. *Biol. Chem.* 1997; **378**: 211–218.
33. Clark LC, Combs GF, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL, Park HK, Sanders BB, Smith CL, Taylor Jr. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin — A randomized controlled trial. *J. Am. Med. Assoc.* 1996; **276**: 1957–1963.
34. Chang JC, Gutenmann WH, Reid CM, Lisk DJ. Selenium content of Brazil nuts from two geographic locations in Brazil. *Chemosphere* 1995; **30**: 801–802.
35. Wrobel K, Kannamkumarath SS, Wrobel K, Caruso JA. Hydrolysis of proteins with methanesulfonic acid for improved HPLC-ICP-MS determination of seleno-methionine in yeast and nuts. *Anal. Bioanal. Chem.* 2003; **375**: 133–138.
36. Cowie DB, Cohen GN. Biosynthesis by *Escherichia coli* of active altered proteins containing selenium instead of sulfur. *Biochim. Biophys. Acta* 1957; **26**: 252–261.
37. Munier R, Cohen GN. Incorporation of structural analogues of amino acids into bacterial proteins during their synthesis *in vivo*. *Biochim. Biophys. Acta* 1959; **31**: 378–390.
38. Koch T, Buchardt O. Synthesis of L-(+)-selenomethionine. *Synthesis* 1993; 1065–1067.
39. Karnbrock W, Weyher E, Budisa N, Huber R, Moroder L. A new efficient synthesis of acetyltelluro- and acetylselenomethionine and their use in the biosynthesis of heavy-atom protein analogues. *J. Am. Chem. Soc.* 1996; **118**: 913–914.
40. Perutz MF, Rossmann MG, Cullis AF, Muirhead H, Will G, North ACT. Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5 Å resolution, obtained by x-ray analysis. *Nature* 1960; **185**: 416–422.
41. Hendrickson WA. Determination of macromolecular structures from anomalous diffraction of synchroton radiation. *Science* 1991; **254**: 51–58.
42. Hendrickson WA, Horton J, LeMaster D. Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD) — A vehicle for direct determination of 3-dimensional structure. *EMBO J.* 1990; **9**: 1665–1672.
43. Budisa N, Minks C, Alefelder S, Wenger W, Dong F, Moroder L, Huber R. Toward the experimental codon reassignment *in vivo*. Protein building with an expanded amino acid repertoire. *FASEB J.* 1999; **13**: 41–51.
44. Budisa N, Moroder L, Huber R. Structure and evolution of the genetic codes viewed from the perspective of the experimentally expanded amino acid repertoire *in vivo*. *Cell. Mol. Life Sci.* 1999; **55**: 1626–1635.
45. Minks C, Alefelder S, Moroder L, Huber R, Budisa N. Towards new protein engineering: *in vivo* building and folding of protein shuttles for drug delivery and targeting by the selective pressure incorporation (SPI) method. *Tetrahedron* 2000; **56**: 9431–9442.
46. Budisa N. Prolegomena to future efforts on genetic code engineering by expanding its amino acid repertoire. *Angew. Chem. Int. Ed.* 2004; **43**: 6425–6463.
47. Smith JL, Thompson A. Reactivity of selenomethionine-dents in the magic bullet? *Structure* 1998; **6**: 815–819.
48. Assmann A, Briviba K, Sies H. Reduction of methionine selenoxide to selenomethionine by glutathione. *Arch. Biochem. Biophys.* 1998; **349**: 201–2003.
49. Sies H, Sharov VS, Klotz LO, Briviba K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations: a new function for selenoproteins as peroxynitrite reductase. *J. Biol. Chem.* 1997; **272**: 27 812–27 817.
50. Sharff AJ, Koronakis E, Luisi B, Koronaki V. Oxidation of selenomethionine: some MADness in the method! *Acta Cryst. D.* 2000; **56**: 785–788.
51. Ali MA, Peisach E, Allen KN, Imperiali B. X-ray structure analysis of a designed oligomeric miniprotein reveals a discrete quaternary architecture. *Proc. Natl Acad. Sci. USA* 2004; **101**: 12 183–12 188.
52. Klayman DL, Günther WHH. *Organic Selenium Compounds: Their Chemistry and Biology*. Wiley-Interscience: New York; 1973.
53. Padmaja S, Squadrito GL, Lemercier JN, Cueto R, Pryor WA. Peroxynitrite-mediated oxidation of D,L-selenomethionine: kinetics, mechanism and the role of carbon dioxide. *Free Radic. Biol. Med.* 1997; **23**: 917–926.
54. Hubbard SJ, Argos P. Cavities and packing at protein interfaces. *Protein Sci.* 1994; **3**: 2194–2206.
55. Gassner NC, Baase WA, Mooers BH, Busam RD, Weaver LH, Lindstrom JD, Quillin ML, Matthews BW. Multiple methionine substitutions are tolerated in T4 lysozyme and have coupled effects on folding and stability. *Biophys. Chem.* 2003; **100**: 325–340.
56. Gassner NC, Baase WA, Hausrath AC, Matthews BW. Substitution with selenomethionine can enhance the stability of methionine-rich proteins. *J. Mol. Biol.* 1999; **294**: 17–20.
57. Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moraefi I. Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* 2000; **101**: 199–210.
58. Roelfes G, Hilvert D. Incorporation of selenomethionine into proteins through selenohomocysteine-mediated ligation. *Angew. Chem. Int. Ed.* 2003; **42**: 2275–2277.
59. Zhou ZS, Smith AE, Matthews RG. L-Selenohomocysteine: one-step synthesis from L-selenomethionine and kinetic analysis as substrate for methionine synthases. *Bioorg. Med. Chem. Lett.* 2000; **10**: 2471–2475.
60. Boles JO, Tolleson WH, Schmidt JC, Dunlap RB, Odom JD. Selenomethionyl dihydrofolate reductase from *Escherichia coli*. Comparative biochemistry and ^{77}Se nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* 1992; **267**: 22 217–22 223.
61. Yuan T, Weljie AM, Vogel HJ. Tryptophan fluorescence quenching by methionine and selenomethionine residues of calmodulin: orientation of peptide and protein binding. *Biochemistry* 1998; **37**: 3187–3195.
62. Siebum AHG, Woo WS, Raap J, Lugtenburg J. Access to any site-directed isotopomer of methionine, selenomethionine, cysteine and selenocysteine. Use of simple, efficient modular synthetic reaction schemes for isotope incorporation. *Eur. J. Org. Chem.* 2004; 2905–2913.

63. Ramadan SE, Razak AA, Ragab AM, El-Meleigy M. Incorporation of tellurium into amino acids and proteins in tellurium-tolerant fungi. *Biol. Trace Element Res.* 1989; **20**: 225–232.
64. Boles JO, Lewinski K, Kunke M, Odom JD, Dunlap RB, Lebioda L, Hatada M. Bio-incorporation of telluromethionine into buried residues of dihydrofolate-reductase. *Nature Struct. Biol.* 1994; **1**: 283–284.
65. Budisa N, Karnbrock W, Steinbacher S, Humm A, Prade L, Neuefeind T, Moroder L, Huber R. Bioincorporation of telluromethionine into proteins. A promising new approach for x-ray structure analysis of proteins. *J. Mol. Biol.* 1997; **270**: 616–623.
66. Knapp FF. Telluroamino acids: synthesis of telluromethionine. *J. Org. Chem.* 1979; **44**: 1007–1009.
67. Silks LA, Boles JO, Modi BP, Dunlap RB, Odom JD. An efficient synthesis of racemic and optically active telluromethionine. *Synth. Commun.* 1990; **20**: 1555–1562.
68. Detty MR. Oxidation of selenides and tellurides with positive halogenating species. *J. Org. Chem.* 1980; **45**: 274–279.
69. Kirsch G, Goodman MM, Knapp FF. Organotellurium compounds of biological interest: unique properties of the *N*-chlorosuccinimide oxidation product of 9-telluroheptadecanoic acid. *Organometallics* 1983; **2**: 357–363.
70. Stadtman TC. Selenocysteine. *Annu. Rev. Biochem.* 1996; **65**: 83–100.
71. Leinfelder W, Zehelein E, Mandrand-Berthelot MA, Böck A. Gene for a novel tRNA species that accepts L-serine and co-translationally inserts selenocysteine. *Nature* 1988; **331**: 723–725.
72. Commans S, Böck A. Selenocysteine inserting tRNAs: an overview. *FEMS Microbiol. Rev.* 1999; **23**: 335–351.
73. Oas TG, Kim PS. A peptide model of a protein folding intermediate. *Nature* 1988; **336**: 42–48.
74. Staley JP, Kim PS. Role of a subdomain in the folding of bovine pancreatic trypsin inhibitor. *Nature* 1990; **344**: 685–688.
75. Staley JP, Kim PS. Complete folding of bovine pancreatic trypsin inhibitor with only a single disulfide bond. *Proc. Natl Acad. Sci. USA* 1992; **89**: 1519–1523.
76. van Mierlo CP, Darby NJ, Neuhaus D, Creighton TE. (14–38, 30–51) Double-disulphide intermediate in folding of bovine pancreatic trypsin inhibitor: a two-dimensional ^{1}H nuclear magnetic resonance study. *J. Mol. Biol.* 1991; **222**: 353–371.
77. van Mierlo CP, Darby NJ, Neuhaus D, Creighton TE. Two-dimensional ^{1}H nuclear magnetic resonance study of the (5–55) single-disulphide folding intermediate of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 1991; **222**: 373–390.
78. van Mierlo CP, Kemmink J, Neuhaus D, Darby NJ, Creighton TE. ^{1}H NMR analysis of the partly-folded non-native two-disulphide intermediates (30–51,5–14) and (30–51,5–38) in the folding pathway of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 1994; **235**: 1044–1061.
79. Cattani-Scholz A, Renner C, Cabrele C, Behrendt R, Oesterhelt D, Moroder L. Photoresponsive cyclic bis(cysteinyl)peptides as catalysts of oxidative protein folding. *Angew. Chem. Int. Ed.* 2002; **41**: 289–292.
80. Renner C, Behrendt R, Heim N, Moroder L. Photomodulation of conformational states. III. Water-soluble bis-cysteinyl-peptides with (4-aminomethyl)phenylazobenzoic acid as backbone constituent. *Biopolymers* 2002; **63**: 382–393.
81. Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid. Redox. Signal.* 2000; **2**: 811–820.
82. Martin JL. Thioredoxin: a fold for all reasons. *Structure* 1995; **3**: 245–250.
83. Ritz D, Beckwith J. Roles of thiol-redox pathways in bacteria. *Annu. Rev. Microbiol.* 2001; **55**: 21–48.
84. Chivers PT, Laboissiere MC, Raines RT. The CXXC motif: imperatives for the formation of native disulfide bonds in the cell. *EMBO J.* 1996; **15**: 2659–2667.
85. Chivers PT, Prehoda KE, Raines RT. The CXXC motif: a rheostat in the active site. *Biochemistry* 1997; **36**: 4061–4066.
86. Lees WJ, Whitesides GM. Equilibrium constants for thiol-disulfide interchange reactions: a coherent, corrected set. *J. Org. Chem.* 1993; **58**: 642–647.
87. Akerboom TP, Bilzer M, Sies H. The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J. Biol. Chem.* 1982; **257**: 4248–4252.
88. Meister A, Anderson ME. Glutathione. *Annu. Rev. Biochem.* 1983; **52**: 711–760.
89. Goldberger RF, Epstein CJ, Anfinsen CB. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. *J. Biol. Chem.* 1963; **238**: 628–635.
90. Freedman RB. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. *Cell* 1989; **57**: 1069–1072.
91. Bardwell JC, McGovern K, Beckwith J. Identification of a protein required for disulfide bond formation *in vivo*. *Cell* 1991; **67**: 581–589.
92. Bardwell JC, Lee JO, Jander G, Martin N, Belin D, Beckwith J. A pathway for disulfide bond formation *in vivo*. *Proc. Natl Acad. Sci. USA* 1993; **90**: 1038–1042.
93. Frand AR, Kaiser CA. Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Mol. Cell* 1999; **4**: 469–477.
94. Tu BP, Ho-Schleyer S, Travers KJ, Weissman JS. Biochemical basis of oxidative folding in endoplasmic reticulum. *Science* 2000; **290**: 1571–1574.
95. Rost J, Rapoport S. Reduction-potential of glutathione. *Nature* 1964; **201**: 185.
96. Millis KK, Weaver KH, Rabenstein DL. Oxidation/reduction potential of glutathione. *J. Org. Chem.* 1993; **58**: 4144–4146.
97. Åslund F, Berndt KD, Holmgren A. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. *J. Biol. Chem.* 1997; **272**: 30 780–30 786.
98. Siedler F, Rudolph-Böhner S, Doi M, Musiol HJ, Moroder L. Redox potentials of active-site bis(cysteinyl)fragments of thiol-protein oxidoreductases. *Biochemistry* 1993; **32**: 7488–7495.
99. Cabrele C, Fiori S, Pegoraro S, Moroder L. Redox-active bis(cysteinyl)peptides as catalysts for *in vitro* oxidative protein folding. *Chem. Biol.* 2002; **9**: 731–740.
100. Lin TY, Kim PS. Urea dependence of thiol-disulfide equilibria in thioredoxin: confirmation of the linkage relationship and a sensitive assay for structure. *Biochemistry* 1989; **28**: 5282–5287.
101. Hawkins HC, de Nardi M, Freedman RB. Redox properties and cross-linking of the dithiol/disulphide active sites of mammalian protein disulphide-isomerase. *Biochem. J.* 1991; **275**: 341–348.
102. Darby NJ, Creighton TE. Characterization of the active site cysteine residues of the thioredoxin-like domains of protein disulfide isomerase. *Biochemistry* 1995; **34**: 16 770–16 780.
103. O'Donnell ME, Williams CH, Jr. Proton stoichiometry in the reduction of the FAD and disulfide of *Escherichia coli* thioredoxin reductase. Evidence for a base at the active site. *J. Biol. Chem.* 1983; **258**: 13 795–13 805.
104. Huber-Wunderlich M, Glockshuber R. A single dipeptide sequence modulates the redox properties of a whole enzyme family. *Fold. Des.* 1998; **3**: 161–171.
105. Krause G, Lundstrom J, Barea JL, Pueyo de la Cuesta C, Holmgren A. Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in *Escherichia coli* thioredoxin, *J. Biol. Chem.* 1991; **266**: 9494–9500.
106. Kortemme T, Darby NJ, Creighton TE. Electrostatic interactions in the active site of the *N*-terminal thioredoxin-like domain of protein disulfide isomerase. *Biochemistry* 1996; **35**: 14 503–14 511.
107. Yang YF, Wells WW. Identification and characterization of the functional amino acids at the active center of pig liver

thioltransferase by site-directed mutagenesis. *J. Biol. Chem.* 1991; **266**: 12 759–12 765.
108. Grauschopf U, Winther JR, Korber P, Zander T, Dallinger P, Bardwell JC. Why is DsbA such an oxidizing disulfide catalyst? *Cell* 1995; **83**: 947–955.
109. Cabrele C, Fiori S, Pegoraro S, Moroder L. In *Peptides: The Wave of the Future*, Lebl M, Houghton RA (eds). Kluwer Academic Publishers: Dordrecht, 2001; 462–463.
110. Mössner E, Wunderlich-Huber M, Glockshuber R. Characterization of *Escherichia coli* thioredoxin variants mimicking the active-sites of other thiol/disulfide oxidoreductases. *Protein Sci.* 1998; **7**: 1233–1244.
111. Kortemme T, Creighton TE. Ionisation of cysteine residues at the termini of model α-helical peptides. Relevance to unusual thiol pK_a values in proteins of the thioredoxin family. *J. Mol. Biol.* 1995; **253**: 799–812.
112. Otto HH, Schirmeister T. Cysteine proteases and their inhibitors. *Chem. Rev.* 1997; **97**: 133–171.
113. Woo HA, Chae HZ, Hwang SC, Yang KS, Kang SW, Kim K, Rhee SG. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* 2003; **300**: 653–656.
114. Schröder E, Littlechil JA, Lebedev AA, Errington N, Vagin AA, Isupov MN. Crystal structure of decameric 2-Cys peroxiredoxin from human erythrocytes at 1.7 Å resolution. *Structure* 2000; **8**: 605–615.
115. Ji Y, Bennett BM. Activation of microsomal glutathione *S*-transferase by peroxynitrite. *Mol. Pharmacol.* 2003; **63**: 136–146.
116. Biteau B, Labarre J, Toledano MB. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 2003; **425**: 980–984.
117. Klaassen CD, Boles JW. Sulfation and sulfotransferases 5: the importance of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in the regulation of sulfation. *FASEB J.* 1997; **11**: 404–418.
118. Hille A, Braulke T, von Figura K, Huttner WB. Occurrence of tyrosine sulfate in proteins — A balance-sheet. 1. Secretory and lysosomal proteins. *Eur. J. Biochem.* 1990; **188**: 577–586.
119. Kimura T. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22b), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 142–161.
120. Xu Y, Wilcox DE. Oxidation of zinc finger cysteines to thiolsulfinate. *J. Am. Chem. Soc.* 1998; **120**: 7375–7376.
121. Musiol HJ, Loidl G, Moroder L. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22b), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 179–183.
122. Sahl HG, Bierbaum G. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu. Rev. Microbiol.* 1998; **52**: 41–79.
123. Süssmuth RD, Jack RW, Jung G. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22b), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 184–213.
124. Fusetani N, Matsunaga S. Bioactive sponge peptides. *Chem. Rev.* 1993; **93**: 1793–1806.
125. Gilon C, Mang C, Lohof E, Friedler A, Kessler H. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22b), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 521–524.
126. Moroder L, Musiol HJ, Schaschke N, Chen L, Hargittai B, Barany G. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22a), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 384–423.
127. Akaji K, Kiso Y. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22b), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 101–141.
128. Babine RE, Bender SL. Molecular recognition of protein-ligand complexes: applications to drug design. *Chem. Rev.* 1997; **97**: 1359–1472.
129. Polgar L, Bender ML. A new enzyme containing a synthetically formed active site. Thiol-subtilisin. *J. Am. Chem. Soc.* 1966; **88**: 3153–3154.
130. Neet KE, Koshland DE. Conversion of serine at the active site of subtilisin to cysteine — A chemical mutation. *Proc. Natl Acad. Sci. USA* 1966; **56**: 1606–1611.
131. Nakatsuka T, Sasaki T, Kaiser ET. Peptide segment coupling catalyzed by the semisynthetic enzyme thiolsubtilisin. *J. Am. Chem. Soc.* 1987; **109**: 3808–3810.
132. Abrahmsen L, Tom J, Burnier J, Butcher KA, Kossiakoff A, Wells JA. Engineering subtilisin and its substrates for efficient ligation of peptide-bonds in aqueous solution. *Biochemistry* 1991; **30**: 4151–4159.
133. Chang TK, Jackson DY, Burnier JP, Wells JA. Subtiligase: a tool for semisynthesis of proteins. *Proc. Natl Acad. Sci. USA* 1994; **91**: 12 544–12 548.
134. Jackson DY, Burnier J, Quan C, Stanley M, Tom J, Wells JA. A designed peptide ligase for total synthesis of ribonuclease A with unnatural catalytic residues. *Science* 1994; **266**: 243–247.
135. Perler FB, Davis EO, Dean GE, Gimble FS, Jack WE, Neff N, Noren CJ, Thorner J, Belfort M. Protein splicing elements — inteins and exteins — a definition of terms and recommended nomenclature. *Nucleic Acids Res.* 1994; **22**: 1125–1127.
136. Chong S, Shao Y, Paulus H, Brenner J, Perler FB, Xu MQ. Protein splicing involving the *Saccharomyces cerevisiae* VMA intein: the steps in the splicing pathway, side reactions leading to protein cleavage, and establishment of an *in vitro* splicing system. *J. Biol. Chem.* 1996; **271**: 22 159–22 168.
137. Brannigan JA, Dodson G, Duggleby HJ, Moody PCE, Smith JL, Tomchick DR, Murzin AG. A protein catalytic framework with an *N*-terminal nucleophile is capable of self-activation. *Nature* 1995; **378**: 416–419.
138. van Poelje PD, Snell EE. Pyruvoyl-dependent enzymes. *Annu. Rev. Biochem.* 1990; **59**: 29–59.
139. Lee JJ, Ekker SC, von Kessler DP, Porter JA, Sun BI, Beachy PA. Autoproteolysis in *hedgehog* protein biosynthesis. *Science* 1994; **266**: 1528–1537.
140. Porter JA, von Kessler DP, Ekker SC, Young KE, Lee JJ, Moses K, Beachy PA. The product of *hedgehog* autoproteolytic cleavage active in local and long-range signalling. *Nature* 1995; **374**: 363–366.
141. Li YM, Milne JC, Madison LL, Kolter R, Walsh CT. From peptide precursor to oxazole and thiazole containing peptide antibiotics: microcin B17 synthase. *Science* 1996; **274**: 1188–1193.
142. Perler FB. Protein splicing of inteins and *hedgehog* autoproteolysis: structure, function, and evolution. *Cell* 1998; **92**: 1–4.
143. Perler FB, Xu MQ, Paulus H. Protein splicing and autoproteolysis mechanisms. *Curr. Opin. Chem. Biol.* 1997; **1**: 292–299.
144. Gogarten JP, Senejani AG, Zhaxybayeva O, Olendzenski L, Hilario E. Inteins: structure, function, and evolution. *Annu. Rev. Microbiol.* 2002; **56**: 263–287.
145. Kawasaki M, Nogami S, Satow Y, Ohya Y, Anraku Y. Identification of three core regions essential for protein splicing of the yeast *Vma1* protozyme. A random mutagenesis study of the entire *VMA1*-derived endonuclease sequence. *J. Biol. Chem.* 1997; **272**: 15 668–15 674.
146. Xu MQ, Perler FB. Identification of three core regions essential for protein splicing of the yeast *Vma1* protozyme — A random mutagenesis study of the entire *Vma1*-derived endonuclease sequence. *EMBO J.* 1996; **15**: 5146–5153.
147. Bodanszky M, Martinez J. *The Peptides: Analysis, Synthesis, Biology*, Gross E, Meienhofer J (eds). Academic Press: New York, 1983; 111–218.

148. Geiger T, Clarke S. Deamidation, isomerization and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* 1987; **262**: 785–794.
149. Blodgett JK, Loudon GM, Collins KD. Specific cleavage of peptides containing an aspartic acid (β-hydroxamic acid) residue. *J. Am. Chem. Soc.* 1985; **107**: 4305–4313.
150. Piszkiewicz D, Landon M, Smith EL. Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. *Biochem. Biophys. Res. Commun.* 1970; **40**: 1173–1178.
151. Wieland T, Bokelmann E, Bauer L, Lang HU, Lau H. Über Peptidsynthesen. 8. Bildung von S-haltigen Peptiden durch intramolekulare Wanderung von Aminoacylresten. *Liebigs Ann. Chem.* 1953; **583**: 129–149.
152. Brenner M, Zimmermann JP, Wehrmüller J, Quitt P, Hardtmann A, Schneider W, Beglinger U. Aminoacyl-Einlagerung. 1. Mitteilung. Definition, Übersicht und Beziehung zur Peptidsynthese. *Helv. Chim. Acta* 1957; **40**: 1497–1517.
153. Brenner M, Zimmermann JP, Wehrmüller J, Quitt P, Photaski I. Eine neue Umlagerungsreaktion und ein neues Prinzip zum Aufbau von Peptidketten. *Experientia* 1955; **11**: 397–399.
154. Kemp DS, Leung SL, Kerkman DJ. Models that demonstrate peptide-bond formation by prior thiol capture. 1. Capture by disulfide formation. *Tetrahedron Lett.* 1981; **22**: 181–184.
155. Dawson PE, Muir TW, Clarke-Lewis I, Kent SBH. Synthesis of proteins by native chemical ligation. *Science* 1994; **266**: 776–779.
156. Tam JP, Lu YA, Liu CF, Shao J. Peptide synthesis using unprotected peptides through orthogonal coupling methods. *Proc. Natl Acad. Sci. USA* 1995; **92**: 12 485–12 489.
157. Yamashiro D, Blake J. Use of thiol acids in peptide segment coupling in non-aqueous solvents. *J. Pept. Prot. Res.* 1981; **18**: 383–392.
158. Hwang PTR, Hancoock CK. Study of the alkaline hydrolysis and nuclear magnetic resonance spectra of some thiol esters. *J. Am. Chem. Soc.* 1973; **38**: 4239–4243.
159. Marahiel MA, Stachelhaus T, Mootz HD. Modular peptide synthetases involved in nonribosomal peptide synthesis. *Chem. Rev.* 1997; **97**: 2651–2673.
160. von Döhren H, Keller U, Vater J, Zocher R. Multifunctional peptide synthetases. *Chem. Rev.* 1997; **97**: 2675–2705.
161. Bang D, Kent SBH. A one-pot total synthesis of crambin. *Angew. Chem. Int. Ed.* 2004; **43**: 2534–2538.
162. Dawson P. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22a), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 627–641.
163. Dawson PE, Kent SBH. Synthesis of native proteins by chemical ligation. *Annu. Rev. Biochem.* 2000; **69**: 923–960.
164. Coltart DM. Peptide segment coupling by prior ligation and proximity-induced intramolecular acyl transfer. *Tetrahedron* 2000; **56**: 3449–3491.
165. Tam JP, Xu JX, Eom KD. Methods and strategies of peptide ligation. *Biopolymers* 2001; **60**: 194–205.
166. Casi G, Hilvert D. Convergent protein synthesis. *Curr. Opin. Struct. Biol.* 2003; **13**: 589–594.
167. Canne LE, Bark SJ, Kent SBH. Extending the applicability of native chemical ligation. *J. Am. Chem. Soc.* 1996; **118**: 5891–5896.
168. Offer J, Dawson PE. N^{α}-2-Mercaptobenzylamine-assisted chemical ligation. *Org. Lett.* 2000; **2**: 23–26.
169. Offer J, Boddy CNC, Dawson PE. Extending synthetic access to proteins with a removable acyl transfer auxiliary. *J. Am. Chem. Soc.* 2002; **124**: 4642–4646.
170. Evans TC, Brenner J, Xu MQ. Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci.* 1998; **7**: 2256–2264.
171. Muir TW, Sondhi D, Cole PA. Expressed protein ligation: a general method for protein engineering. *Proc. Natl Acad. Sci. USA* 1998; **95**: 6705–6710.
172. Evans TC Jr, Xu MQ, Intein-mediated protein ligation: harnessing nature's escape artists. *Biopolymers* 1999; **51**: 333–342.
173. Muir TW. Semisynthesis of proteins by expressed protein ligation. *Annu. Rev. Biochem.* 2003; **72**: 249–289.
174. David R, Richter MPO, Beck-Sickinger AG. Expressed protein ligation — Method and applications. *Eur. J. Biochem.* 2004; **271**: 663–677.
175. Creighton TE. Disulfide bonds and protein stability. *BioEssays* 1988; **8**: 57–63.
176. Anfinsen CB, Haber E, Sela M, White FH. Kinetics of formation of native ribonuclease during oxidation of reduced polypeptide chain. *Proc. Natl Acad. Sci. USA* 1961; **47**: 1309–1314.
177. Anfinsen CB. Principles that govern the folding of protein chains. *Science* 1973; **181**: 223–230.
178. Gething MJ, Sambrook J. Protein folding in the cell. *Nature* 1992; **355**: 33–45.
179. Hartl FU. Molecular chaperones in cellular protein folding. *Nature* 1996; **381**: 571–580.
180. Freedman RB, Hirst TR, Tuite MF. Protein disulfide-isomerase — Building bridges in protein folding. *Trends Biochem. Sci.* 1994; **19**: 331–336.
181. Schmid FX. Protein folding: prolyl isomerases join the fold. *Curr. Biol.* 1995; **5**: 993–994.
182. Hwang C, Sinskey AJ, Lodish HF. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* 1992; **257**: 1496–1502.
183. Tu BP, Weissman JS. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J. Cell Biol.* 2004; **164**: 341–346.
184. Wetlaufer DB, Saxena VP. Formation of three-dimensional structure in proteins. I. Rapid nonenzymic reactivation of reduced lysozyme. *Biochemistry* 1970; **9**: 5015–5023.
185. Creighton TE. Protein folding coupled to disulphide bond formation. *Biol. Chem.* 1997; **378**: 731–744.
186. Rumbley J, Hoang L, Mayne L, Englander SW. An amino acid code for protein folding. *Proc. Natl Acad. Sci. USA* 2001; **98**: 105–112.
187. Lazaridis TZ, Karplus M. 'New view' of protein folding reconciled with the old through multiple unfolding simulations. *Science* 1997; **278**: 1928–1931.
188. Onuchic JN, Luthey-Schulten Z, Wolynes PG. Theory of protein folding: the energy landscape perspective. *Annu. Rev. Phys. Chem.* 1997; **48**: 545–600.
189. Huyghues-Despointes BM, Nelson JW. Stabilities of disulfide bond intermediates in the folding of apamin. *Biochemistry* 1992; **31**: 1476–1483.
190. Chau MH, Nelson JW. Cooperative disulfide bond formation in apamin. *Biochemistry* 1992; **31**: 4445–4450.
191. Zimmerman SS, Scheraga HA. Local interactions in bends of proteins. *Proc. Natl Acad. Sci. USA* 1977; **74**: 4126–4129.
192. Creighton TE. An empirical approach to protein conformation stability and flexibility. *Biopolymers* 1983; **22**: 49–58.
193. Creighton TE. Disulphide bonds as probes of protein folding pathways. *Methods Enzymol.* 1986; **131**: 83–106.
194. Zhang RM, Snyder GH. Dependence of formation of small disulfide loops in two-cysteine peptides on the number and types of intervening amino acids. *J. Biol. Chem.* 1989; **264**: 18 472–18 479.
195. Wedemeyer WJ, Xu X, Welker E, Scheraga HA. Conformational propensities of protein folding intermediates: distribution of species in the 1S, 2S, and 3S ensembles of the [C40A,C95A] mutant of bovine pancreatic ribonuclease A. *Biochemistry* 2002; **41**: 1483–1491.
196. Thornton JM. Disulfide bridges in globular proteins. *J. Mol. Biol.* 1981; **151**: 261–287.
197. Dobson CM, Sali A, Karplus M. Protein folding: a perspective from theory and experiment. *Angew. Chem. Int. Ed. Engl.* 1998; **37**: 868–893.

198. Capaldi AP, Kleanthous C, Radford SE. Im7 folding mechanism: misfolding on a path to the native state. *Nat. Struct. Biol.* 2002; **9**: 209–216.
199. Hunter HN, Fulton DB, Ganz T, Vogel HJ. The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. *J. Biol. Chem.* 2002; **277**: 37 597–37 603.
200. Wang XH, Connor M, Smith R, Maciejewski MW, Howden MEH, Nicholson GM, Christie MJ, King GF. Discovery and characterization of a family of insecticidal neurotoxins with a rare vicinal disulfide bridge. *Nat. Struct. Biol.* 2000; **7**: 505–513.
201. Capasso S, Mattia C, Mazarella L, Puliti R. Structure of a cis-peptide unit: molecular conformation of the cyclic disulphide L-cysteinyl-L-cysteine. *Acta Crystallogr.* 1977; **B33**: 2080–2083.
202. Čemažar M, Zahariev S, Lopez JJ, Carugo O, Jones JA, Hore PJ, Pongor S. Oxidative folding intermediates with nonnative disulfide bridges between adjacent cysteine residues. *Proc. Natl Acad. Sci. USA* 2003; **100**: 5754–5759.
203. Rudolph R, Böhm G, Lilie H, Jaenicke R. *Protein Function: A Practical Approach*, Creighton TE (ed.). IRL Press/Oxford University Press: Oxford, 1997; 57–99.
204. Daugherty DL, Rozema D, Hanson PE, Gellman SH. Artificial chaperone-assisted refolding of citrate synthase. *J. Biol. Chem.* 1998; **273**: 33 961–33 971.
205. Rattenholl A, Ruoppolo M, Flagiello A, Monti M, Vinci F, Marino G, Lilie H, Schwarz E, Rudolph R. Prosequence assisted folding and disulfide bond formation of human nerve growth factor. *J. Mol. Biol.* 2001; **305**: 523–533.
206. Weissman JS, Kim PS. The pro-region of BPTI facilitates folding. *Cell* 1992; **711**: 841–851.
207. Moroder L, Besse D, Musiol HJ, Rudolph-Böhner S, Siedler F. Oxidative folding of cystine-rich peptides *vs* regioselective cysteine pairing strategies. *Biopolymers* 1996; **40**: 207–234.
208. Dimarcq JL, Bulet P, Hetru C, Hoffmann J. Cysteine-rich antimicrobial peptides in invertebrates. *Biopolymers* 1998; **47**: 465–477.
209. García-Olmedo F, Molina A, Alamillo JM, Rodríguez-Palenzuéla P. Plant defense peptides. *Biopolymers* 1998; **47**: 479–491.
210. Terlau H, Olivera BM. Conus venoms: a rich source of novel ion channel-targeted peptides. *Physiol. Rev.* 2004; **84**: 41–68.
211. Loughnan ML, Alewood PF. Physico-chemical characterization and synthesis of neuronally active α-conotoxins. *Eur. J. Biochem.* 2004; **271**: 2294–2304.
212. Tamaoki H, Miura R, Kusunoki M, Kyogoku Y, Kobayashi Y, Moroder L. Folding motifs induced and stabilized by distinct cystine frameworks. *Protein Eng.* 1998; **11**: 649–659.
213. Cornet B, Bonmatin JM, Hetru C, Hoffmann JA, Ptak M, Vovelle F. Refined 3-dimensional solution structure of insect defensin-A. *Structure* 1995; **3**: 435–448.
214. Narasimhan L, Singh J, Humblet C, Guruprasad K, Blundell T. Snail and spider toxins share a similar tertiary structure and 'cystine motif'. *Nat. Struct. Biol.* 1994; **1**: 850–852.
215. Carugo O, Lu SY, Luo JC, Gu XC, Liang SP, Strobl S, Pongor S. Structural analysis of free and enzyme-bound amaranth alpha-amylase inhibitor: classification within the knottin fold superfamily and analysis of its functional flexibility. *Protein Eng.* 2001; **14**: 639–646.
216. Tang YQ, Yuan J, Ösapay G, Ösapay K, Tran D, Miller CJ, Quellette AJ, Selsted ME. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated α-defensins. *Science* 1999; **286**: 498–502.
217. Trabi M, Schirra HJ, Craik DJ. Three-dimensional structure of RTD-1, a cyclic antimicrobial defensin from rhesus macaque leukocytes. *Biochemistry* 2001; **40**: 4211–4221.
218. Altamirano MM, Golbik R, Zahn R, Buckle AM, Fersht AR. Refolding chromatography with immobilized mini-chaperones. *Proc. Natl Acad. Sci. USA* 1997; **94**: 3576–3578.
219. Karadimitris A, Gadola S, Altamirano M, Brown D, Woolfson A, Klenerman P, Chen JL, Koezuka Y, Roberts IAG, Price DA, Dusheiko G, Milstein C, Fersht A, Luzzatto L, Cerundolo V. Human CD1d-glycolipid tetramers generated by *in vitro* oxidative refolding chromatography. *Proc. Natl Acad. Sci. USA* 2001; **98**: 3294–3298.
220. Woycechowsky KJ, Hook BA, Raines RT. Catalysis of protein folding by an immobilized small-molecule dithiol. *Biotechnol. Prog.* 2003; **19**: 1307–1314.
221. Annis I, Chen L, Barany G. Novel solid-phase reagents for facile formation of intramolecular disulfide bridges in peptides under mild conditions. *J. Am. Chem. Soc.* 1998; **120**: 7226–7238.
222. Woycechowsky KJ, Wittrup KD, Raines RT. A small-molecule catalyst of protein folding *in vitro* and *in vivo*. *Chem. Biol.* 1999; **6**: 871–879.
223. 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**: 613–619.
224. Gilbert HF. Protein disulfide isomerase and assisted protein folding. *J. Biol. Chem.* 1997; **272**: 29 399–29 402.
225. Bergmann M, Brand ME, Weinmann F. Umlagerungen peptidähnlicher Stoffe. *Hoppe Seyler's Z. Physiol. Chem.* 1923; **131**: 1–17.
226. Sakakibara S, Shin KH, Hess GP. An approach to specific cleavage of peptide bonds. 1. Acyl migration in dipeptides containing hydroxyamino acids in anhydrous hydrogen fluoride. *J. Am. Chem. Soc.* 1962; **84**: 4921–4928.
227. Reissmann S, Steinmetzer T, Greiner G, Seyfart L, Besser D, Schumann C. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22a), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 347–378.
228. Merrifield RB, Vizioli LD, Boman HG. Synthesis of the antibacterial peptide cecropin-A(1–33). *Biochemistry* 1982; **21**: 5020–5031.
229. Fujino M, Wakimasu M, Shinagawa S, Kitada C, Yajima H. Synthesis of nonacosapeptide corresponding to mammalian glucagon. *Chem. Pharm. Bull.* 1978; **26**: 539–548.
230. Kent SB, Michell AR, Engelhard M, Merrifield RB. Mechanisms and prevention of trifluoroacetylation in solid-phase peptide synthesis. *Proc. Natl Acad. Sci. USA* 1979; **76**: 2180–2184.
231. Quibell M, Turnell WG, Johnson T. Synthesis of azapeptides by the Fmoc *tert*-butyl polyamide technique. *J. Chem. Soc. Perkin Trans. I* 1993; 2843–2849.
232. Hübener G, Göhring W, Musiol HJ, Moroder L. N^{α}-Trifluoroacetylation of *N*-terminal hydroxyamino acids: a new side reaction in peptide synthesis. *Pept. Res.* 1992; **5**: 287–292.
233. Kemp DS. The amine capture strategy for peptide-bond formation — An outline of progress. *Biopolymers* 1981; **20**: 1793–1804.
234. Johnson T, Quibell M, Sheppard RC. *N*,*O*-bisFmoc derivatives of *N*-(2-hydroxy-4-methoxybenzyl)-amino acids: useful intermediates in peptide synthesis. *J. Pept. Sci.* 1995; **1**: 11–25.
235. Thamm P, Kolbeck W, Musiol HJ, Moroder L. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22a), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 260–275.
236. Hurley TR, Colson CE, Hicks G, Ryan MJ. Orally active water-soluble *N*,*O*-acyl transfer products of a β,γ-bishydroxyl amide containing renin inhibitor. *J. Med. Chem.* 1993; **36**: 1496–1498.
237. Hamada Y, Matsumoto H, Yamaguchi T, Kimura T, Hayashi Y, Kiso Y. Water-soluble prodrugs of dipeptide HIV protease inhibitors based on $O \rightarrow N$ intramolecular acyl migration: design, synthesis and kinetic study. *Bioorg. Med. Chem.* 2004; **12**: 159–170.
238. Horikawa M, Shigeri Y, Yumoto N, Yoshikawa S, Nakajima T, Ohfune Y. Synthesis of potent Leu-enkephalin analogues possessing β-hydroxy-α,α-disubstituted α-amino acid and their characterization to opioid receptors. *Bioorg. Med. Chem. Lett.* 1998; **8**: 2027–2032.

239. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Novel and efficient synthesis of difficult sequence-containing peptides through $O \rightarrow N$ intramolecular acyl migration reaction of *O*-acyl isopeptides. *Chem. Commun.* 2004; 124–125.
240. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Design and synthesis of a novel water-soluble Aβ1–42 isopeptide: an efficient strategy for the preparation of Alzheimer's disease-related peptide, Aβ1–42, via *O–N* intramolecular acyl migration reaction. *Tetrahedron Lett.* 2004; **45**: 5965–5968.
241. Carpino LA, Krause E, Sferdan CD, Schümann M, Fabian H, Bienert M, Beyermann M. Synthesis of 'difficult' peptide sequences: application of a depsipeptide technique to the Jung-Redemann 10- and 26-mers and the amyloid peptide Aβ(1–42). *Tetrahedron Lett.* 2004; **45**: 7519–7523.
242. Mutter M. Chandravarkar A, Boyat C, Lopez J, Dos Santos S, Mandal B, Mimna R, Murat K, Patiny L, Saucede L, Tuchscherer G. Switch peptides in statu nascendi: induction of conformational transitions relevant to degenerative diseases. *Angew. Chem. Int. Ed.* 2004; **43**: 4172–4178.
243. Kryukov GV, Castellano S, Novoselov SV, Lobanov V, Zehtab O, Guigó R, Gladyshev VN. Characterization of mammalian selenoproteomes. *Science* 2003; **300**: 1439–1443.
244. Köhrle J, Brigelius-Flohé R, Böck A, Gartner R, Meyer O, Flohé L. Selenium in biology: facts and medical perspectives. *Biol. Chem.* 2000; **381**: 849–864.
245. Hatfield DL, Gladyshev VN. How selenium has altered our understanding of the genetic code. *Mol. Cell. Biol.* 2002; **22**: 3565–3576.
246. Huber RE, Criddle RS. Comparison of chemical properties of selenocysteine and selenocystine with their sulfur analogues. *Arch. Biochem. Biophys.* 1967; **122**: 164–173.
247. Arnold AP, Tan KS, Rabenstein DL. Nuclear-magnetic-resonance studies of the solution chemistry of metal complexes. 23. Complexation of methylmercury by selenohydryl-containing amino-acids and related molecules. *Inorg. Chem.* 1986; **25**: 2433–2437.
248. Köhrle J. Local activation and inactivation of thyroid hormones: the deiodinase family. *Mol. Cell. Endocrinol.* 1999; **151**: 103–119.
249. Szajewski RP, Whitesides GM. Rate constants and equilibrium constants for thiol-disulfide interchange reactions involving oxidized glutathione. *J. Am. Chem. Soc.* 1980; **102**: 2011–2026.
250. Singh R, Whitesides GM. Selenols catalyze the interchange reactions of dithiols and disulfides in water. *J. Org. Chem.* 1991; **56**: 6931–6933.
251. Besse D, Moroder L. Synthesis of selenocysteine-peptides and their oxidation to diselenide-bridged compounds. *J. Pept. Sci.* 1997; **3**: 442–453.
252. 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**: 883–885.
253. Tanaka H, Soda K. Selenocysteine. *Methods Enzymol.* 1987; **143**: 240–243.
254. Stocking EM, Schwarz JN, Senn H, Salzmann M, Silks LA. Synthesis of L-selenocystine, L-[^{77}Se-]selenocystine and L-tellurocystine. *J. Chem. Soc. Perkin Trans. I.* 1997; 2443–2447.
255. Gieselman MD, Xie L, van der Donk WA. Synthesis of a selenocysteine-containing peptide by native chemical ligation. *Org. Lett.* 2001; **3**: 1331–1334.
256. Tamura T, Oikawa T, Ohtaka A, Fujii N, Esaki N, Soda K. Synthesis and characterization of the selenium analogue of glutathione disulfide. *Anal. Biochem.* 1993; **208**: 151–154.
257. Koide T, Itoh H, Otaka A, Yasui H, Kuroda M, Esaki N, Soda K, Fujii N. Synthetic study on selenocystine-containing peptides. *Chem. Pharm. Bull.* 1993; **41**: 502–506.
258. Oikawa T, Esaki N, Tanaka H, Soda K. Metalloselenonein, the selenium analogue of metallothionein: synthesis and characterization of its complex with copper ions. *Proc. Natl Acad. Sci. USA* 1991; **88**: 3057–3059.
259. Chocat P, Esaki N, Tanaka H, Soda K. Synthesis of L-selenodjenkolate and its degradation with methionine γ-lyase. *Anal. Biochem.* 1985; **148**: 485–489.
260. Pegoraro S, Moroder L. *Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics* (E 22b), Goodman M, Felix A, Moroder L, Toniolo C (eds). Georg Thieme Verlag: Stuttgart, 2002; 214–223.
261. Rajarathnam K, Sykes BD, Dewald B, Baggiolini M, Clark-Lewis I. Disulfide bridges in interleukin-8 probed using non-natural disulfide analogues: dissociation of roles in structure from function. *Biochemistry* 1999; **38**: 7653–7658.
262. Koide T, Itoh H, Otaka A, Furuya M, Kitajima Y, Fujii N. Synthesis and biological activities of selenium analogues of α-rat atrial-natriuretic peptide. *Chem. Pharm. Bull.* 1993; **41**: 1596–1600.
263. Ralle M, Berry SM, Nilges MJ, Gieselman MD, van der Donk WA, Lu Y, Blackburn NJ. The selenocysteine-substituted blue copper center: spectroscopic investigations of Cys112SeCys *Pseudomonas aeruginosa* azurin. *J. Am. Chem. Soc.* 2004; **126**: 7244–7256.
264. Moroder L, Marchiori F, Borin G, Scoffone E. Studies on cytochrome c. Part II. Synthesis of the protected heptapeptide (sequence 17–23) of bakers yeast iso-I-cytochrome c. *Biopolymers* 1973; **12**: 493–505.
265. Arnér ESJ, Sarioglu H, Lottspeich F, Holmgren A, Böck A. High-level expression in *Escherichia coli* of selenocysteine-containing rat thioredoxin reductase utilizing gene fusions with engineered bacterial-type SECIS elements and co-expression with the selA, selB and selC genes. *J. Mol. Biol.* 1999; **292**: 1003–1016.
266. Wu ZP, Hilvert D. Conversion of a protease into an acyl transferase — selenosubtilisin. *J. Am. Chem. Soc.* 1989; **111**: 4513–4514.
267. Chu Sh, Mautner HG. Analogues of neuroeffectors. V. Neighboring-group effects in reactions of esters, thiolesters and selenolesters. Hydrolysis and aminolysis of benzoylcholine, benzoylthiolcholine, benzoylselenolcholine and of their dimethylamino analogues. *J. Org. Chem.* 1966; **31**: 308–312.
268. Mautner HG, Chu SH, Günther WHH. Aminolysis of thioacyl and selenoacyl analogues. *J. Am. Chem. Soc.* 1963; **85**: 3458–3462.
269. Müller S, Senn H, Gsell B, Vetter W, Baron C, Böck A. The formation of diselenide bridges in proteins by incorporation of selenocysteine residues: biosynthesis and characterization of $(Se)_2$-thioredoxin. *Biochemistry* 1994; **33**: 3404–3412.
270. Boschi-Müller S, Müller S, van Dorsselaer A, Böck A, Branlant G. Substituting selenocysteine for active site cysteine 149 of phosphorylating glyceraldehyde 3-phosphate dehydrogenase reveals a peroxidase activity. *FEBS Lett.* 1998; **439**: 241–245.
271. Hondal RJ, Nilsson BL, Raines RT. Selenocysteine in native chemical ligation and expressed protein ligation. *J. Am. Chem. Soc.* 2001; **123**: 5140–5141.
272. Quaderer R, Sewing A, Hilvert D. Selenocysteine-mediated native chemical ligation. *Helv. Chim. Acta* 2001; **84**: 1197–1206.
273. Berry SM, Gieselman MD, Nilges MJ, van der Donk WA, Lu Y. An engineered azurin variant containing a selenocysteine copper ligand. *J. Am. Chem. Soc.* 2002; **124**: 2084–2085.
274. Gieselman MD, Zhu Y, Zhou H, Galonic D, vander Donk WA. Selenocysteine derivatives for chemoselective ligations. *ChemBioChem* 2002; **3**: 709–716.
275. Harpp DN, Gleason JG. Organic sulfur chemistry. 10. Selective desulfurization of disulfides — scope and mechanism. *J. Am. Chem. Soc.* 1971; **93**: 2437–2445.
276. Fukase K, Kitazawa M, Sano A, Shimbo K, Horimoto S, Fujita H, Kubo A, Wakamiya T, Shiba T. Synthetic study on peptide antibiotic nisin. 5. Total synthesis of nisin. *Bull. Chem. Soc. Jpn* 1992; **65**: 2227–2240.
277. 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 [Sec^3,Sec^{11},Nle^7]-endothelin-1. *J. Mol. Biol.* 1998; **284**: 779–792.

278. 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* 2000; **53**: 550–564.
279. Müller JCD, Graf von Roedern E, Grams F, Nagase H, Moroder L. Non-peptidic cysteine derivatives as inhibitors of matrix metalloproteinases. *Biol. Chem.* 1997; **378**: 1475–1480.
280. Pegoraro S, Fiori S, Cramer J, Rudolph-Böhner S, Moroder L. The disulfide-coupled folding pathway of apamin as derived from diselenide-quenched analogues and intermediates. *Prot. Sci.* 1999; **8**: 1605–1613.
281. Bae JH, Alefelder S, Kaiser JT, Friedrich R, Moroder L, Huber R, Budisa N. Incorporation of β-selenolo[3,2-b]pyrrolyl-alanine into proteins for phase determination in protein x-ray crystallography. *J. Mol. Biol.* 2001; **309**: 925–936.
282. Boles JO, Henderson J, Hatch D, Silks LAP. Synthesis and incorporation of [6,7]-selenatryptophan into dihydrofolate reductase. *Biochem. Biophys. Res. Commun.* 2002; **298**: 257–261.
283. Sanchez JF, Hoh F, Strub MP, Aumelas A, Dumas C. Structure of the cathelicidin motif of protegrin-3 precursor: structural insights into the activation mechanism of an antimicrobial protein. *Structure* 2002; **10**: 1363–1370.
284. Strub MP, Hoh F, Sanchez JF, Strub JM, Böck A, Aumelas A, Dumas C. Selenomethionine and selenocysteine double labeling strategy for crystallographic phasing. *Structure* 2003; **11**: 1359–1367.
285. Besse D. Synthese and Redoxeigenschaften von Selenocysteinpeptiden. Dissertation, Technical University of Munich, Munich 1997.
286. Chen Y, Barkley MD. Toward understanding tryptophan fluorescene in proteins. *Biochemistry* 1998; **37**: 9976–9982.
287. Neves-Petersen MT, Gryczynski Z, Lakowicz J, Fojan P, Pedersen S, Petersen E, Petersen SB. High probability of disrupting a disulfide bridge mediated by an endogenous excited tryptophan residue. *Protein Sci.* 2002; **11**: 588–600.
288. Lapidus LJ, Steinbach PJ, Eaton WA, Szabo A, Hofrichter J. Effects of chain stiffness on the dynamics of loop formation in polypeptides. Appendix: Testing a 1-dimensional diffusion model of peptide dynamics. *J. Phys. Chem. B* 2002; **106**: 11628–11640.
289. Weljie AM, Vogel HJ. Tryptophan fluorescence of calmodulin binding domain peptides interacting with calmodulin containing unnatural methionine analogues. *Protein Engineer.* 2000; **13**: 59–66.
290. Garberg P, Engman L, Tolmachev V, Lundqvist H, Gerdes RG, Cotgreave IA. Binding of tellurium to hepatocellular selenoproteins during incubation with inorganic tellurite: consequences for the activity of selenium-dependent glutathione peroxidase. *Int. J. Biochem. Cell Biol.* 1999; **31**: 291–301.
291. Clive DLJ, Anderson PC, Moss N, Singh A. New method for coupling allylic halides: use of Te^{2-} species. *J. Am. Chem. Soc.* 1982; **47**: 1641–1647.
292. Esaki N, Soda K. *Te*-phenyltellurohomocysteine and *Te*-phenyltellutrocysteine. *Methods Enzymol.* 1987; **143**: 245.
293. Rooseboom M, Vermeulen NPE, Durgut F, Commandeur JNM. Comparative study on the bioactivation mechanisms and cytotoxicity of *Te*-phenyl-L-tellurocysteine, *Se*-phenyl-L-selenocysteine and S-phenyl-L-cysteine. *Chem. Res. Toxicol.* 2002; **15**: 1610–1618.
294. Müller S. *Design neuer Selenoproteine*. Dissertation, Technical University of Munich, Munich, 1997.
295. Srinivasan N, Sowdhamini R, Ramakrishnan C, Balaram P. Conformation of disulfide bridges in proteins. *Int. J. Pept. Protein Res.* 1991; **36**: 147–155.
296. Zahn H, Otten HG. Über unsymmetrische, offenkettige Cystinpeptide. *Liebigs Ann. Chem.* 1992; **653**: 139–148.

