Oxidative Folding of Peptides with Cystine-Knot Architectures: Kinetic Studies and Optimization of Folding Conditions

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Oxidative Folding of Peptides with Cystine-Knot Architectures: Kinetic Studies and Optimization of Folding Conditions

Michael Reinwarth, Bernhard Glotzbach, Michael Tomaszowski, Sebastian Fabritz, Olga Avrutina,* and Harald Kolmar*[a]

Bioactive peptides often contain several disulfide bonds that provide the main contribution to conformational rigidity and structural, thermal, or biological stability. Among them, cystine-knot peptides—commonly named "knottins"—make up a subclass with several thousand natural members. Hence, they are considered promising frameworks for peptide-based pharmaceuticals. Although cystine-knot peptides are available through chemical and recombinant synthetic routes, oxidative folding to afford the bioactive isomers still remains a crucial step. We therefore investigated the oxidative folding of ten protease-inhibiting peptides from two knottin families, as well as that of an HIV entry inhibitor and of aprotinin, under two conventional sets of folding conditions and by a newly developed procedure. Kinetic studies identified folding conditions that resulted in correctly folded miniproteins with high rates of conversion even for highly hydrophobic and aggregation-prone peptides in concentrated solutions.

Introduction

Natural polypeptides containing multiple disulfide bonds with mechanically interlocked topologies are found in diverse organisms: arthropoda, mollusca, porifera, vertebrata, fungi, and plantae.[1] These biomolecules, known as cystine-knot peptides or "knottins", usually possess peptide backbones of about 30 amino acid residues.[2] Being rather small, they nevertheless demonstrate the properties of full-sized proteins due to their unique architectures, each defined by three β-strands that are interconnected by three disulfide bonds, in which CysI of the sequence is connected to CysIV, CysII to CysV, and CysIII to CysVI (Figure 1).[2] The interlocked topology is formed by two disulfide bonds that, together with the connecting loops, form a ring through which the third disulfide bond is threaded. This results in a rigid structural core with exceptional thermal and biological stability. Knottins can be endowed with new bioactivities by variation of surface-exposed loops and are therefore considered to be promising peptide scaffolds for the generation of tailor-made bioactive compounds for various diagnostic and therapeutic applications.[3] Several miniproteins have already been applied as frameworks for the development of peptide-based pharmaceuticals, among them cyclotides, spider toxins, scyllatoxin, and squash trypsin inhibitors.[4] Knottins that act as, for example, potent inhibitors of human mast cell tryptase, an enzyme that plays a role in inflammatory diseases, especially in asthma, have been designed.[5] An engineered form of the human agouti-related protein (AgRP), a cystine-knot peptide with four disulfide bonds, was successfully used in radiolabeling for tumor imaging.[3b] Moreover, Ziconotide, a cystine-knot peptide from cone snails, has been on the market for treatment of severe and chronic pain since 2004.[6]

Thanks to their small sizes, knottins are readily accessible through solid-phase peptide synthesis.[2c] However, their chemical synthesis is often hampered by low folding yields due to the formation of incorrect intra- and intermolecular disulfide bonds.[2c] Although the investigative focus on the pathways of cystine knot formation is currently set on cyclotides—cyclic cystine-knot peptides in which the amino and the carboxy termini are linked by single peptide bonds—detailed studies on acyclic knottins have been carried out for only a few members of this large family.[7] In cases in which folding conditions have been systematically optimized for a particular knottin of interest, it has remained unclear whether these conditions are also applicable to other cystine-knot peptides.[2c,8]

In this study, we have investigated the folding of cysteine-rich peptides from different knottin families, as well as that of aprotinin, a three-disulfide trypsin inhibitor of the Kunitz-type family similar to basic pancreatic trypsin inhibitor (BPTI).[9] Peptides **1–8** (Figure 1 A) are based on the *Momordica cochinchinensis* trypsin inhibitor II (MCoTI-II) and therefore share the architecture of an inhibitor cystine knot (ICK) with the functional loop located between CysI and CysII.[10] During the last decade, ICK peptides have been intensively used as engineered frameworks in pharmaceutically inspired research.[1a,3c,d,5,11]

Recently reported three-disulfide peptides isolated from *Spinacia oleracea* seeds (SOTI I–III) also attracted our attention as

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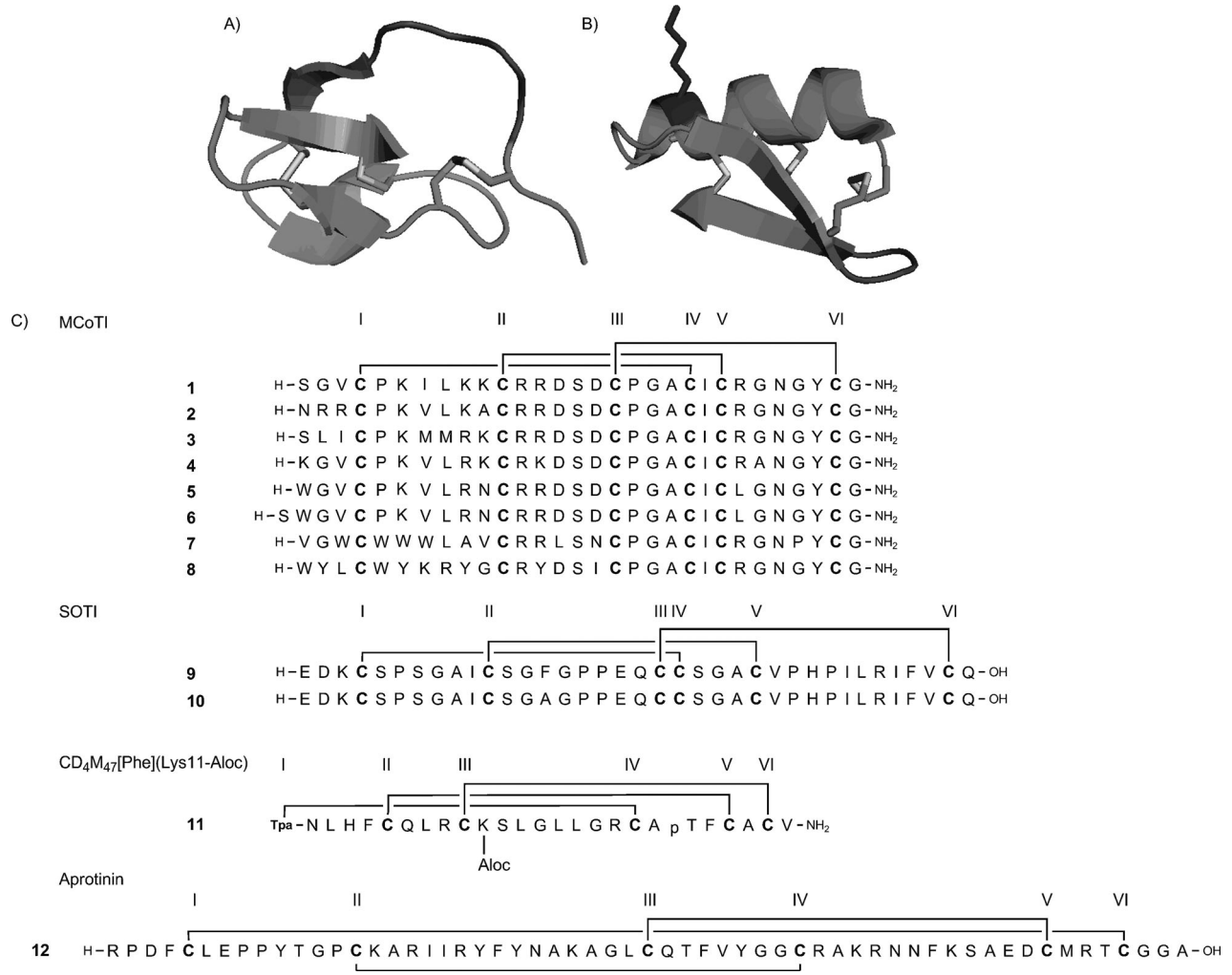

Figure 1. Overview of the miniproteins investigated in this work. A) Structure of an open-chain variant of MCoTI-II.[3a] B) Structure of CD4M47.[14b] C) Amino acid sequences of bioactive miniproteins presented in this work. The inhibitory loop can be seen top in (A) and bottom right in (B); Lys11 of CD4M47 is top left in (B); three disulfide bridges are shown in each. Tpa stands for thiopropiolic acid, p for D-proline, and Aloc for allyloxycarbonyl. Cysteines (and Tpa) are marked bold and numbered according to their appearance in the sequence. Cystine connectivities are indicated for each family.

potential scaffolds for the design of miniprotein libraries directed towards new protease binders against members of the type II serine protease family (compounds **9** and **10**).[1b,3b,5,12] Surprisingly, trypsin inhibitor SOTI I shows similarity not to any of the known plant protease inhibitors, but to a class of antimicrobial peptides from the seeds of *Mirabilis jalapa* with the inhibitory loop located between CysV and CysVI.[12b,13]

CD4M47[Phe] (**11**, Figure 1 B) was derived from scyllatoxin of *Leiurus quinquestriatus hebraeus*.[4d,14] This peptide has been optimized for binding to HIV-1 glycoprotein gp120 through several rounds of protein design and directed evolution, resulting in peptides with dissociation constants in the low nanomolar range towards the viral transmembrane protein.[4d,14a,b] Interestingly, this peptide is amino-terminally capped with dithiopropiolic acid (diTpa); this results in amino-terminal thiopropiolic acid (Tpa) that mimics homocysteine upon folding.

Aprotinin is a trypsin inhibitor obtained from bovine lung, and is identical to the more common bovine pancreatic trypsin inhibitor (BPTI), despite their different origins (bovine pancreas or lung, respectively). This inhibitor consists of 58 amino acids, among them six cysteine residues, and possesses a high proportion of basic amino acid residues; this makes the peptide a very potent inhibitor of trypsin.[9b,c,15] Folded BPTI contains three disulfides with a I–VI, II–IV, III–V connectivity that is different from that found in cystine-knot peptides **1**–**11**. Nevertheless, this peptide was also included in this study because in terms of oxidative folding and structural biology, BPTI is one of the most thoroughly studied trypsin inhibitors with clinical relevance. It reduces blood clotting through the inhibition of plasmin and until 2007 was sold under the registered trade name "Trasylol" by Bayer AG.[9c,15–16]

Although cystine-knot peptides are available through an abundant repertoire of peptide synthesis methods, oxidative folding still remains the crucial synthetic step.[2c,5,17] Very often, the formation of authentic disulfide connectivities requires a sophisticated regioselective strategy implying orthogonal thiol protection, polymer-supported oxidation, and isosteric disulfide replacements to ensure the correct crosslinking in the

presence of redox-favored species, or even deletion of a non-critical cystine pair or some combination of these methods.[2c,18] Moreover, folding yields are usually tightly correlated with high dilutions of the peptide, thus complicating workup of the folding mixtures.[2c] To date, no implementable generic approach to cystine bridging has been described.[2c]

Here we report the oxidative folding kinetics of a set of cystine-knot miniproteins, as well as the three-disulfide protease inhibitor aprotinin, and present a generic folding system that, in combination with ultrasonically assisted presolvation, allows one to obtain correctly disulfide-bonded knottins in high yields.

Results

Miniproteins of different origin were subjected to oxidative folding. Among them were the MCoTI-based open-chain protease inhibitors **1**–**8** (Figure 1), obtained from high-throughput screening experiments against matriptase or hepsin, which are known to be overexpressed in tumor cells, SOTI III and its Ala14 mutant (compounds **9** and **10**, respectively), and the scyllatoxin-derived CD 4 mimetic **11**, which contains a solubility-decreasing allyloxycarbonyl (Aloc) protecting group for downstream bioconjugation.[4d,13,14b,19] Although bioactive peptides of the same families have very similar backbone structures, differences in their individual sequences can result in remarkable differences in aggregation and folding behavior. The efficiency of our new folding system was determined by using reduced multicysteine peptide precursors that differed in their overall charges, polarities, and three-dimensional structures. Eight slightly basic cystine-knot peptides of the MCoTI family, some of them with large patches of hydrophobic or aromatic residues, two acidic knottins of the SOTI family, and one basic miniprotein derived from scyllatoxin, as well as the highly basic trypsin inhibitor aprotinin, were thus subjected to oxidative folding.

Optimization of oxidative folding conditions

Folding kinetics were followed by reversed-phase HPLC (Figures 2, 3, 4, and 5), as exemplified by folding of knottins **2** and **9**. Different oxidative folding conditions were applied. Air oxygen, as well as mixtures of reduced and oxidized glutathione or of DMSO/TFE/acetonitrile/Gu·HCl (DTAG) were used to establish redox conditions that drive oxidative folding.

Because the product yields were rather low when the previously reported standard air oxygen or the glutathione redox pair in aqueous buffer were used (Table 1), folding conditions were further optimized with use of DMSO as oxidant and co-solvent.[2c,4a,8c,10] Variation of DMSO concentration revealed that folding in 20% DMSO resulted in reaction rates equal to those of the glutathione system, but at the cost of the formation of side products. Higher concentrations of DMSO (up to 50%) correlated with better solubility and even faster reaction rates, but gave large amounts of non-reducible byproducts (data not shown). Use of less DMSO (down to 5%) led to reduced formation of side products but to longer reaction times and decreased solubility; in some cases even aggregation of peptides and precipitation was observed (data not shown). From the various folding experiments it was concluded that a mixture of organic solvents containing 10% acetonitrile, 10% DMSO as an oxidation agent and solvent, 10% trifluoroethanol (TFE) as secondary structure stabilizing and solvating adjuvant, and 1 M

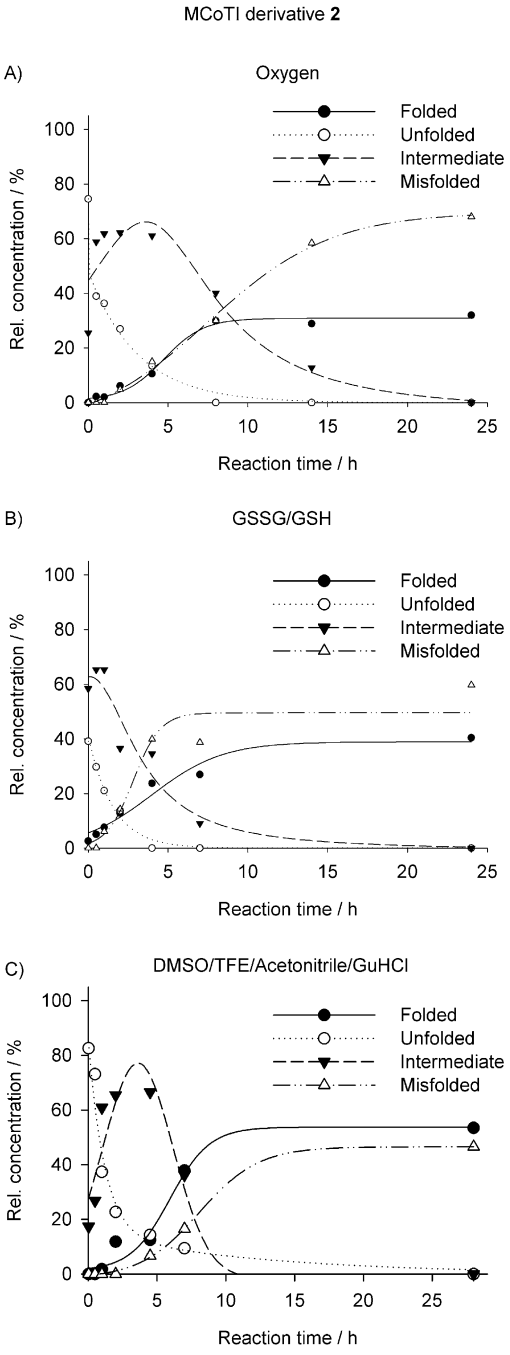

Figure 2. Kinetic investigation of MCoTI derivative **2** under different sets of folding conditions. A) Stirring in ammonium acetate buffer (pH 9.1) containing acetonitrile (10%). B) Acetonitrile (10%), oxidized/reduced glutathione (5 and 10 mM, respectively) in ammonium acetate buffer (pH 9.1). C) Acetonitrile (10%), DMSO (10%), TFE (10%), and guanidinium chloride (1 M) in aqueous phosphate buffer (pH 7.0). Data points were obtained by RP-HPLC analysis of the folding mixture in question at different time points. Relative concentrations were determined by peak integration.

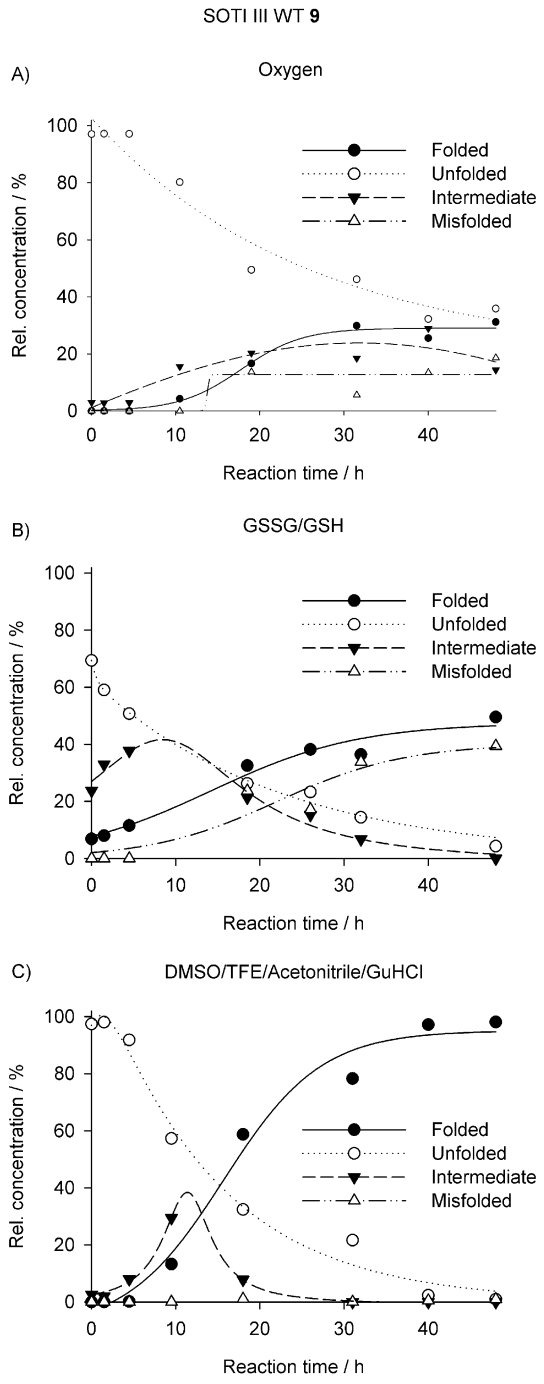

Figure 3. Kinetic investigation of SOTI derivative **9** under different sets of folding conditions. A) Stirring in ammonium acetate buffer (pH 9.1) containing acetonitrile (10%). B) Acetonitrile (10%), oxidized/reduced glutathione (5 and 10 mM, respectively) in ammonium acetate buffer (pH 9.1). C) DMSO (10%), acetonitrile (10%), TFE (10%), guanidinium chloride (1 M) in aqueous phosphate buffer (pH 7.0). Data points were obtained by RP-HPLC analysis of the folding mixture in question at different time points. Relative concentrations were determined by peak integration.

aqueous guanidinium hydrochloride (Gu·HCl) as aggregation-disrupting and solvating additive at pH 7 gave the highest yields relative to commonly used folding conditions both on analytical and on multimilligram scales (Figure 4, Table 1).[3b,8c,20] Except for the folding of **12**, each experiment was conducted with a peptide concentration of 10 mg mL^{-1}.

Table 1. Comparison of folding yields after 24 h under different sets of oxidative conditions at peptide concentrations of 10 mg mL^{-1} in each experiment, except in the case of **12**, which was folded at 5 mg mL^{-1}.

Mini-protein	HPLC conversion [%] 1	2	3	Yield[d] [%]
1	17.5	22.6	49.1	22.0
2	32	40.4	53.4	19.1
3	9.7	16.2	80.6	27.8
4	0	20.2	34.9	18.2
5	0[a,b]	0[a,b]	72.1[b]	14.1[b]
6	41.1[b]	43.1[b]	50.5[b]	11.5[b]
7	0[a,b]	1.4[a,b]	42.5[b]	9.6[b]
8	0[a,b]	0[a,b]	48.7[b]	9.5[b]
9	31.1[c]	49.6[c]	98.1[c]	23.8[c]
10	42.7[c]	59.8[c]	85[c]	16.2[c]
11	0[a]	0[a]	54.4	7.0
12	0[a,c]	48.7[a,c]	72.4[a,c]	13.3[c]

Conditions: 1: air oxygen; 10% MeCN in 200 mM NH_4OAc, pH 9.1; 2: GSSG/GSH (5/10 mM) in 10% MeCN in 200 mM NH_4OAc, pH 9.1; 3: DMSO, TFE, MeCN (10% each) in 1 M Gu·HCl in 50 mM Na_2HPO_4, pH 7. [a] Aggregation as a white precipitate observed. [b] Reaction time of seven days. [c] Reaction time of two days. [d] On semipreparative scale with DMSO/TFE/Gu·HCl; yields refer to crude linear precursor.

The folding process was usually completed within 24 h, although in some difficult cases complete conversion took several days. Surprisingly, suspension of the peptide in acetonitrile

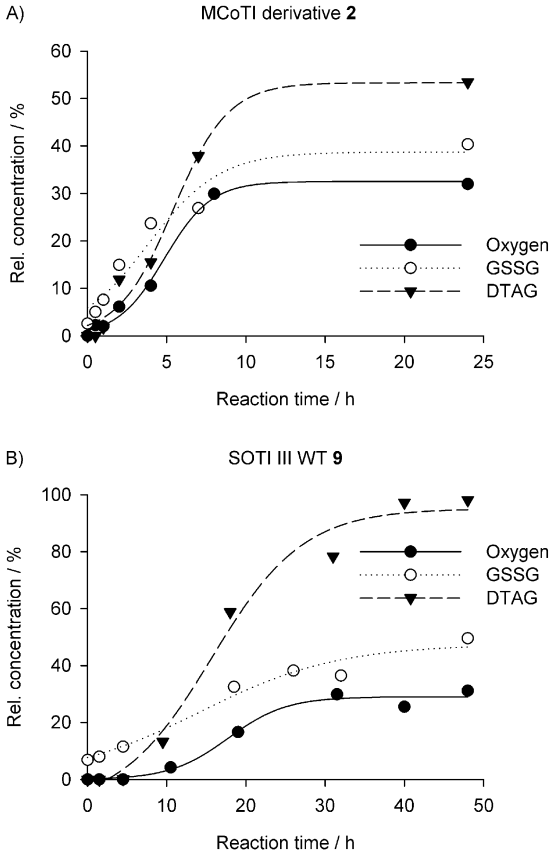

Figure 4. Comparison of product formation under the different sets of folding conditions. A) MCoTI derivative **2**. B) SOTI III WT (**9**).

in an ultrasonic bath was found to be a crucial step to avoid peptide aggregation in the folding process, especially during experiments on semipreparative scales; this is likely due to solvation of aggregation-prone regions. In particular, peptide **7**, with its unusually large number of tryptophan residues, was found to be very prone to aggregation in aqueous buffer systems. This peptide was obtained from screening experiments as an inhibitor (most likely allosteric) of the serine protease hepsin. Nevertheless, for aprotinin (**12**), with its numerous patches of basic amino acids, the DTAG folding conditions had to be slightly modified due to the fact that reduced aprotinin is almost completely insoluble in neutral or basic aqueous folding buffer (Table 1, Figure S40 in the Supporting Information). In this case, the peptide was predissolved in more concentrated (2.4-fold) DTAG, followed by dilution to 5 mg mL^{-1} final concentration.

Comparison of folding kinetics in DTAG folding buffer

Figure 5 shows RP-HPLC traces of folding reactions for three peptides, each a representative example of a knottin family, in the optimized DTAG-based folding buffer. Interestingly, folding intermediates of trypsin inhibitors **2** and **9** strongly differ with respect to their hydrophobicity. For MCoTI derivative **2** the observed folding intermediate—most likely the CysI–CysIV, CysII–CysV two-disulfide version—displays intermediate hydrophobicity relative to the native and the unfolded state (Figure 5, left).[2c,7b,21] During the folding of SOTI, an intermediate that displayed considerably higher hydrophobicity than the peptide in its unfolded state was accumulated (Figure 5, middle). The nature of this intermediate still has to be elucidated.

Biological activities of folded peptides

Although different disulfide connectivities can be distinguished by comparison of RP-HPLC retention times on a C18 column, it remained to be investigated whether the obtained oxidized species possessed the correctly knotted pattern.[2c,8c,22] For knottins that act as protease inhibitors it is known that inhibitory activities are strictly dependent on particular disulfide bond topologies.[5,23]

Wild-type sequences of MCoTI and SOTI with known K_i values against trypsin were used as references for determination of the correct disulfide patterns. Similarity between the K_i values obtained in this work and those previously reported indicated the formation of correct disulfide patterns.[10,12b,13] Additionally, the grafted miniproteins displayed behavior analogous to that of their wild-type counterparts upon HPLC monitoring during folding. For miniproteins **7** and **8** the inhibition constants were determined not only for the final products but also for the major folding byproducts, revealing K_i values in the micro- to millimolar range, hence clarifying their misfolded natures (data not shown). A combination of both methods is thus a valuable tool to test native disulfide bond formation. All 11 protease inhibitors examined in this study showed inhibition constants in the nanomolar range against their corresponding target proteases (Table 2). Correct folding of **11** was verified by CD spectroscopy (Figure S37).

N-terminal aldehyde installation in a one-pot reaction

Peptides **1**, **3**, and **6** each contain an N-terminal serine residue that can be used for selective periodate oxidation to generate

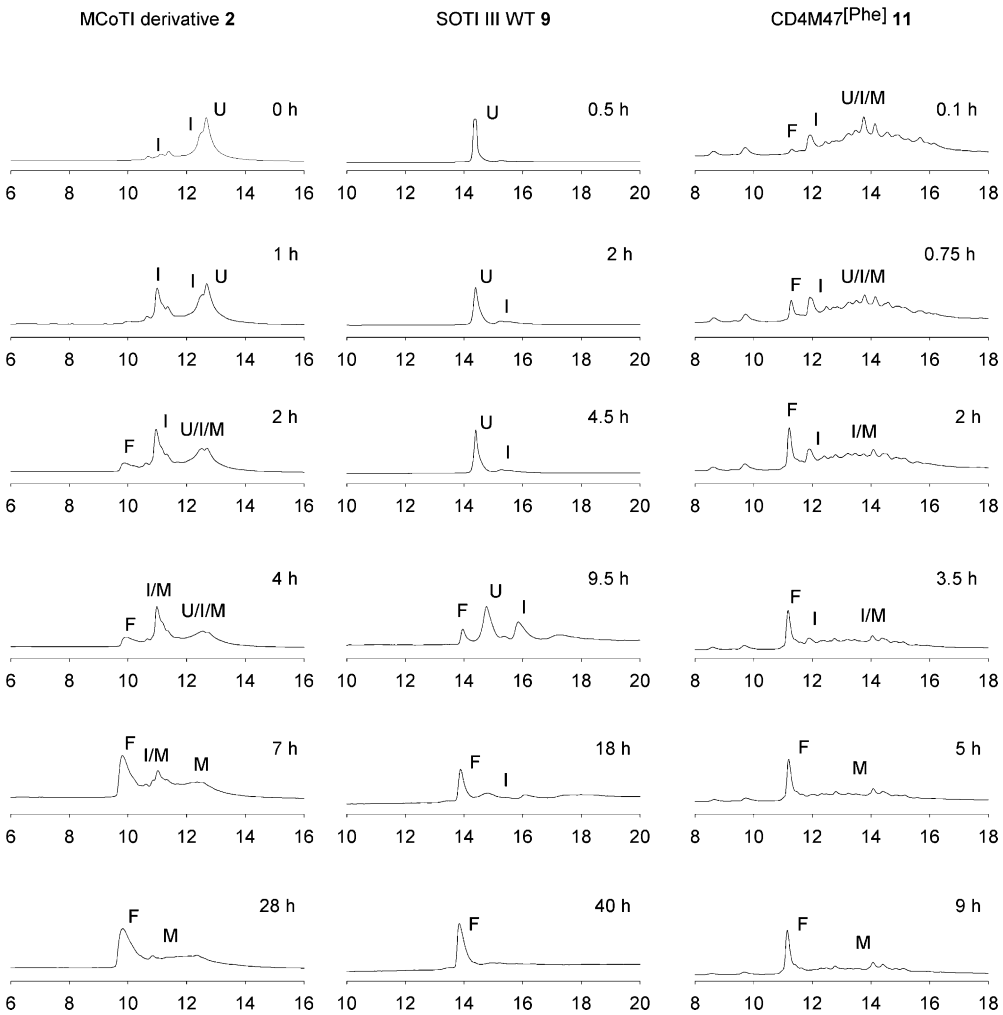

Figure 5. HPLC monitoring of folding of **2**, **9**, and **11** in DTAG folding buffer at different reaction times. F: folded products, M: misfolded byproducts, I: folding intermediates, U: unfolded precursor.

Table 2. Average inhibition of folded miniprotein variants against trypsin or hepsin.

Miniprotein	K_i [nM]	Miniprotein	K_i [nM]
1	0.15	**6** (glyoxylyl)	24
1 (glyoxylyl)	0.5	**7**	131[a]
2	2.3	**8**	100[a]
3	1.2	**9**	58
3 (glyoxylyl)	589	**10**	194
4	2.3	**12** (lung isolate)	0.11
5	2.2	**12** (refolded)	0.18
6	32		

[a] Determined against hepsin as a double determination.

a glyoxylyl moiety for subsequent site-specific conjugation as, for example, a fluorescent label or a radiolabel.[24] Addition of 10 equiv periodate to the DTAG mixture containing **1** or **6** resulted in complete conversion to the desired glyoxal or its corresponding hydrated form (Figure 6).[25] Periodate oxidation of MCoTI variant **3** also resulted in the generation of an N-terminal aldehyde with DTAG, but side reactions due to an unspecified oxidation of the vicinal methionine residues were observed and dramatically decreased inhibitory efficiency (Table 2). Under periodate-supplemented glutathione-based folding conditions, less or no N-terminal conversion (peptides **1**, **3**) or precipitation of peptide (**6**) was observed. For the oxidation of **1** and **3** increased amounts of sodium periodate (up to 50 equiv, based on peptide) caused no improvement and no oxidized peptide was noticeable. In conclusion, oxidation of amino-terminal serine in high yield and purity can easily be accomplished in a stepwise (serine oxidation after folding) one-pot reaction.

Discussion

In recent years, knottins have frequently been used as rigid and stable structural scaffolds for the introduction of new biological activities by varying the lengths and sequences of loops and also by substituting the framework residues.[5, 14b, c, 26] Many natural inhibitor cystine-knot peptides were found to be aggregation-prone, particularly in their reduced forms. For engineered variants this tendency can increase with increasing amounts of amino acid variation relative to the wild types. In this study, folding of cysteine-rich peptides derived from the trypsin inhibitors MCoTI II and SOTI, as well as a scyllatoxin derivative, was investigated. Variants displaying minor polarity changes within their sequences (**2**, **3**, **4**, **6**) demonstrated folding behavior similar to that of the wild type (**1**), although significant polarity changes—exchange of arginine for leucine or more hydrophobic residues (**5**, **7**, **8**), for example—resulted in highly aggregation-prone peptides hardly soluble in aqueous media. Yields of correctly folded knottins correlated with the solubilities of the corresponding linear precursors, as is highlighted by comparison of peptides **5** and **6**, in which N-terminal elongation with serine resulted in considerable increases of correctly folded miniprotein in all three folding systems (Figure 1, Table 1). Alteration of the pH in the glutathione-based systems revealed no relevant changes in conversion to the correctly folded products or their yields, as determined representatively for knottin **2** (data not shown).

Although the explicit folding mechanism of SOTI could not be investigated in the context of this project, RP-HPLC indicates a kinetically trapped intermediate of relatively hydrophobic character (Figure 5). This intermediate might involve an established CysII–CysV and CysIII–CysVI connection, because this is the common energetic trap for peptides of the inhibitor cystine-knot family.[2c, 7b, 27] In contrast to what might be intuitively expected, a longer RP-HPLC retention time than for the unfolded form was observed for this intermediate with hydrophobic residues present on the outer shell. The rate-limiting final folding step requires compaction and the reorientation of the hydrophobic and hydrophilic residues towards the inner core or the outer shell, respectively. The most demanding knottin in this series with respect to folding is the HIV entry inhibitor **11**, because the presence of an Aloc protecting group at Lys11 dramatically reduces solubility in aqueous buffers, so the unfolded peptide could not be dissolved in the ammonium acetate buffer and air oxygen or a glutathione mixture could not be used as ox-

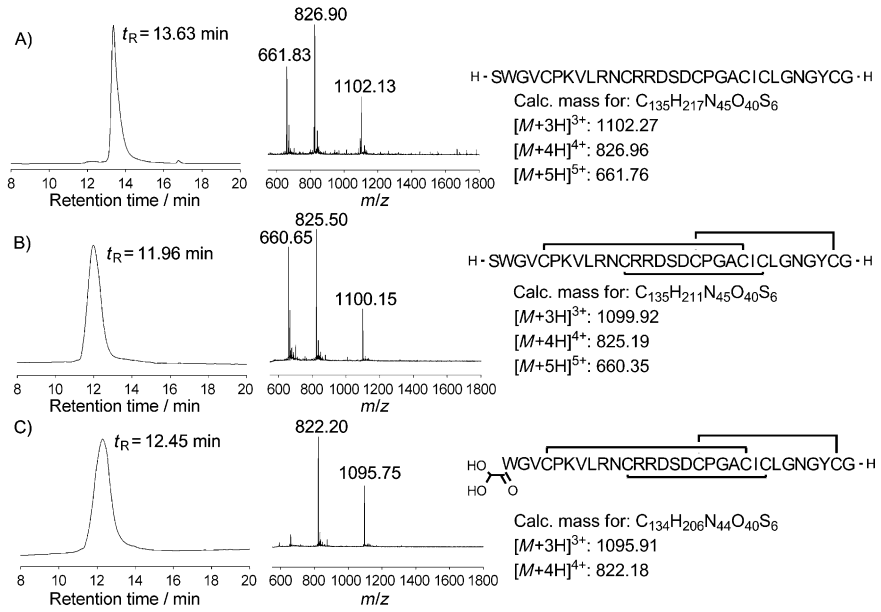

Figure 6. One-pot folding and oxidation of MCoTI variant **6**. A) HPLC trace and ESI-MS of the linear precursor of MCoTI derivative **6**. B) HPLC trace and ESI-MS of folded miniprotein **6**. C) HPLC trace and ESI-MS of folded and N-terminally oxidized MCoTI derivative **6**. t_R: RP-HPLC retention time.

idants. The fast folding in the DTAG folding cocktail—within five hours—indicates that this mixture is particularly useful for oxidative folding of hydrophobic and aggregation-prone peptides (Figure 5). For aprotinin, which is usually produced recombinantly fused to a polar propeptide that assists folding, the experimental procedure with the DTAG system had to be altered because of the high content of basic amino acid residues in the sequence that resulted in reduced solubility at pH 7 and pH 9.1 (see the Experimental Section).[28] As a consequence, the reduced peptide was directly dissolved in a 2.4-fold concentrated DTAG mixture followed by dilution with phosphate buffer pH 7.0 to 5 mg mL^{-1} and 2.5 M Gu·HCl, resulting in folding conditions nearly identical to those used for all other peptides.

Oxidation of cysteine residues is usually mediated by air, cystines, or dimethyl sulfoxide.[29] In the case of air oxidation of knottins, slow reaction rates and requirements for high dilution, especially for aggregation-prone peptides, are two major problems.[2c] Use of stronger oxidizing agents can cause significant side reactions through incorrect bridging or overoxidation of sulfur-containing residues. Both undesirable processes lead to the loss of particular architecture and biological activity. The necessity to conduct oxidative folding of hydrophobic peptides under conditions of very high dilution significantly complicates their post-synthetic purification, so the use of a polar organic co-solvent such as TFE, which solubilizes hydrophobic side chains and also assists in the formation and stabilization of α-helices, especially in combination with a mild oxidizing agent as DMSO, is often an alternative approach.[30] Additionally, some structure-disrupting blocks—proline or proline-like surrogates, for example—are known to reduce aggregation and have been used as additives in several folding buffers.[31] Other systems involving ionic liquids and platinum-based adjuvants are quite laborious, impractical, or narrow in scope for common use.[32]

The DTAG knottin folding cocktail developed in this study contains DMSO as oxidizing agent, TFE as cosolvent, and acetonitrile as solvent, together with aqueous guanidinium hydrochloride as chaotropic agent. The absence of guanidinium hydrochloride frequently resulted in detectable aggregation and precipitation that was not observed in the presence of 1 M guanidinium hydrochloride. At this concentration the oxidation process itself can be expected not to be influenced negatively by this denaturing agent, whereas disfavored peptide aggregation, particularly at relatively high peptide concentrations in the DTAG folding cocktail (usually 10 mg mL^{-1}), can be suppressed until the peptide becomes more hydrophilic and less aggregation-prone by compaction through oxidative folding.

The aggregation tendencies of the peptides, which are closely correlated with but not equal to their polarities, were found to be a key factor upon oxidative folding. Aggregation under oxidative conditions most likely results in the formation of disulfide-crosslinked polymers that are very hard to reduce or to redissolve, even by treatment with reducing agents such as dithiothreitol at high concentrations or with Zn as reducing agent in TFA/H_2O.[33] Ultrasonic-assisted presolvation in acetonitrile in combination with organic solvents and chaotropic salts is likely to reduce the aggregation tendency during the oxidation reaction.

Oxidative folding of multidisulfide-containing peptides by, for example, air oxygen without any reducing agent often results to large extent in misfolded byproducts, because cysteine thiols, once incorrectly bridged, lack the capability for spontaneous reduction.[2c] Addition of a redox pair such as ox./red. glutathione or cystine/cysteine to the folding mixture usually increases yields and purities of the correctly folded products.[2c,20,26,34] Interestingly, the DTAG mixture that provided the highest folding yields did not contain any reducing agent. However, inaccurately connected cysteine residues have low thermodynamic barriers to their reduction, so unpaired thiols in the peptide backbone might serve as reducing agents in a rearrangement reaction; the relatively low reduction potential of DMSO/DMS (160 mV) relative to GSSG/GSH (240 mV) or ($^1/_2O_2 + H_2$)/H_2O (410 mV) at pH 7 likely provides a favorable redox environment.[11,35] The side products of DMSO-based folding might therefore act as mild oxidants, thereby allowing for rearrangements, whereas side products of oxygen- or glutathione-based folding are often trapped in energetically uncorrectable states (Table 1).

Very often, bioactive peptides need incorporation of functional moieties with orthogonal reactivity to act as coupling sites for further modifications, such as for introduction of labeling compounds, conjugation with biomolecules, grafting onto surfaces, ligation with hybrid inorganic targets, or multivalent presentation on different scaffolds for enhanced bioactivity and bioavailability.[36] Although a variety of orthogonal active groups and conjugation techniques are available for peptidic molecules, the majority of ligation methods are not suitable for reactions involving cysteine-rich peptides.[37] Chemoselective oxidation of N-terminal serine, however, yields a reactive glyoxylyl functionality that can easily react with aminooxy, hydrazine, hydrazide, or thiosemicarbazide groups to form stable covalent bonds under mild, disulfide-friendly conditions.[38] Although periodate oxidation is a fully elaborated method, it needs an additional purification step, thus decreasing the final yield of the desired peptide.[37c,38a]

An additional benefit of the DTAG folding cocktail is the option to add sodium periodate to the reaction mixture directly for oxidation of an N-terminal serine unit to an aldehyde without affecting already bridged cystine pairs. Unwanted oxidation of methionine residues can occur, however (Figure S12), so periodate concentration and reaction time require careful adjustment.[39] Yields of oxidation product similar to those achieved with commonly used aqueous solvents were obtained for DTAG mixtures.[25a] Notably, subsequent in situ oxidation of the aldehyde group to the acid was not observed.[40]

Conclusions

Oxidative folding of cysteine-rich peptides is still a challenging matter because individually tuned folding conditions are often necessary. Particularly demanding is the formation of three disulfides in small peptides possessing cystine-knot architectures.[2c] These kinetic studies have identified folding conditions

that have resulted in correctly folded products even in cases of hydrophobic peptides containing multiple thiol units with strong aggregation tendencies in concentrated solutions (10 mg mL^{-1}) with excellent conversion rates. As demonstrated for aprotinin, this method might not be limited to the oxidative folding of peptides of the cystine-knot family but might also provide a starting point for optimizing folding conditions of other peptides with high cysteine contents and different disulfide patterns.

Experimental Section

All reagents were used without further purification as received from commercial sources. Fmoc-protected amino acids and activators for solid-phase peptide synthesis (SPPS) were obtained from IRIS Biotech (Marktredwitz, Germany); common reagents and solvents were purchased from Sigma–Aldrich, Carl Roth, or Fisher Scientific.

Synthesis of linear precursors: Peptides were assembled by standard Fmoc SPPS chemistry with a fully automated microwave-assisted CEM Liberty peptide synthesizer. Peptide amides were generated with a ChemMatrix Fmoc-Rink amide resin (Agilent Technologies, Böblingen, Germany). Peptide acids were synthesized on Fmoc-Gln preloaded TentaGel resin (Rapp Polymere, Tübingen, Germany). Amino-terminal capping of the HIV entry inhibitor **11** with Tpa was conducted by a standard coupling strategy with Tpa (4 equiv), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate (HBTU; 3.9 equiv) and *N*,*N*-diisopropylethylamine (DIEA; 8 equiv). Cleavage from the solid support was generally achieved with TFA (94%, *v*/*v*), H_2O (2%, *v*/*v*), dithiothreitol (DTT, 2%, *w*/*v*), triethylsilane (1%, *v*/*v*), and anisole (1%, *v*/*v*). After precipitation of the crude peptide in methyl *tert*-butyl ether, the pellet was dissolved in a mixture of acetonitrile and water (2:8, *v*/*v*), lyophilized, and analyzed by RP-HPLC and LC-ESI-MS.

RP-HPLC, LC-ESI-MS, and CD spectroscopy: Analytical RP-HPLC was performed with a Varian LC 920 system and a Phenomenex Synergi 4μ Hydro-RP 80 Å (250×4.6 mm, 4 μm) column with application of linear gradients of acetonitrile at a flow rate of 1 mL min^{-1}. Semipreparative RP-HPLC was performed with a Varian LC 940 system and an axia-packed Phenomenex Luna C18 (250×21.2 mm, 5 μm, 100 Å) column with application of linear acetonitrile gradients at a flow rate of 18 mL min^{-1}. Isocratic elution [10% eluent B for 2 min (analytical scale) or 5 min (semipreparative scale)] was followed by a linear gradient of 10→60% B or 10→80% B, respectively, over 20 min.

LC-MS was performed with a Shimadzu LC-MS 2020 instrument and a Phenomenex Jupiter C4 (50×1 mm, 5 μm, 300 Å) column with linear acetonitrile gradients and a flow rate of 0.2 mL min^{-1} (see Figures S1–S40). Isocratic elution (2% eluent B for 2 min) was followed by a linear gradient of 2→100% B over 10 min.

CD spectroscopy was performed as previously reported.[14b] Peptide **11** was thus dissolved in aqueous Na_2HPO_4 (pH 7, 2 mM) to a final concentration of 50 μM. The resulting spectrum (Figure S37) was obtained through accumulation of 100 spectra with use of a 0.1 mm quartz cuvette and 0.5 nm steps.

Integration of HPLC chromatograms was performed with Varian Galaxie chromatography software (Version 1.9.302.952). The graphic display was achieved with the "dynamic plot" function of Sigma Plot 11.

Stepwise one-pot folding and amino-terminal oxidation of cystine-knot peptides: Oxidative folding on analytic scale was performed by three different approaches. In each case the appropriate purified and lyophilized peptide (1 mg) was suspended in acetonitrile (10 μL) and treated in an ultrasonic bath for 5 min. Afterwards, the appropriate folding mixture (90 μL) was added: mixture A was composed of aqueous ammonium acetate (200 mM, pH 9.1), mixture B of oxidized/reduced glutathione (5/10 mM) in aqueous ammonium acetate (200 mM, pH 9.1), and mixture C of DMSO (10%, *v*/*v*), TFE (10%, *v*/*v*), and Gu·HCl (1 M) in aqueous sodium phosphate buffer (pH 7, 50 mM). Oxidative folding was usually complete within 24 h at room temperature; slow-folding peptides were kept for two or seven days (Table 1). Reaction was stopped through direct injection into analytical HPLC. On a semipreparative scale, folding of mixture C was applied as described with a final concentration of the crude peptide of 10 mg mL^{-1}, while the ratios of the individual folding assistants used were kept equal in the system.

Oxidation of N-terminal serine was performed by direct addition of $NaIO_4$ (10 equiv with respect to the peptide) to the folding mixture. Reaction was terminated by direct injection into the HPLC or LC-ESI-MS system for purification or analysis, respectively.

Unfolding and refolding of aprotinin: For reductive unfolding, aprotinin (Carl Roth) was dissolved in Gu·HCl (6 M). After addition of DTT (10 equiv) the reaction mixture was kept at ambient temperature for 2 h. It was directly injected into semipreparative HPLC.

Oxidative refolding of the reduced precursor was performed as for the cystine-knot peptides with slight modifications of the DTAG-based method. The reduced peptide was suspended in acetonitrile and treated in an ultrasonic bath for 5 min. Afterwards, the prefolding mixture containing DMSO (25%, *v*/*v*), TFE (25%, *v*/*v*), and Gu·HCl (6 M) in aqueous sodium phosphate buffer (pH 7, 50 mM) was added at 12.5 mg mL^{-1}. After additional treatment in an ultrasonic bath until the peptide was completely dissolved, the mixture was diluted 2.5 times with aqueous sodium phosphate buffer (pH 7, 50 mM). Oxidative folding was complete within 48 h at room temperature; formation of a small amount of insoluble white precipitate was observed.

Inhibition assays: The substrate-independent inhibition constants were determined as described previously.[10,41] Generally, data were collected in triplicate with a Tecan Genios ELISA reader. In the case of hepsin, measurements were performed in duplicate. The normalized residual proteolytic activities (v/v_0) of trypsin (Sigma–Aldrich), or hepsin (1 nM) towards the chromogenic substrates Boc-QAR-pNA (250 μM) or Boc-QAR-AMC (250 μM, Bachem, Weil am Rhein, Germany), respectively, in the presence of inhibitors at different concentrations were determined after 30 min by monitoring the absorbance at 405 or 465 nm for 30 min. Apparent inhibition constants (K_i^{app}) were calculated by fitting the data with use of the Morrison equation [Eq (1)] for tight-binding inhibitors with the Marquardt–Levenberg algorithm of SigmaPlot 11.[42]

$$\frac{v}{v_0} = 1 - \frac{(E_0 + I_0 + K_i^{app}) - \sqrt{E_0 + I_0 + K_0} - 4\,E_0 I_0}{2\,E_0} \quad (1)$$

Substrate-independent inhibition constants K_i were calculated from K_i^{app} and K_M of the enzyme according to Equation (2).

$$K_i = \frac{K_i^{app}}{1 + [S]/K_M} \quad (2)$$

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