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REVIEW

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Amino acid chalcogen analogues as tools in peptide and protein research[¥]

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The chalcogen elements oxygen, sulfur, and selenium are essential constituents of side chain functions of natural amino acids. Conversely, no structural and biological function has been discovered so far for the heavier and more metallic tellurium element. In the methionine series, only the sulfur-containing methionine is a proteinogenic amino acid, while selenomethionine and telluromethionine are natural amino acids that are incorporated into proteins most probably because of the tolerance of the methionyl-*t*RNA synthetase; so far, methoxinine the oxygen analogue has not been discovered in natural compounds. Similarly, the chalcogen analogues of tryptophan and phenylalanine in which the benzene ring has been replaced by the largely isosteric thiophene, selenophene, and more recently, even tellurophene are fully synthetic mimics that are incorporated with more or less efficiency into proteins via the related tryptophanyl- and phenylalanyl-*t*RNA synthetases, respectively. In the serine/cysteine series, also selenocysteine is a proteinogenic amino acid that is inserted into proteins by a special translation mechanism, while the tellurocysteine is again most probably incorporated into proteins by the tolerance of the cysteinyl-*t*RNA synthetase. For research purposes, all of these natural and synthetic chalcogen amino acids have been extensively applied in peptide and protein research to exploit their different physicochemical properties for modulating structural and functional properties in synthetic peptides and *r*DNA expressed proteins as discussed in the following review.

KEYWORDS

methoxinine, peptides, proteins, redox potentials, selenocysteine and tellurocysteine, selenomethionine and telluromethionine, β-selenienylalanine and β-tellurienylalanine, β-thienylalanine, β-(thienopyrrolyl)- and β-(selenolopyrrolyl)-alanine

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1 | INTRODUCTION

In early days of X-ray crystallography of proteins, one of the main difficulties was the trial and error soaking procedure for the production of heavy atom derivatives. It was with the fast and efficient advances in *r*DNA technology that using methionine-auxotrophic *Escherichia coli* strains quantitative biosynthetic replacement of methionine by selenomethionine was used by Hendrickson et al[1] to generate atomic mutants of proteins as a new approach to solve the phase problem in protein crystallography with the multiwavelength anomalous dispersion method.[2] This new procedure led to increasing interest in chalcogen analogues of amino acids in the methionine and serine/cysteine series as reviewed in previous literature.[3,4] These analogues are shown in Figure 1 and differ in their physicochemical properties such as electronegativity, atom volume, and carbon-metal bond length depending upon the chalcogen atoms in their side chains.

Additional fully synthetic chalcogen amino acids have been synthesized as isosteric tryptophan (**1**-**4**)[5,6] and phenylalanine (**5**-**8**)[7-9] analogues, respectively, and were used for in vivo protein synthesis of chalcogen analogues of proteins for X-ray analysis and other purposes (Figure 2).

2 | CHALCOGEN ANALOGUES OF METHIONINE

In the methionine series, only methionine is a proteinogenic amino acid, whereas selenomethionine and telluromethionine are natural amino acids that are inserted into natural proteins because of the tolerance of the methionyl-*t*RNA synthetase.[10-12]

2.1 | Selenomethionine and telluromethionine protein variants for X-ray crystallography

Intensive optimization of the expression conditions in *Escherichia coli* was required for quantitative replacement of methionine in proteins with the related chalcogen analogues to produce the highly isomorphous protein variants. Moreover, it was noticed that the proteins have to be biosynthesized in the folded form since oxidation of the selenium and even more of the tellurium occurs in the unfolded states as exists in inclusion bodies.[3,13-16] The chalcogen protein analogues are isomorphous and can be considered as atomic mutants of the native methionine proteins. Therefore, no significant changes in the overall shape, steric complementarity, and occupation volume are gen-

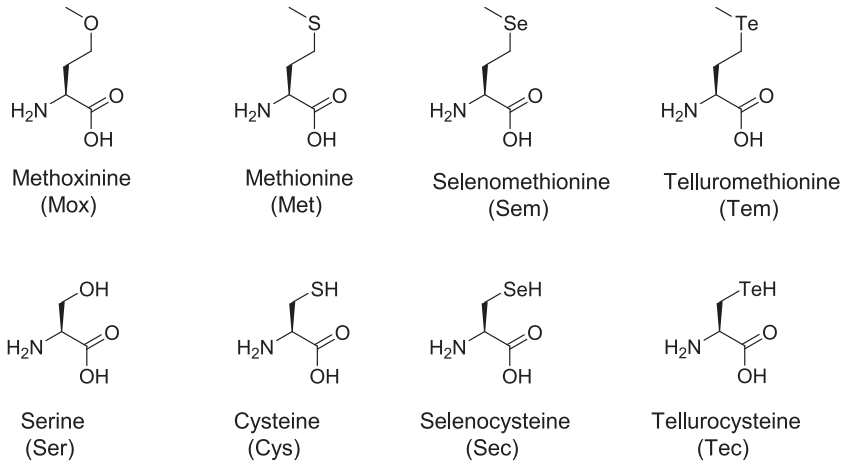

FIGURE 1 Chalcogen amino acids discovered in natural compounds except methoxinine

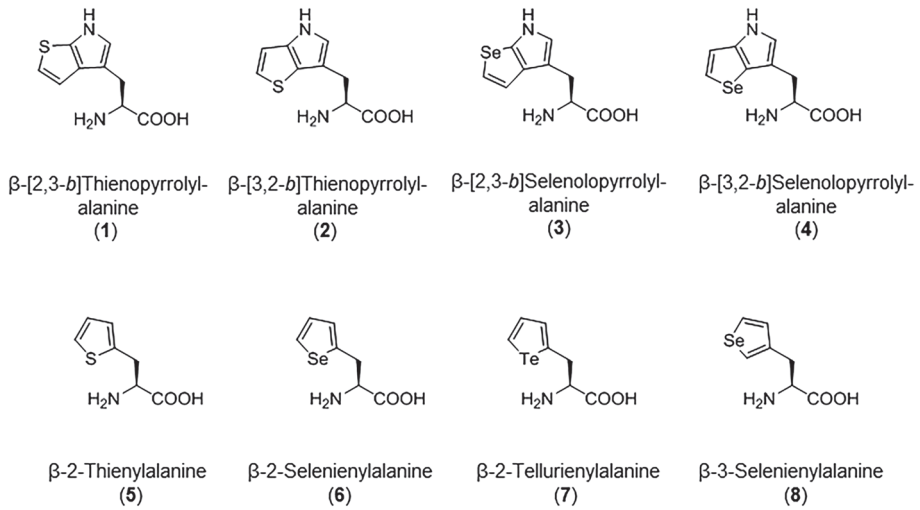

FIGURE 2 Isosteric synthetic chalcogen analogues of tryptophan and phenylalanine

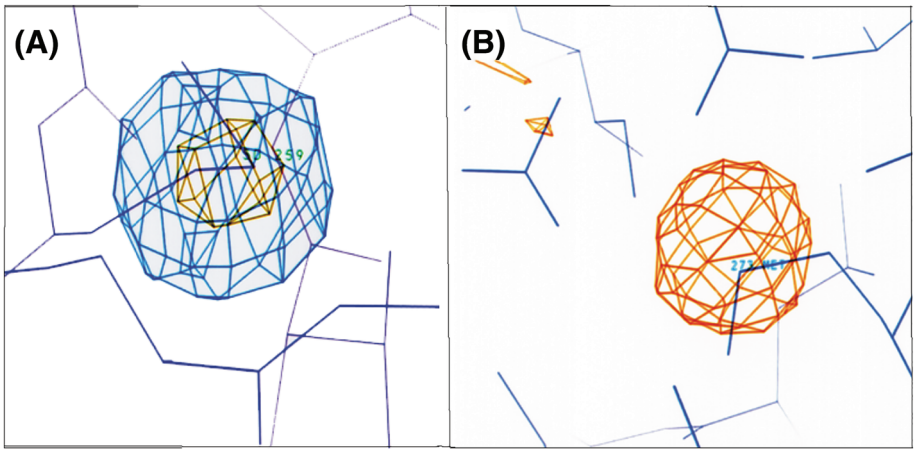

FIGURE 3 Crystallographic mapping of selenomethionine and telluromethionine residues in the spatial structure of recombinant human annexin V by the difference electron density maps (Fo-Fc) for (A) Met259 → Sem259 (contouring level 3.0 σ) and for (B) Met273 → Tem273 (contouring level 5.0 σ) replacement[16]

erated. Minor steric effects cannot be excluded as shown by the electron difference maps (Figure 3), but the protein folds show generally sufficient plasticity to accommodate such minor differences even in the case of tellurium. On the other hand, the telluro-analogues provide directly heavy atom derivatives for the classical multiple isomorphous replacement method, but the production is more difficult. Conversely, the seleno-mutants in most cases are not suitable for this method and are more appropriate for multiwavelength anomalous dispersion experiments that require, however, synchrotron radiation with precise beam wavelength control.

2.2 | Methoxinine as methionine-oxide mimic

This additional chalcogen analogue of methionine with sulfur replaced by oxygen differs from methionine particularly because of its strong hydrophilicity compared with methionine, selenomethionine, and telluromethionine. With this hydrophilic property, it resembles the methionine-oxide. Because of this particular property, it was reasonable to expect that a related prion protein (*hr*PrP-[125-231]) Mox-analogue could be useful for analysis of the proposed but still not clearly documented hypothetical role of Met oxidation as the main cause of the prion protein conversion to its scrapie form.[17,18] Indeed particularly Met-205 and Met-206 oxidation was suggested to be responsible for the conformational transition of the α-helix into β-sheet and thus for the aggregation and the neurotoxic amyloid formation (see Figure 4).[20] The in vitro aggregation propensity of the Mox-*hr*PrP^{c}-[125-231] analogue was found to be significantly increased compared with that of the native Met-protein (Figure 5).[19] Even the content of α-helix as derived from the circular dichroism (CD) spectrum was visibly decreased with concomitant shift to β-sheet. With the large number of methionine residues in the PrP-[125-231] protein, it is difficult to confirm that the oxidation of a few selected methionine residues initiates the conformational transition. However, this is not only supported by the results obtained with the Mox-*rh*PrP^{c}-[125-231] analogue but also by experimental results obtained with model peptide of Dado and Gellman[22] where a

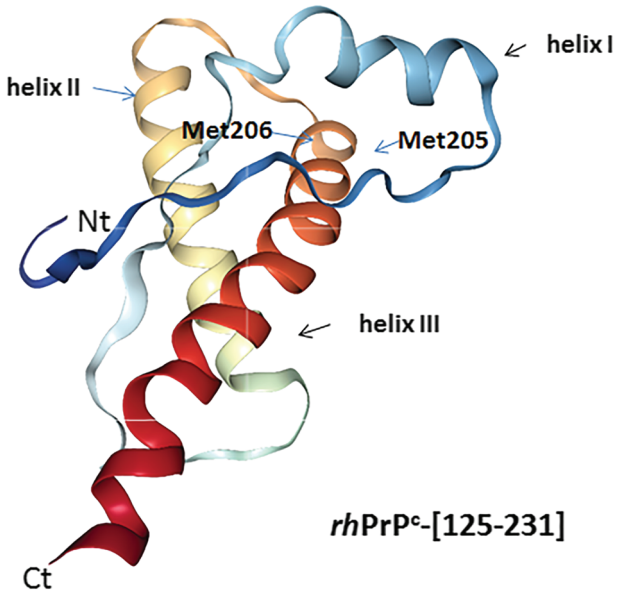

FIGURE 4 Spatial structure of *rh*PrP^{c}-[125-231] (PDB accession no. 1QM0). Except for Met-205/Met-206, all other methionine residues of this protein are surface exposed. In the bioexpressed Mox-*hr*PrP^{c}-[125-231] protein, all nine Met residues were replaced by methoxinine[19]

significant conformational shift from α-helix to β-sheet could be observed by replacing the hydrophobic norleucine residues as methionine analogues with the hydrophilic Mox residues.[19]

3 | SULFUR- AND SELENIUM-CONTAINING TRYPTOPHAN ANALOGUES

The β-(thienopyrrolyl)- and β-(selenolopyrrolyl)-L-alanine (compounds **1**-**4** in Figure 2) are synthetic mutually isosteric amino acids that mimic tryptophan with the benzene ring in the indole moiety replaced by thiophene and selenophene, respectively. These tryptophan mimics are prepared enzymatically from the [3,2-*b*]- and [2,3-*b*]-fused

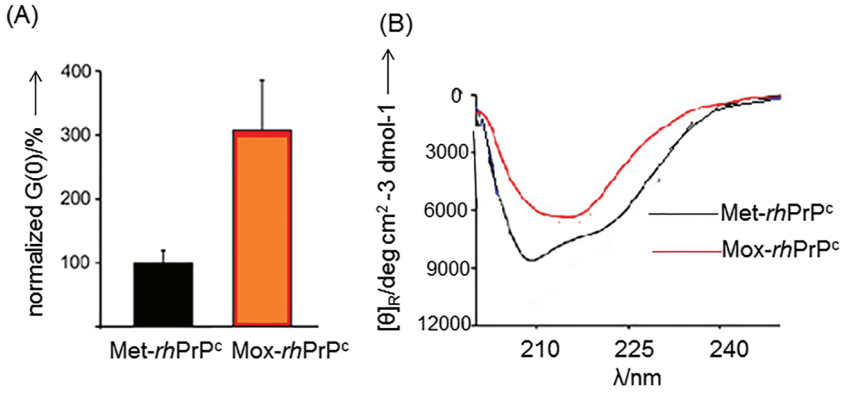

FIGURE 5 Comparative in vitro aggregation properties of Met-*hr*PrP^c-[125-231] and Mox-*hr*PrP^c-[125-231] (A) as determined by confocal single molecule analysis with fluorescence cross-correlation spectroscopy[21]; CD spectra of Met-*hr*PrP^c-[125-231] and Mox-*hr*PrP^c-[125-231] (B) recorded in 10mM 2-(*N*-morpholino) ethanesulfonic acid buffer at pH 6.0 and in the presence of 0.2% SDS to prevent aggregation

selenolopyrroles and thienopyrroles[23] with the *Salmonella typhimurium* tryptophan synthase[5,6] as shown in Figure 6.

Tryptophan represents a suitable target for its replacement in proteins with synthetic isosteric analogues of near-equal volumes and approximately identical electron densities. Moreover, Trp is known to occur rather rarely in proteins as it represents only about 1% of all residues of globular proteins.[24] Correspondingly, chalcogen-containing Trp analogues provide quasi-site–specific probes for studying protein structure, dynamics, and function via their incorporation into recombinant proteins in response to the Trp UGG codons by fermentation in Trp-auxotrophic *E coli* host strains and applying the selective pressure incorporation (SPI) method.[25-27] Under routine bioexpression protocols, an almost quantitative incorporation of both β-(thienopyrrolyl)alanines in annexin V that contains only one Trp residue and in the pseudo-wild–type barstar mutant C40A/C82A/P27A/W38F (b*) with two Trp residues was achieved.[28] The barstar mutant is obtained in inclusion bodies and is refolded into the native form during the purification procedure. Both the annexin V and barstar Trp analogues proved to be stable, and despite exposure to air and light for days, a degradation of the thienopyrrolyl moieties was not observed. Both proteins were found to exhibit reduced thermal stability but full retention of the biological activities. Although these protein mutants retain the secondary structure of the native proteins, they were found to differ significantly in optical and thermodynamic properties.

In view of these positive results, the β-selenolo[3,2-*b*]pyrrolyl-L-alanine was incorporated into human annexin V and barstar mutant b* C40A/C82A/P27A as model proteins in Trp-auxotrophic *E coli* using the SPI method. The seleno-proteins were obtained in yields comparable with those of the wild-type proteins and the β-(thienopyrrolyl)-L-alanine mutants.[29] Both selenium-containing mutants were found to exhibit crystallographic isomorphism with the parent proteins. Although the selenium-containing Trp analogue replaces in annexin V the fully exposed Trp-187 and in the barstar mutant the two Trp residues 44 and 38 partially or fully exposed to solvent, their exceptional stability can be regarded as a considerable advantage over selenomethionine in replacement experiments for crystallographic phasing. The most probable explanation for this observed high stability is a resonance stabilization of the Se atoms in aromatic rings where the chalcogen atom is less prone to oxidation than in dialkylselenides, such as selenomethionine. Moreover, in cases where Sem is less suitable for diffraction analysis because of the atomic mobility factors, the use of the selenotryptophan analogue could be an alternative.

4 | SULFUR-, SELENIUM-, AND TELLURIUM-CONTAINING PHENYLALANINE ANALOGUES

On the basis of early reports of du Vigneaud et al[30] on the incorporation of β-2-thienylalanine (**5** in Figure 2) into proteins, already in the early 1960s, it could be demonstrated that this phenylalanine analogue is inserted into the enzyme β-galactosidase in a Phe-requiring

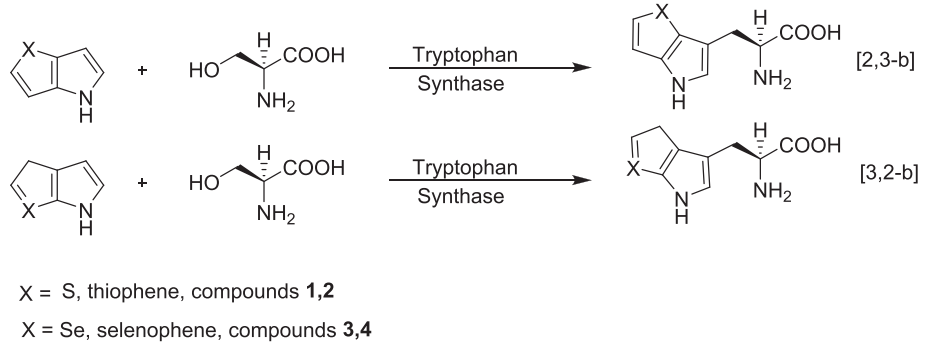

FIGURE 6 Enzymatic synthesis of β-(thienopyrrolyl)- (**1**,**2**) and β-(selenolopyrrolyl)-L-alanine (**3**,**4**)[5,6]

auxotroph of *E coli*.[31] This result confirms the largely isosteric character of the thiophene ring with the benzene ring although of less aromatic character, having a resonance energy of 25 kcal/mol as compared with 36 kcal/mol for benzene. The catalytic properties of the enzyme with about 95% of the phenylalanine residues replaced by the analogue were retained; however, the enzyme was found to be significantly less stable to heat, urea, and enzymatic degradation. Later in the 1970s, the β-2-thienylalanine has also been incorporated synthetically in various peptide hormones such as vasopressin and bradykinin leading to full retention or even increased bioactivities.[32,33]

Conversely, the β-2- and β-3-selenienyl-L-alanines (**6** and **8** in Figure 2) have been synthesized as potential diagnostic agents for disorders in the pancreas[8] but so far did not find any application in replacement experiments for crystallographic phasing. However, very recently, a detailed study on the stability of various organotellurium compounds revealed for 2-alkyl-tellurophenes high stability to aerobic oxidation in both organic and aqueous solutions and a limited toxicity in cell-based assays ($IC_{50} \geq 200\mu M$)[34] making such tellurophenes ideal probes for mass cytometry. Correspondingly, β-2-tellurienyl-L-alanine was synthesized knowing that the related sulfur- and selenium-containing phenylalanine mimics are efficiently incorporated into proteins because of the tolerance of the phenylalanine-*t*RNA synthetase.[9]

The telluro-phenylalanine analogue was found to behave as an excellent phenylalanine isostere since it is incorporated into proteins via the native translation machinery without phenylalanine starvation in vitro and in vivo allowing its measurements by mass cytometry techniques. It looks also as a promising probe in X-ray crystallography as the electron density of tellurium is known to generate isomorphous and anomalous difference Patterson maps. In this context, its properties should be superior to those of telluromethionine that suffers from a more facile oxidation when exposed on the protein surface (see Section 2.1) but with the drawback of a generally higher number of Phe residues in proteins compared with Met.

5 | SERINE AND CYSTEINE CHALCOGEN ANALOGUES

In the serine/cysteine series, also selenocysteine is a proteinogenic amino acid that is inserted into proteins by a special translation mechanism,[35,36] while the tellurocysteine is again only tolerated by the cysteinyl-*t*RNA synthetase.[11]

In this series of chalcogen amino acids, the related hydroxyl, thiol, and selenol side chain groups of serine (pK_a 13), cysteine (pK_a 8.25), and selenocysteine (pK_a 5.24-5.63) differ significantly in the nucleophilicity leading to different structural and functional properties.[37-39] While the cysteine residues form the known important structural disulfide crosslinks for stabilization of tertiary structures in peptides and proteins,[40,41] the peroxides of serine would be biologically too dangerous because of their oxidation power. Although serine and cysteine differ considerably in the nucleophilicity, both residues play a central role in proteases. In the catalytic triad of serine proteases, an aspartyl residue is required to increase via hydrogen bonding the nucleophilicity of the serine hydroxyl group, while such a helping residue is not required in the cysteine proteases.

Besides the established role of disulfides crosslinks in cysteine-rich peptides and proteins for stabilizing related tertiary structures, vicinal disulfides in specific sequence motifs such as the Cys-Xaa-Yaa-Cys act as highly efficient active sites of thiol-protein oxidoreductases.[42,43] This active-site motif of thioredoxins and glutaredoxins is involved in the reduction of intermolecular and intramolecular disulfide bonds and other forms of oxidized cysteines, and in protein disulfide isomerases, this bis-cysteinyl motif catalyzes the oxidative folding of secretory proteins into their native structures.[44] This has, therefore, been called a "rheostat at the active center." Indeed, changes of the residues that separate the two cysteines affect the redox potentials, thus structuring the proteins for a particular redox function.[45,46] Besides the effect of the dipeptide intervening sequence,[47] which is also well supported by the redox potentials of synthetic fragments related to their active sites[48] (Table 1), the redox properties of these active sites are strongly governed by the conformational restrictions imparted by the overall almost identical thioredoxin-like structure of the oxidoreductases.[42,47] These structural effects were further evidenced by the redox potentials of conformationally restricted cyclic active-site (bis-cysteinyl)hexapeptides related to oxidoreductases,[56] when compared with those of the linear unconstrained peptides (Table 1).

5.1 | Selenocysteine as isosteric replacement of cysteine

Selenium and sulfur possess very similar atomic sizes, bond lengths to carbon, homodimeric bond lengths, and electronegativity. Correspondingly, selenocysteine represents an isosteric analogue of cysteine.[3,4,57,58] This was already demonstrated with early synthetic selenocysteine/selenocystine analogues of bioactive peptides such as oxytocin, somatostatin, and rat α-natriuretic peptide, which retained the bioactivities of the parent Cys-peptides.[58] The isosteric character was then fully confirmed by comparing the NMR structures of [$Sec^{3,11}$,Nle^{7}]-endothelin,[59] [Sec^{7}]- and [Sec^{34}]-IL-8,[60] [$Sec^{1,11}$]-apamin,[61] and Sec-analogues of the α-conotoxins ImI[62] and AuIB[63] with those of the parent Cys-peptides but even more accurately by the superposition of the X-ray structures of α-PnIA and α-[$Sec^{3,16}$,Leu^{10}]-PnIA[63] and X-ray diffraction analysis of selenocysteine-mutant proteins.[64,65]

5.2 | Synthesis of selenocysteine/selenocystine peptides and proteins

The most efficient strategies for the synthesis of selenocysteine/selenocystine peptides have been comprehensively reviewed.[4,57,66] From the large experimental work performed so far, mainly two synthetic strategies have emerged on the basis of the Fmoc/tBu and Boc/Bzl protection schemes, respectively.

TABLE 1 Apparent redox potentials (E'_0, mV) of thiol/disulfide oxidoreductases, their linear active-site fragments, and related cyclic hexapeptides as extracted by the Nernst equation from the equilibrium constants of exchange reactions with the reference redox systems

Enzyme/Active-Site Fragment		E'_0	Ref
Thioredoxin	(Trx)	−270	[49]
Ac-Trp-Cys-Gly-Pro-Cys-Lys-His-Ile-NH_2	[His^{37}]-trx-$^{32-38}$	−190	48[a]
c[Trp-Cys-Gly-Pro-Cys-Lys]	*c*[Trx-31-36]	−152	49[b]
Glutaredoxin-1	(Grx1)	−233	[50]
Ac-Gly-Cys-Pro-Tyr-Cys-Val-Arg-Ala-NH_2	Grx1-$^{10-17}$	−215	48[a]
c[Gly-Cys-Pro-Tyr-Cys-Val]	*c*[Grx1-10-15]	−178	49[b]
Protein disulfide isomerase	(PDI)	−110/145	[51/52]
Ac-Trp-Cys-Gly-His-Cys-Lys-Ala-Leu-NH_2	PDI-$^{35-45}$	−205	48[a]
c[Trp-Cys-Gly-His-Cys-Lys]>	*c*[PDI-35-40]	−130	49[b]
Thioredoxin reductase	(Trr)	−254/271	[53]
Ac-Ala-Cys-Ala-Thr-Cys-Asp-Gly-Phe-NH_2	Trr-[134-141]	−210	48[a]
c[Ala-Cys-Ala-Thr-Cys-Asp]	*c*[Trr-134-139]	−204	49[b]

[a]GSH/GSSG (E'_0 = −240).[54]

[b]Cysteine/cystine (E'_0 = −223).[55]

For the Fmoc strategy, the selenol function is protected by the methoxybenzyl (Mob) group that is removed by treatment with TFA containing 10% DMSO yielding directly the oxidized selenocystine peptides.[67,68] Alternatively, the Sec (Mob) is deprotected with TFA containing thioanisole and 2,2′-dithiobis(5-nitropyridine) (DTNP) leading to the adduct of Sec with thio-5-nitropyridine. The Sec(5-Npys) adduct can be cleaved by thiolysis,[69] but it can also be reduced with ascorbate.[70] An even more gentle and facile method for deprotection of Sec (Mob) was recently proposed and is based on the reductive cleavage of the protecting group with a combination of TFA/TIS/thioanisole (96:2:2) that provides complete removal yielding the peptide mainly in the diselenide form.[71]

With the Boc strategy where the selenol function is protected by the methylbenzyl group, the facile deselenization of the Sec (Mob) in the iterative piperidine-mediated Fmoc-deprotection steps is avoided.[68] Moreover, the facile racemization of Sec residues can readily be suppressed by the in situ neutralization protocol of solid-phase peptide synthesis.[66] The final HF deprotection step at 0°C for all protecting groups leads to the Sec residues already oxidized to the expected diselenide or mixed selenosulfide bonds even under the very strong acid conditions.[63,72]

Alternatively, a cost-efficient approach can be applied for the synthesis of short selenocystine peptides by reacting the related β-chloroalanyl-peptides with Na_2Se_2[73] in protic solvents such as methanol avoiding by this way protection and deprotection of the selenocysteine residues.[74]

Using prokaryotes incorporation of selenocysteine into recombinant natural Sec-proteins is rather inefficient[75] and requires the Sec-expression machinery.[76] Therefore, recombinant expression of natural and designed seleno-proteins is not yet a routinely applied method for the production of seleno-peptides and -proteins.[77] Conversely, recombinant expression of selenocysteine variants of Cys-containing proteins in Cys-auxotrophic *E coli* was found to proceed more successful as exemplarily shown for $(Se)_2$-thioredoxin[78] and subsequently also for various other Sec-mutants of proteins.

However, a very efficient alternative to this recombinant technology proved to be the synthetic approach to natural Sec-proteins or Sec-mutants of Cys-proteins as comprehensively reviewed more recently.[77,79] This synthetic strategy relies mainly on the assembly of suitable fragments by the native chemical ligation (NCL) and expressed protein ligation (EPL) where because of the similar nucleophilic properties of Sec to those of Cys, the reaction of N-Sec-peptide segments with synthetic or expressed peptide thioester is exploited to produce seleno-proteins.[80-83]

5.3 | Redox potential of selenocysteine

While the highly isomorphous character of disulfides and diselenides is perfectly suited for atomic mutants in X-ray crystallography, the selenium and sulfur chemistry diverges most prominently in the redox properties.

Using cyclic voltammography with a dropping mercury working electrode, a redox potential of selenocystine of E'_0 = −488 mV vs NHE was determined.[84] This very negative value differs significantly from that of selenocysteamine (E'_0 = −352/−368 mV)[85,86] determined by exchange reaction with DTT (E'_0 = −323/327 mV).[87,88] This difference may likely derive from absorption of the selenol on the dropping mercury electrode. Therefore, alternatively, the redox potentials of diselenide and mixed selenide/sulfide were determined for the $Sec^{11,14}$- and Sec^{11},Cys^{14}-Grx1-$^{10-17}$ octapeptides related to the active site of glutaredoxin-1 (Grx1) (Table 2).[92] This choice was based on the observation that this short active-site fragment shows only minimal effects imparted by the 3D fold of the protein on the apparent redox potential of the bis (cysteinyl)-octapeptide $Cys^{11,14}$-Grx1-$^{10-17}$ (E'_0 = −215 mV).[48] Moreover, it was only minimally

TABLE 2 Redox potentials of disulfide-, diselenide-, and selenosulfide amino acids, peptide, and proteins

Compounds		Reference Redox System	E'_0, mV, (Ref)
GSH/GSSG		Lipoamide (−288 mV)	−240[54]
GSeH/GSeSeG		DTT (−327 mV)	−407[89]
Arg-vasopressin		GSH/GSSG (−262 mV)	−228[90]
Oxytocin		GSH/GSSG (−262 mV)	−216[90]
Somatostatin		GSH/GSSG (−262 mV)	−228[91]
Glutaredoxin-1	(Grx1)	Trx (−270 mV)	−233[50]
Ac-Gly-Cys-Pro-Tyr-Cys-Val-Arg-Ala-NH_2	Grx1-$^{10\text{-}17}$	GSH/GSSG (−240 mV)	−215[48]
Ac-Gly-Sec-Pro-Tyr-Sec-Val-Arg-Ala-NH_2	Sec^{11},Sec^{14}-Grx1-$^{10\text{-}17}$	DTT (−323 mV)	−381[86]
Ac-Gly-Sec-Pro-Tyr-Cys-Val-Arg-Ala-NH_2	Sec^{11},Cys^{14}-Grx1-$^{10\text{-}17}$	DTT (−323 mV)	−326[92]
Glutaredoxin-3 (Grx3)		Trx (−270 mV)	−198[50]
[Sec^{11},Cys^{14}]-Grx3		Trx (−270 mV)	−260[72]
[Cys^{11},Sec^{14}]-Grx3		Trx (−270 mV)	−275[72]
[Sec^{11},Sec^{14}]-Grx3		Trx (−270 mV)	−309[72]
Cys/Cys_2		GSH/GSSG (−240 mV)	−223[93]
Sec/Sec_2		DTT (−332 mV)	−386[94]

affected by the neighbouring amino acid residues compared with the redox potential of cysteine as amino acid (E'_0 = −223 mV).[93] It also was found to be similar to the values previously determined for other unconstrained linear bis (cysteinyl)-peptides such as Arg-vasopressin and oxytocin[90] as well as somatostatin.[91]

As shown in Table 2, the redox potential of the bis (selenocysteinyl)-peptide (E'_0 = −381 mV)[92] was found to be remarkably lower than that of DTT (E'_0 = −323/327 mV)[87,88] but very similar to the electrode potential of selenocysteine determined by using a graphite electrode (E'_0 = −386 mV).[94] Conversely, the redox potential of the mixed Sec,Cys-peptide (E'_0 = −326 mV) was similar to that of DTT but still significantly more reducing than that of the parent bis (cysteinyl)-peptide.

By comparing the redox potentials of the bis (cysteinyl)-peptides related to the active sites of various thiol-protein oxidoreductases, significant effects of the structural constraints of the intact enzymes were clearly observed. Very similar structural effects were imparted even on the redox potentials of the diselenide and sulfenylselenide groups in the Sec-analogues of Grx3 (Table 2).[72]

5.4 | Synthesis of cysteine-rich peptides by the selenocysteine strategy

The strong differences of more than 100 mV between the redox potentials of disulfides and diselenides and to lesser extents between disulfides and selenosulfide groups make formation of a diselenide and even mixed selenosulfide highly favored over that of a disulfide bond. It was known that oxidative folding of cystine-rich peptides already with two disulfide bonds rarely produces quantitatively the native disulfide isomer like in apamin[95] but more often mixtures of the native disulfide isomer contaminated at differing extents by the nonnative isomer like in endothelin-1.[96]

The ability of selenocysteine to act as an internal "chaperone" and thus to dictate the folding pathway of cysteine-rich peptides was first confirmed by the synthesis of seleno-endothelin-1.[59] Indeed, air oxidation of its fully reduced Sec^{3,11},Cys^{1,15},Nle^{7}-analogue produced quantitatively the native diselenide$^{3\text{-}11}$,disulfide$^{1\text{-}15}$ ribbon isomer. Conversely, in the case of apamin, not only the tetrathiol-precursor folds quantitatively into the native [Cys$^{1\text{-}11}$,Cys$^{3\text{-}15}$]-globule structure[95] but even the nonnative [Cys$^{1\text{-}15}$,Cys$^{3\text{-}11}$]-ribbon and [Cys$^{1\text{-}3}$, Cys$^{11\text{-}15}$]-beads isomer, prepared as des-(16-18)-apamin analogues by regioselective disulfide pairing procedures, were shown to convert quantitatively into the native fold when equilibrated in the GSH/GSSG (15.0/2.3mM) redox buffer at pH 7.4.[97] As expected from the redox potential of the selenocysteine, air oxidation of the fully reduced [Sec$^{1\text{-}11}$,Cys$^{3\text{-}15}$]-, [Cys$^{1\text{-}15}$, Sec$^{3\text{-}11}$]-, and [Sec$^{1\text{-}}$3, Cys$^{11\text{-}15}$]-apamin analogues was found to generate exclusively the wt and the two nonnative ribbon and beads diselenide/disulfide isomers, respectively (Figure 7).[98]

Despite the different disulfide/diselenide crossbridging in the three isomers, the NMR derived structures revealed a surprising persistence of the main structural elements: These are the C-terminal α-helix and the N-terminal β-bend as shown in Figure 7.[61] This would suggest a rather low difference in free energy of folding of the three isomers (for the native isomer, a value of −4.5 kcal/mol at pH 7.0 and 20°C was reported[99]) and thus also explain why equilibration of the two nonnative diselenide-quenched isomers with GSH/GSSG mixtures at different ratios under exclusion of air did not result in a reshuffling even at minor extents into the native isomer with generation of mixed selenosulfide bonds. Because of this modest stability of apamin in its native fold, the intrinsically higher stability of a nonnative diselenide bond is sufficient to offset the preferential conformational stabilization of the native crossbridging, thus allowing formation of the nonnative isomers. This, however, may not be the case in peptides

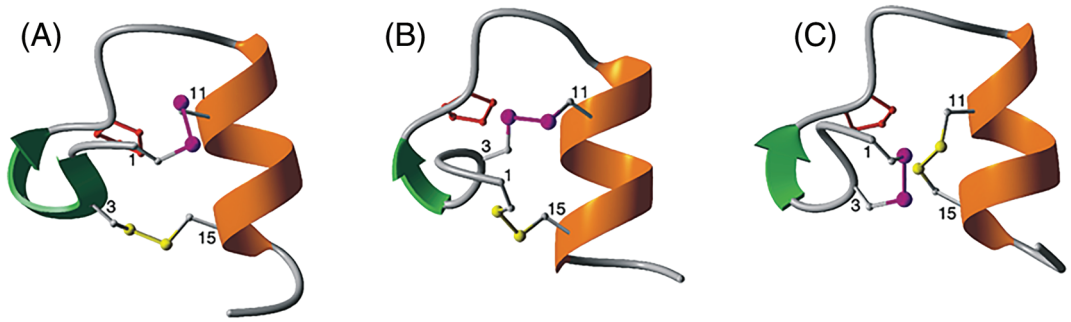

FIGURE 7 Solution structure of [Sec[1-11],Cys[3-15]]-apamin (wt globular fold), [Cys[1-15],Sec[3-11]]-apamin (ribbon isomer), and [Sec[1-3],Cys[11-15]]-apamin (beads isomer) as determined by NMR[61]

and proteins where structural disulfides can be exceptionally stable with redox potentials ranging from −350 to −470 mV,[100] thus competing or even offsetting that of a diselenide as in the case of bovine pancreatic trypsin inhibitor (BPTI) with a nonnative diselenide bridge (vide infra).[101,102]

This latter problem should not be encountered when diselenides are used to replace native disulfides. In such cases, even the redox potential of the diselenide should become more negative and, correspondingly, further stabilize the native side chain bridging framework even in reducing environments such as GSH/GSSG. Indeed, this was fully confirmed by the very high yields of correct folds obtained in a large series of conotoxins with two disulfide bonds by replacing one with a diselenide.[62,63]

The selenocysteine approach proved to be of great help also in the synthesis of cysteine-rich peptides containing more than two disulfide groups such as the inhibitory cystine knot (ICK) with one disulfide threaded through the other two. This structural motif is found in large classes of peptides from various natural sources including conus peptide families. Among these toxins, the μO-conotoxin MrVIB was considered unfoldable. But applying the selenocysteine strategy, the folding of the related [Sec[9-25]]-, [Sec[2-20]]-, and [Sec[19-30]]-MrVIB diselenide precursors was found to generate the correctly folded Sec-analogues in rather high yields of 61%, 42%, and 47%, respectively.[103] Since enhanced kinetics of disulfide formation was observed in selenopeptides, the rate increase has been in first instance attributed to conformational effects. However, diselenides as additives were known to efficiently catalyze oxidative protein folding.[104,105] Correspondingly, the kinetics of oxidative folding of the μ-conotoxin SIIIA and ω-conotoxin GVIA, both containing the cystine knot ICK, were compared with the kinetics of formation of the correctly folded [Sec[4-19]]-SIIIA and [Sec[8-19]]-GVIA. The results clearly revealed an efficient catalytic effect of even intramolecular diselenides in the formation of the correct disulfides.[106]

This can be explained by the fact that the diselenide bond is weaker than the corresponding disulfide bond and thus is easier to break and to be cleaved by thiols. The way that selenium accelerates in the case of a thiol/diselenide exchange reaction is that the electrophilic selenium atom being attacked is a much better electrophile than sulfur and accelerates the reaction rate 100-fold more in comparison with sulfur.[107] Correspondingly, diselenides can catalyze thiol-disulfide exchange reactions required to reach the thermodynamic most favored framework.[101,102]

Another interesting application was reported by Metanis and Hilvert who used a nonnative diselenide to catalyze the correct oxidative folding of BPTI.[101,102] Very intensive studies on the oxidative folding pathway of BPTI had disclosed the formation of a significant amount of a kinetically trapped intermediate (N*) containing the two native disulfide bonds [Cys[5]-Cys[55]] and [Cys[14]-Cys[38]].[108] As mentioned above, because of the higher polarizability of selenium than of sulfur, even nonnative diselenides could catalyze correct folding if sufficient conformational energy is available to neutralize the thermodynamic benefit of a diselenide bond. With this hypothesis in mind, two selenocysteines were placed in the positions 5 and 14 of BPTI to induce in first instance formation of the nonnative Sec[5]-Sec[14] diselenide bond, which then was found to undergo thiol diselenide exchange reactions bypassing the kinetic trapped intermediate N* and generating in high yields correctly folded BPTI with two

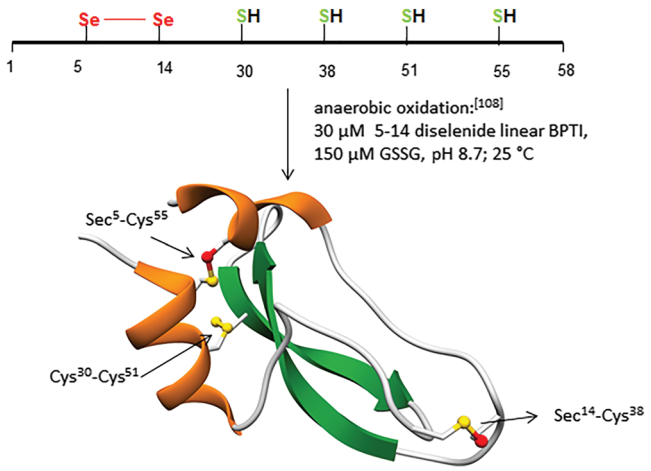

FIGURE 8 Anaerobic oxidation of linear diselenide [Sec[5]-Sec[14]]-BPTI with the four residual Cys residues as free thiols with a fivefold excess of GSSG leads to the native intramolecular [Cys[30]-Cys[51]]-disulfide (yellow) and the two [Sec[5]-Cys[55]]- and [Sec[14]-Cys[38]]-selenosulfide bonds (pink-yellow) in the native tertiary structure and in more than 70% yield after 30 h.[101,102] A similar behavior was observed at neutral pH; air oxidation in the absence of GSSG leads to 70% to 80% seleno-BPTI, while native reduced BPTI folds extremely slowly and inefficiently (12% after 28 h) under such conditions

selenosulfide bridges [Sec[5]-Cys[55]] and [Sec[14]-Cys[38]] in addition to the disulfide [Cys[30]-Cys[51]] (Figure 8).[101,102]

This surprising strong effect of intramolecular catalysis of diselenide groups on the folding of cysteine-rich peptides was compelling to apply the selenocysteine strategy in the attempt to advance in the still existing problem of efficient insulin syntheses as recently reviewed.[109] Indeed, acceptable yields in the correct assembly of the A- and B-chain into native insulin with the intramolecular A^6-A^{11} and the two intermolecular A^7-B^7 and A^{20}-B^{19} disulfides could mainly be achieved applying the most advanced regioselective Cys-protection schemes to guarantee in optimal yields the correct native disulfide pairing. While these synthetic approaches imply extensive experimental work, the direct oxidative assembly of the A- and B-chain failed in terms of yields unless very optimized conditions (A-chain: B-chain 2:1; GSSG/GSH and catalytic amounts of PDI) were applied.[110] Alternatively, yields similar to those of the oxidative folding of native proinsulin (70%-80%) could be achieved with various biomimetic single-chain proinsulin constructs in which the natural 35-residue C-peptide that connects the C-terminus of the B-chain with the N-terminus of the A-chain was replaced by peptidic and even nonpeptidic tethers of varying sizes and chemical structure. Among these artificial single-chain insulin precursors, the most appealing in terms of atom economy was the "Ester-Insulin" of Kent and associates in which the crosslinking of the A- and B-chain is achieved by esterification of the β-hydroxyl group of Thr[B30] with the γ-carboxyl group of Glu[A4] yielding an insulin precursor of a foldability similar to that of natural proinsulin; a simple saponification of the ester group produces the insulin in correctly folded structure and yields similar to those obtained with optimized single-chain proinsulins (Figure 9).[111-113]

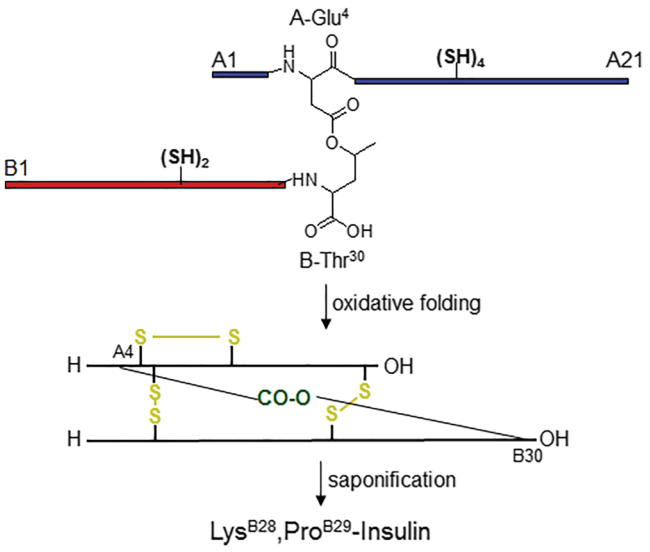

FIGURE 9 Conversion of ester-insulin to Lys^{B28},Pro^{B29}-insulin variant via oxidative folding and saponification according to Kent and associates[111,112]

This experimental evidence clearly confirms that a simple crosslinking of the A- and B-chain to the heterodimer via an ester linkage would suffice for reducing the entropic penalty in the oxidative folding of the two chains in a manner similar to the proinsulin precursor. These findings were strongly suggesting an application of the selenocystine strategy to produce an A7-B7 or A20-B19 diselenide as interchain crosslink with similar atom economy as the ester-insulin but with the advantage of the intramolecular folding catalysis by the diselenide (Figure 10).

With this working hypothesis in mind and knowing about the facile deselenization of the protected Sec (Mob) residues during the multistep synthesis of larger peptide chains with the Fmoc/tBu strategy,[68] the laboratories of Hojo and Iwaoka combined their efforts in the synthesis of the A- and B-chains containing the Sec (Mob) residues in position A7 and B7 of bovine insulin, respectively.[114] For the required handling of the peptides in the purification step, both the Cys and Sec residues were kept in the cysteine(S-pyridylsulfanyl) and selenosulfide forms, which were then reduced with DTT (4 equivalents for the total number of Cys and Sec residues) prior to folding in a 1:1 mixture of the A- and B-chain at 200μM concentration under thermodynamic control by air oxygen in a buffer at pH 10 (4°C) in the presence of 0.5M urea to solubilize the A-chain (Figure 11). The oxidative assembly was monitored by high-performance liquid chromatography (HPLC) and was found to be finished after 24 hours yielding upon HPLC purification 27% insulin. Slightly higher yields were obtained with TCEP as reducing agent. Compared with the assembly of the A- and B-chain under similar conditions with about 3% yield after 4 days, the selenocysteine strategy proved to be very successful in terms of reaction rates and yields by largely preventing the entropic penalty in the chain crosslinking at those low micromolar concentrations. The synthetic bovine seleno-insulin was found to exhibit the identical tertiary structure as the wt insulin, practically the identical bioactivities in terms of phosphorylation levels of Akt and GSK3β in Hela cells, but significantly increased resistance to the insulin degrading enzyme, a very positive property for a fully active insulin analogue.[114] The highly beneficial effect of the catalysis by the intermolecular diselenide and the significant reduction of the entropic penalty by the highly favored interchain-diselenide crosslink was fully confirmed.

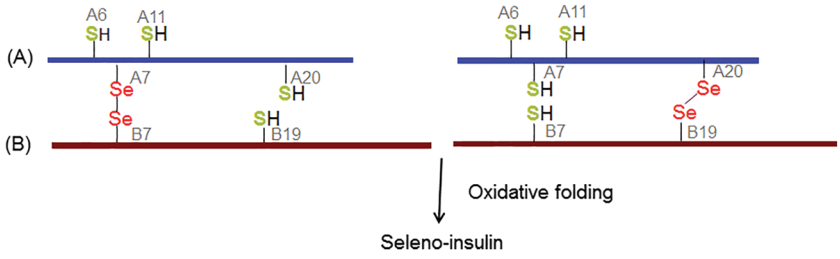

FIGURE 10 Possible placement of the two selenocysteines in synthetic linear A- and B-chains for induction of diselenide-crossbridged heterodimeric insulin-folding precursors

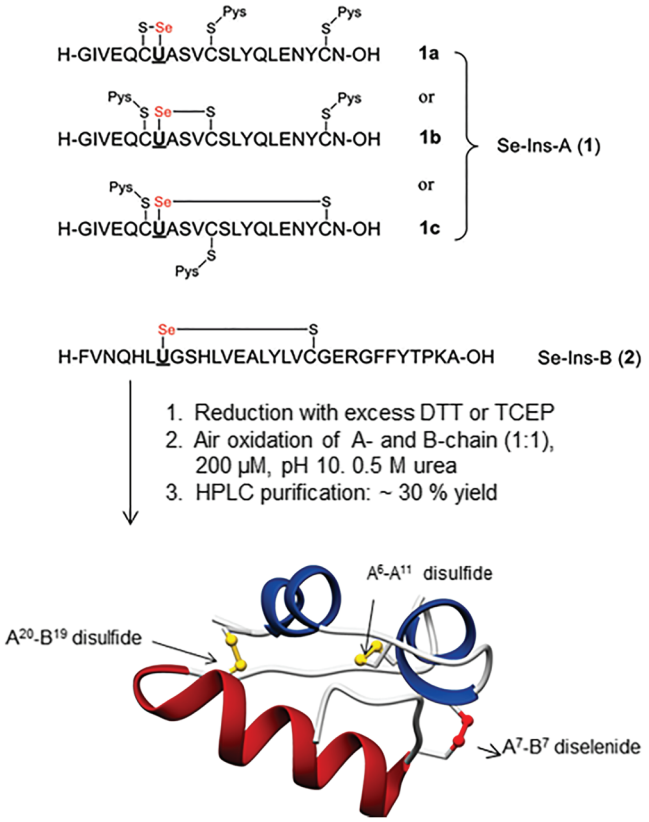

FIGURE 11 Oxidative assembly of the bovine [Sec^{A7},Sec^{B7}]-insulin from the two chains into the native insulin tertiary structure[114]

From extensive studies on folding of insulin and proinsulin, it was known that the intrachain disulfide Cys^{A6}-Cys^{A11} is important for induction of the overall insulin structure, even if not essential.[115] However, this disulfide if preferentially installed leads to conformational constraints that structurally preorganize the A-chain facilitating the correct formation of the remaining interchain disulfides.[116] On the basis of this information, Metanis and associates approached the synthesis of human seleno-insulin by placing the diselenide in the A-chain as intramolecular crossbridge to restrict the conformational space of the A-chain and thus to possibly improve a correct A- and B-chain oxidative assembly.[117-119] Unexpectedly, the synthetic linear [Sec^{A6}-Sec^{A11}]-A-chain proved to be highly soluble similarly to the sulfitolized A- and B-chains and thus with no expressed tendency to aggregation.[120] This beneficial solubility property allowed its use in the oxidative assembly reaction together with the sulfitolized B-chain readily prepared by oxidative sulfitolysis of commercial human insulin. The air oxidation was performed at significantly higher concentrations than in the preceding procedure with the Sec^{A7}- and Sec^{B7}-chains. As shown in Figure 12, upon reduction of the B-chain sulfonate with DTT in stoichiometric equivalents to the concentration of the sulfonate groups, but with the Sec^{A6}-Sec^{A11}-A-chain as a mixture of oxidized products (selenylsulfides or disulfide/diselenide), the two chains at a 1:1 molar ratio and 1.2mM concentration were exposed to air oxidation. After 8 hours, the product was isolated by HPLC in about 30% yield confirming the strong catalytic effect of the intrachain diselenide in both the recombination rates and final yields. This human seleno-

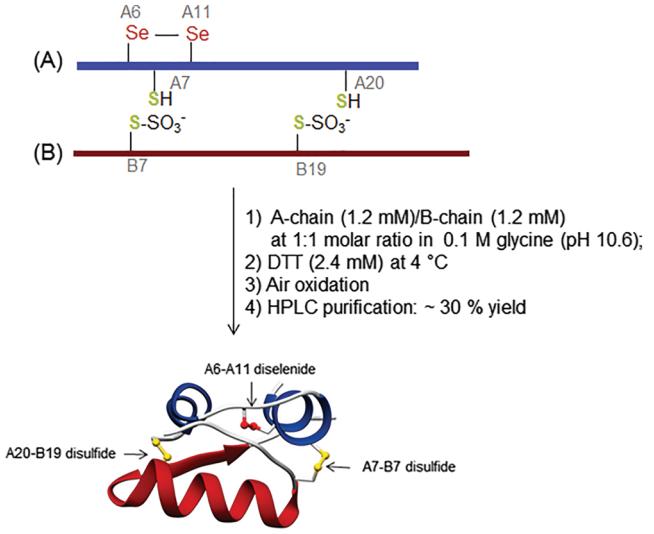

FIGURE 12 Oxidative assembly of the human [Sec^{A6},Sec^{A11}]-insulin from the two chains into the native insulin tertiary structure[117-119]

insulin variant was found to exhibit the identical tertiary structure as the native insulin. Identical bioactivities as the wt human insulin and resistance to both reductive and enzymatic degradation were realized.[117-119]

Both examples of using selenocystine as catalysts in the oxidative assembly of bovine and human seleno-insulin from designed Sec-variants of the A- and B-chain clearly revealed significantly increased rates of oxidative folding and yields of the desired products with native-like structures and activities. These data provide new approaches for the efficient generation of insulin analogues containing even unnatural amino acid residues in view of developing of new-insulin variants with improved therapeutic values.

5.5 | Tellurocysteine as isosteric replacement of cysteine

Unlike the chalcogens oxygen, sulfur, and selenium that find biological use, for tellurium, no biological function is known so far. However, in rare cases, bacteria and fungi grown in the presence of sodium tellurite and in the absence of sulfur were found to produce tellurium-containing amino acids (tellurocysteine, tellurocystine, and telluromethionine) and to bioincorporate these into proteins,[11,121] a process that has to occur by the tolerance of the methionyl- and cysteinyl-tRNA synthetases. On the basis of experience with the accumulation of selenium in proteins of *Spirulina platensis*, a blue-green

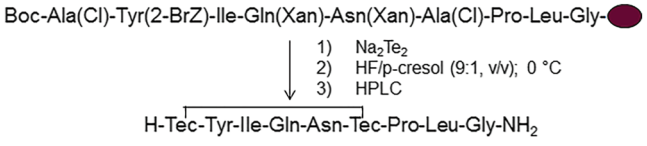

FIGURE 13 Synthesis of [Tec^{1},Tec^{6}]-oxytocin on MBHA-polystyrene resin[129]

TABLE 3 A comparison of bioactivities and resistance to digestion of seleno- and telluro-oxytocin with the wild-type hormone[131]

Oxytocin (OT)	Human OT Receptor		Human Serum
	binding K_i (nM)	functional EC_{50} (nM)	half-life (h)
wt-OT	0.79 ± 0.16	15.0 ± 2.5	11.6 ± 1.9
[Se-Se]-OT	11.8 ± 4.1	18.0 ± 12	25.3 ± 5.9
[Te-Te]-OT	7.6 ± 2.7	27.3 ± 7.4	24.2 ± 2.2

algae, in the form of selenomethionine and selenocysteine,[122,123] accumulation of tellurium into the phycobiliproteins phycocyanin and allophycocyanin of *S platensis* was found to proceed successfully leading to enhanced antioxidant activities of both proteins.[124] These results strongly support such novel organic tellurium species for application in antioxidation for the treatment of diseases related to oxidative stress.

On the basis of these results, even recombinant expression of the tellurocysteine-containing glutathione-*S*-transferase from *Lucilia cuprina* (LuGST-1) was found to occur in good yields with a Cys-auxotrophic expression system in the presence of synthetic tellurocysteine,[125] although tellurocysteine is apparently on the borderline in terms of the substrate tolerance of cysteinyl-tRNA from *E coli*.[126] Alternatively, the Tec amino acid was inserted into subtilisin to form semisynthetic tellurosubtilisin[127,128]; however, this chemical modification has the disadvantage that it can be applied only at active-site serine residues like Ser^{221} in subtilisin. Thus, the development of a general strategy for the incorporation of Tec into proteins so far remains a challenging task.

On the other side, the redox potential of tellurocysteine/tellurocystine determined by cyclic voltammography using mercury film coated glass carbon electrode is characterized by an intrinsically lower redox potential (−850 mV versus Ag/AgCl) than that of selenocysteine/selenocystine (−640 mV versus Ag/AgCl) making it an interesting mutant of Cys- and Sec-containing proteins because of its considerably more reducing properties.[126]

The synthesis of tellurocysteine has been reported[125,129] as well as the synthesis of $(Boc\text{-}Tec\text{-}OH)_2$[130] and H-Tec (Meb)-OH[129] as potential intermediates for the synthesis of Tec-peptides. However, oxytocin as the only reported synthetic Tec-containing peptide so far was prepared according to the synthetic route shown in Figure 13.[129]

A comparison of the oxytocin ditelluride with its wild-type and diselenide variant clearly revealed a visibly reduced receptor affinity and functional property (Table 3).[131] However, it was found to be surprisingly stable in view of general observations with organotellurium compounds where the ditelluride group was found to be light-sensitive with tendency to hydrolyze and to decompose forming telluroxides, tellurite, tellurate, and even elemental tellurium.[132] In addition, also the carbon-Te bond (around 200 kJ mol^{-1}) is less stable than the carbon-Se bond (234 kJ mol^{-1}) and the carbon-S bond (272 kJ mol^{-1}) with the carbon-O bond being the most stable (358 kJ mol^{-1}). In this context, the Te-alkyl compounds were found to be generally less stable than the Te-aryl or Te-alkylaryl compounds, a fact that was confirmed in the case of telluromethionine-protein analogues (see Section 2.1 and Besse et al[3]) in comparison with the stability of simple organotellurium compounds.[132] The higher stability of the alkylaryl tellurides could well be exploited for the synthesis of Tec-peptides with Tec (Mob) or Tec (Meb) protected tellurocysteine.

The promising therapeutic properties already reported for simple organotellurium derivatives with antimicrobial, antioxidant, immunomodulatory, and anticancer activities should foster intense research also with small purposely designed Tec-containing low-mass peptides and related derivatives.

6 | CONCLUSION AND REMARKS

As among the members of the chalcogen elements, oxygen, sulfur, and selenium play vital roles in the chemistry of life, the apparent lack of any biochemical function for tellurium is rather surprising also in view of its properties that are similar to that of sulfur and selenium. While oxygen is essential for life on Earth, sulfur exerts crucial roles in cellular processes, biocatalysis, and protein structures. This may well be the reason why great attention was paid to study and exploit the biochemistry and properties of sulfur as well as more recently of selenium, the latter because of its two faces as it is both toxic to all organisms, but also essential to many bacteria and animal species.[133,134] Thereby, the redox chemistry was found to represent the largest difference between the two chalcogens, a fact that has been exploited in nature to enhance and adjust redox properties of selenium-containing enzymes and proteins, but also in the laboratory to direct oxidative folding of cysteine-rich peptides and enhance their thermodynamic stability in view of potential therapeutic applications (see Section 5). On the basis of the superb results obtained by replacing sulfur with selenium particularly in methionine, but also in cysteine for producing heavy atom analogues with considerable advantages in protein and peptide X-ray crystallography, a similar approach has been proposed also for bioincorporation of telluromethionine (see Section 2.1). This approach has not found the due attention particularly because of the facile oxidation and decomposition of the telluromethionine. Such drawback can, however, be overcome by using the Te-containing phenylalanine and tryptophan analogues. Indeed, on the basis of optimized synthesis of β-selenolo[3-2*b*]pyrrolyl-L-alanine and β-selenolo[2-3*b*]pyrrolyl-L-alanine compared with the previously known methods (see Section 3 and Phillips et al[5] and Welch and Phillips[6]) to generate enzymatically [4,5]Se-Trp and [6,7]Se-Trp for their incorporation into dihydropholate reductase,[135,136] the synthesis of 6*H*-telluro[2-3*b*]pyrrolo for its enzymatic

conversion to the Te-Trp analogue has been reported.[137] Such a tellurium-containing Trp analogue as well as the β-2-tellurienyl-L-alanine as Phe analogue[9] where in both cases the tellurium is incorporated into the aromatic ring system should be significantly more stable to oxidation and decomposition than the alky-Te-alkyl compounds like telluromethionine.[34,130] This observation may well help raising the pharmacological perspective of tellurium compounds even at the level of peptides and proteins from a forgotten to an emerging new chalcogen-containing class of compounds with promising bioactivities.

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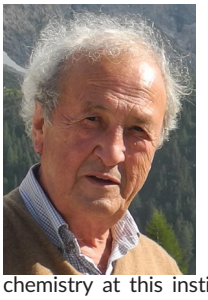

Luis Moroder After his training in peptide chemistry with E. Scoffone at the University of Padua and with Klaus Hofmann in Pittsburgh as postdoc, Luis Moroder became senior scientists at the Max-Planck-Institute of Biochemistry in Martinsried/Munich in 1975 and then head of the research group in Bioorganic chemistry at this institute. He was awarded the Max Bergmann Medal 1995, Josef Rudinger Memorial Lectureship in 2004, and the Akabori Memorial Lectureship in 2018. He is author and coauthor of over 500 publications and coeditor of the five-volume Houben-Weyl treatise "Synthesis of Peptides and Peptiodomimetics" and of the monograph "Oxidartive Folding of Peptides and Proteins". Presently he is Editor-in-Chief of Journal of Peptide Science.

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