ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 1, 2008

DOI: 10.1089/ars.2007.1849

Forum Review

Oxidative Folding of Cyclic Cystine Knot Proteins

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ABSTRACT

Cyclic cystine knot proteins are small but topologically complex molecules that occur naturally in plants and have a wide range of bioactivities that make them interesting from a pharmaceutical perspective. Their remarkable stability is dependent on the correct formation of a knotted arrangement of disulfide bonds. This review reports on studies that have deciphered the pathways to the "tying of the knot." These studies have involved a range of biophysical techniques and suggest that the major intermediate species presented on these pathways are two disulfide native species, which are not necessarily the precursors of the native protein. Structural elucidations of one analogue and one such intermediate have been reported, and they both show highly native-like conformation and native disulfide bond connectivity. Cyclic cystine knot formation has also been shown to be assisted by protein disulfide isomerase. The points summarized in this review will be important to consider in the design of novel pharmaceutically interesting biomolecules based on the cyclic cystine knot motif, which has shown potential as a molecular scaffold because of its exceptional stability. *Antioxid. Redox Signal.* 10, 103–111.

INTRODUCTION

The formation of disulfide bonds is crucial to the folding, stability, and biological activity of many cysteine-containing proteins. The process of oxidative folding refers to the combination of native disulfide bond formation and conformational folding (*i.e.*, formation of the native three-dimensional fold of a protein) and is of interest not only because it is essential to the functional production of proteins, but also because deciphering the interplay between conformational folding and disulfide formation at different stages of the folding process is a challenging problem. In principle, the number of potential pathways and transient intermediate species present in the oxidative folding of disulfide-rich proteins increases as the number of cysteine residues increases. However, even some small proteins containing as few as three disulfide bonds have the potential to form topologically complex motifs. Despite this complexity, disulfide-rich proteins often fold with remarkable efficiency and specificity for a particular disulfide connectivity. How does this occur? Of particular interest in this review is the folding process leading to formation of the cystine knot motif—a structure in which two disulfide bonds and their connecting backbone segments form an embedded ring that is threaded by a third disulfide bond. Cystine knots are found in a broad spectrum of organisms, including insects, plants, and animals (18, 43, 44) and occur in a wide range of physiologically important molecules, including growth factors (38), inhibitory proteins, and toxins (43).

This article focuses on a specific subclass of cystine knot molecules, namely cyclic cystine knots, that have the additional distinguishing feature of being embedded within a head-to-tail cyclized peptide backbone. Cyclic cystine knot molecules are exemplified by the cyclotides (16, 18) a large family of plant proteins that comprise 28–37 amino acids with six conserved cysteine residues forming three knotted disulfide bonds with the connectivity I–IV, II–V, and III–VI, with the latter being the "threading" disulfide bond. Needless to say, the combination of a macro-cyclic backbone and a knotted cross-bracing arrangement of disulfide bonds makes cyclotides exceptionally stable (2, 10). Indeed the prototypical member of the family, kalata B1, was orig-

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inally discovered as the active agent in a uterotonic medicine used to accelerate childbirth by women in central Africa. The medicine was prepared by boiling the plant *Oldenlandia affinis* in water to make a tea which was ingested during childbirth (28, 29). The fact that the active peptide could apparently withstand high temperature during boiling and oral ingestion provided a first indication of its thermal stability and resistance to enzymatic digestion. The structure was not fully elucidated at the time of its discovery but it is clear from the knotted and macro-cyclic structure reported in 1995 (47) that this unique structure is responsible in large part for the remarkable stability of kalata B1.

Reports in the mid-1990s of several other macro-cyclic peptides from plants (35, 48, 56) sparked interest in the field and prompted systematic discovery efforts to see if other related peptides existed (16, 26). The answer was a clear yes, with nearly 100 sequences now published. Cyclotides have so far been discovered in plants of the Rubiaceae, Violaceae, and Cucurbitaceae families, and it is estimated that the family may ultimately contain >9,000 members (49). Cyclotides have been divided into three subfamilies—the Möbius, bracelet, and trypsin inhibitor subfamilies—as outlined in Fig. 1. The first two subfamilies are by far larger and the third contains only two members: *Momordica cochinchinensis* trypsin inhibitors I and II, MCoTI-I and MCoTI-II (37), with the latter peptide being more extensively studied (24, 36). Because of its homology to a range of linear cystine knot proteins called knottins, MCoTI-II is also referred to as a cyclic knottin (8).

Cyclotides have been reported to have a wide range of biological activities, including anti-HIV, antimicrobial, neurotensin antagonism, antifouling, hemolytic, and other activities (8, 17, 27, 34). However, their natural function in plants appears to be as defense molecules, as judged by their potent activity at retarding the growth of *Helicoverpa spp*. species when incorporated into the diets of these insects (30, 39, 40). Interestingly, a range of other peptides with head-to-tail cyclic backbones are found in animals, plants, and bacteria, and all are involved in host defense (11, 53). Some of these, namely RTD-1 (51) and retrocyclin (42), also contain exactly three disulfide bonds and a cyclic backbone, but are not knotted. We will return to them later.

As well as having potential agricultural applications for crop protection arising from their native insecticidal activity, cyclotides have been proposed as templates for drug design applications (12, 14, 19). In particular, due to their exceptional stability, they offer the potential as a peptide drug delivery vehicle. Figure 2 shows the concept of how bioactive peptide epitopes could be grafted into a cyclic cystine knot framework, a concept that has already been demonstrated for acyclic cystine knot molecules such as *Ecballium elatarium* trypsin inhibitor-II (EETI-II) (9). The latter molecule is very similar to the trypsin inhibitor cyclotide MCoTI-II. Overall, it is clear that cyclic cystine knot molecules are interesting not only from a fundamental topological perspective, but also because of their potential applications. Thus, we have been interested in deciphering their folding pathways; in the remainder of this article we provide an overview of these studies.

Oxidative folding of the model cyclotide kalata B1

As noted in the introduction, kalata B1 is the prototypic cyclotide and was first discovered in the African plant *Oldenlandia affinis* (28, 29). Although many of the studies involving the structures (15, 46) and bioactivities (1, 39, 50) of this molecule involved native peptide extracted from the plant, methods for its synthesis have been developed (22, 33, 50), along with methods for folding it *in vitro*. Reduced cyclic kalata B1, either synthetic or extracted from plant material, can be refolded into the native conformation in 50% isopropyl alcohol (v/v) in 0.1 *M* ammonium bicarbonate, pH 8.5, and 1 m*M* reduced glutathione at room temperature overnight (22). Essentially only one product is obtained that is manifested by a late eluting peak on RP–HPLC with a yield >95%. Acid-quench RP–HPLC was used to analyse the oxidative folding mechanism of kalata B1 in detail by removing aliquots from the folding solution at selected time points (20). In addition to the native peptide, several partially oxidized intermediate species were identified in the process, although not all could be characterized in terms of their disulfide content and disulfide connectivity. Of particular interest, apart from the reduced and native species, was a major sharp peak that turned out to be a two-disulfide native intermediate, which was named II_a (20).

Intermediate II_a has two native disulfides of the cyclic cystine knot (II–V and III–VI) and two reduced cysteine residues (I and IV) (20). It was initially tempting to speculate that since this is a two-disulfide intermediate with two native disulfides, that it would be the last on-pathway intermediate in the oxidation process to the native conformation. However, when this species was isolated and incubated in the folding buffer without disulfide shuffling reagents, it did not form the native peptide and it was therefore concluded that it is not the direct precursor of the native peptide. A later study (21) identified more one- and two-disulfide intermediate species in the folding pathway of kalata B1 by using mass spectrometry and proteolytic digestion, and an additional two-disulfide species was identified that appears to be the direct precursor of the native species. This species lacks the II–V disulfide bond of native kalata B1, which corresponds to one of the outer disulfide bonds that form the embedded ring.

The same study also showed that the quantity of one-disulfide species reaches a plateau at ~50–60% of the total peptide content after 3–5 min from the start of the oxidative folding reaction and remains around this level for ~2 h. The proportion of two-disulfide species reaches a maximum at 30 min, after which it slowly declines until at ~5 h, where there is a rapid decrease (21). In summary, in the oxidative folding of kalata B1 the major intermediate species II_a with disulfides II–V and III–VI is not a direct precursor of the native species but represents a kinetic trap with two native disulfide bonds (Fig. 3). Another two-disulfide species, which is the precursor of the native species, is present in only minor amounts. Hence, the conversion from II_a to the immediate precursor of the native state is the slow, rate-determining step.

Reductive unfolding of kalata B1

The reductive unfolding of kalata B1 was studied recently (20, 21). No intermediates are observed when kalata B1 is reduced at neutral or basic pH with DTT, but several partially reduced species appear when it is reduced at acidic pH with TCEP [Tris(2-carboxyethyl)phosphine]. The species isolated from the reductive unfolding of kalata B1 with TCEP were characterized

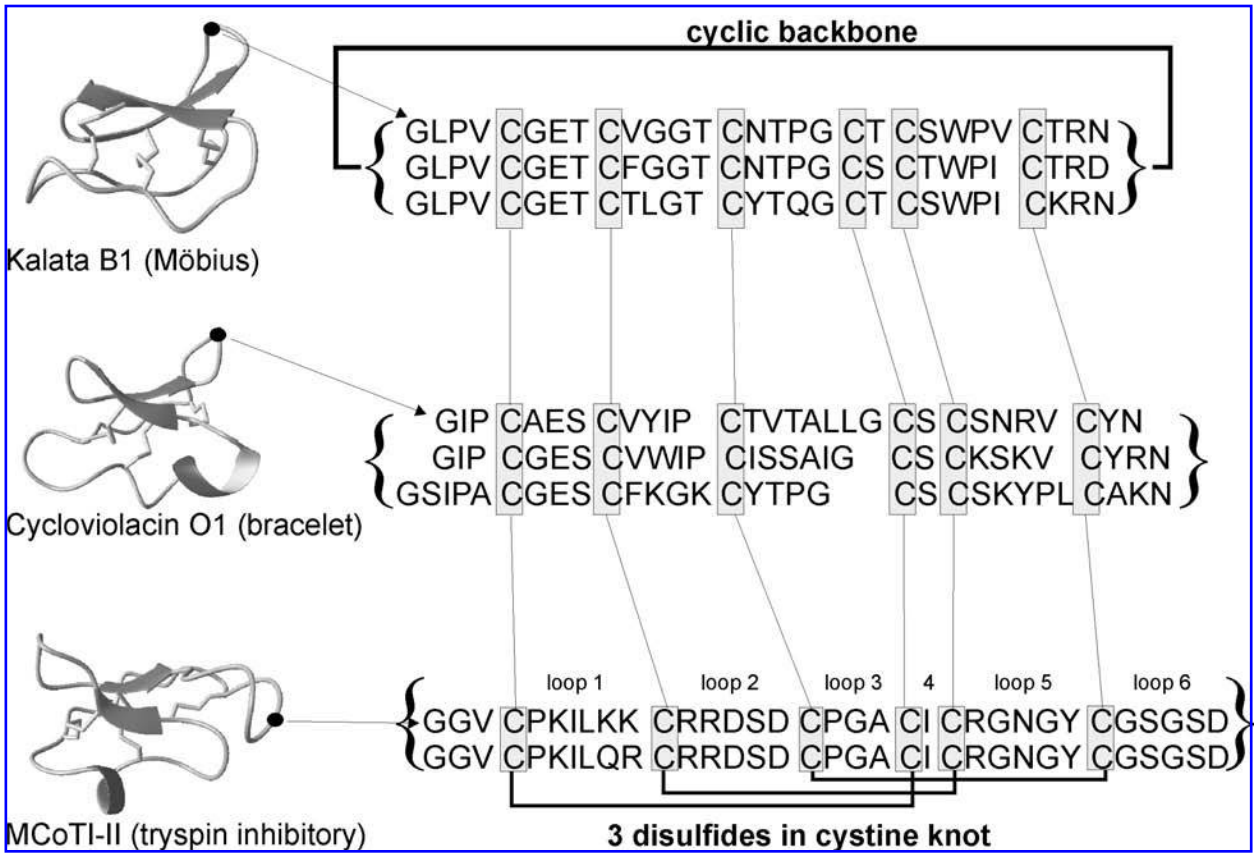

FIG. 1. Cyclic cystine knot motif and cyclotide subfamilies. This figure shows the structures of representative peptides from each of the cyclotide subfamilies: Möbius, bracelet, and the trypsin inhibitory family. Next to each of the structures are representative sequences of peptides from the same subfamily. For the Möbius subfamily, the sequences of kalata B1, kalata B2, and kalata B7 are shown; for the bracelet family, the sequences of cycloviolacin O1, cycloviolacin O2, and cycloviolacin O14 are shown, and for the trypsin inhibitory subfamily MCoTI-II and MCoTI-I are shown, respectively, *from top to bottom*. All peptides incorporate the unique cyclic cystine knot motif, including the cyclic backbone and the three disulfide bonds. These two features are shown at the *top* and the *bottom* of the figure, respectively. The conserved six cysteine residues (I–VI) are shown, along with the names of the intracysteine loops (1–6). The *black circles* on the backbone and the *arrows* indicate the places in the three structures corresponding to glycine residues that occur first in the sequence of the linear precursor proteins.

with a combination of specific labeling, proteolytic digestion, and mass spectrometry (21, 25). This was achieved by isolating and labeling the partially reduced intermediate species with *N*-ethylmaleimide, followed by reduction and aminoethylation of the remaining cysteine residues. Enzymatic cleavage of the modified peptides yielded fragments directly corresponding to the position of the different alkylators, as aminoethylated cysteine is a target site for proteolytic digestion with trypsin (25).

The partially reduced species that were isolated on the reductive unfolding pathway included a one-disulfide species

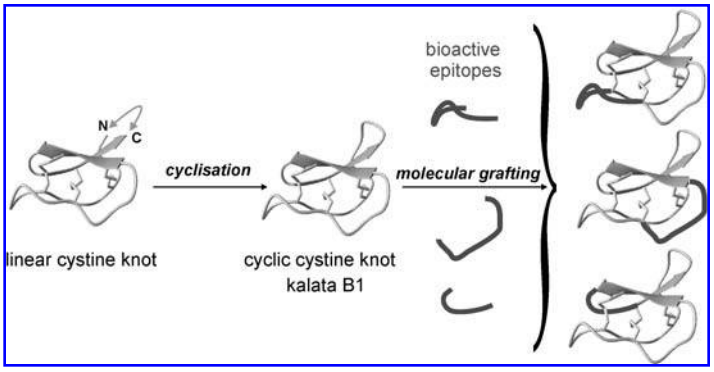

FIG. 2. Applications of cyclic cystine knot motif. Two applications of the cystine knot motif to the design of molecules with potential pharmaceutical applications are shown (12). On the *left-hand side* is a linear cystine knot peptide, whose stability can be increased by joining its N and C-terminal (*cyclization* of the backbone). Alternatively, cyclic cystine knot molecules may be used as molecular scaffolds for the design of molecules with novel activities by including a bioactive epitope into the intracysteine loops on its scaffold (*molecular grafting*).

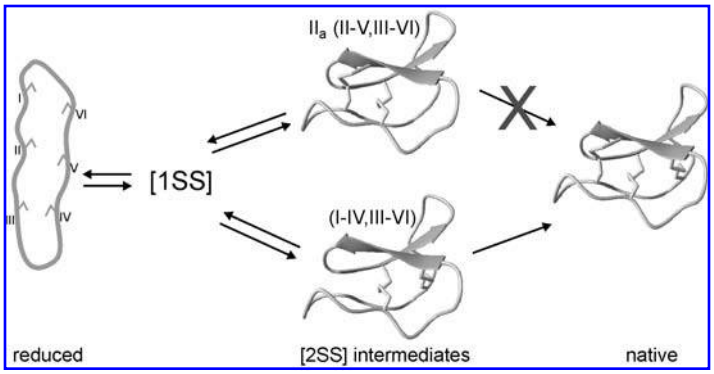

FIG. 3. Oxidative folding of kalata B1: This schematic representation shows a summary of the oxidative folding process for the representative peptide of the Möbius subfamily of cyclotides, kalata B1. From the fully reduced peptide on the *left-hand side*, several one and two-disulfide species are formed, including a major species that can be isolated corresponding to a native two-disulfide intermediate with the disulfide bonds II–V and III–VI. Interestingly, this intermediate is not the direct precursor of the native state and has to be partially or completely reduced to convert to another species, which is the direct precursor of the native species [*i.e.*, the two disulfide species (I–IV, III–VI) shown on the figure].

containing the III–VI disulfide bond and a two-disulfide species incorporating III–VI and I–IV disulfide bonds. The disulfides remaining in these partially reduced species suggest that the first disulfide to be reduced during unfolding is II–V. This strengthens the view that the two-disulfide intermediate lacking the II–V disulfide is likely to be the precursor of the native species during oxidative folding. It also appears that the III–VI disulfide, which is the penetrating disulfide in the original cystine knot, is the most stable to reduction, possibly reflecting its low accessibility from the solvent and its location in the core of the knot with respect to the other two disulfide bonds. Analysis of the rates of unfolding revealed that the rate determining step is the reduction of the disulfide II–V, whereas the formation of one-disulfide species appears rapid after this slow step (21, 25).

In general, it is recognized that there are several types of reductive unfolding mechanisms for disulfide-rich proteins, ranging from an *all-or-none* mechanism, where all disulfide bonds are reduced simultaneously, to other cases where intermediate species can be detected, including ones with non-native disulfide bonds (7). These mechanisms reflect two general ways that disulfide bonds are stabilized in proteins: either in a concerted or an independent fashion. The studies reported for kalata B1 suggest that its disulfide bonds are essentially interdependent and are stabilized in a concerted manner. This is consistent with the compact three-dimensional structure of kalata B1, where the three disulfides are in close proximity in the core of the protein (45).

Oxidative folding of MCoTI-II

MCoTI-II is readily obtained via extraction from the seeds of *M. cochinchinensis* and purified over several rounds of RP–HPLC (8, 24, 36, 37). *M. cochinchinensis* is from the Cucurbitaceae (squash) family of plants, and other plants from the same family also contain trypsin inhibitors having an acyclic cystine knot motif in their structures (8). MCoTI-II has also been produced synthetically (52).

The oxidative folding of MCoTI-II has been recently studied as a first representative of the trypsin inhibitory subfamily of cyclotides and the results compared with those for kalata B1 of the Möbius subfamily (3). The oxidative folding pathway of MCoTI-II is homogeneous, with only two species detected in significant amounts, namely the native species (N) and a folding intermediate (II_a). The native species accumulates to >90% of the overall protein content at pH 8.5 in 0.1 *M* ammonium acetate buffer with 2 m*M* reduced glutathione in just over 4 h, indicating highly efficient disulfide formation (Fig. 4). The oxidative folding was also examined at a higher concentration of reduced glutathione (5 m*M*) and the mechanism did not change with the increased reducing potential of the folding buffer. Increasing the reducing potential does increase the rate of formation of the native species, but not the overall yield (3).

Apart from the native and II_a species, there are several other partially reduced species apparent in the oxidative folding process, but their abundance is very small, as can be seen on the left-hand side of Fig. 4. In particular, a small peak appearing just left of the native species is apparent on the HPLC trace. Previously it has been shown (24) that MCoTI-II is prone to α-/β-aspartyl isomerization, which introduces an extra -CH_2- group into the peptide backbone at the Asp31–Gly32 linkage in loop 6. The isomerization reaction is accelerated by alkaline pH and the β-aspartyl isomer of MCoTI-II is separately detectable, eluting just before the α-isomer on RP–HPLC. From the α-aspartyl isomer, small amounts of the β-aspartyl isomer are obtained, while the complementary reaction is evident as well: from purified reduced β-aspartyl isomer small amounts of the oxidized α-aspartyl are obtained (3). The two isomers have been separated on RP–HPLC and their structures have been studied via 2D NMR (24). The β-aspartyl isomer of MCoTI-II follows the same oxidative folding mechanism as the α-aspartyl isomer with a similar rate constant. This shows that small structural perturbations in loop 6 are not crucial for the folding pathway.

The disulfide bond connectivity of the II_a intermediate species has been determined and reflects a two-disulfide native species with disulfides II–V and III–VI intact and cysteines I and IV reduced (3). This is analogous to the major intermediate species found for the Möbius subfamily representative, kalata B1. Both intermediates lack the I–IV disulfide bond. To determine whether II_a is a direct precursor of native MCoTI-II, the oxidative folding and reductive unfolding of this intermediate were studied (3). During the oxidative folding process, no other species are present in significant amounts and II_a directly interconverts to the native species. Furthermore, we found that II_a readily interconverts to the native species even in the absence of oxidative conditions and at low pH. It is clear that even at low pH, where the rate of disulfide formation and exchange is usually low, the native species is readily formed from II_a

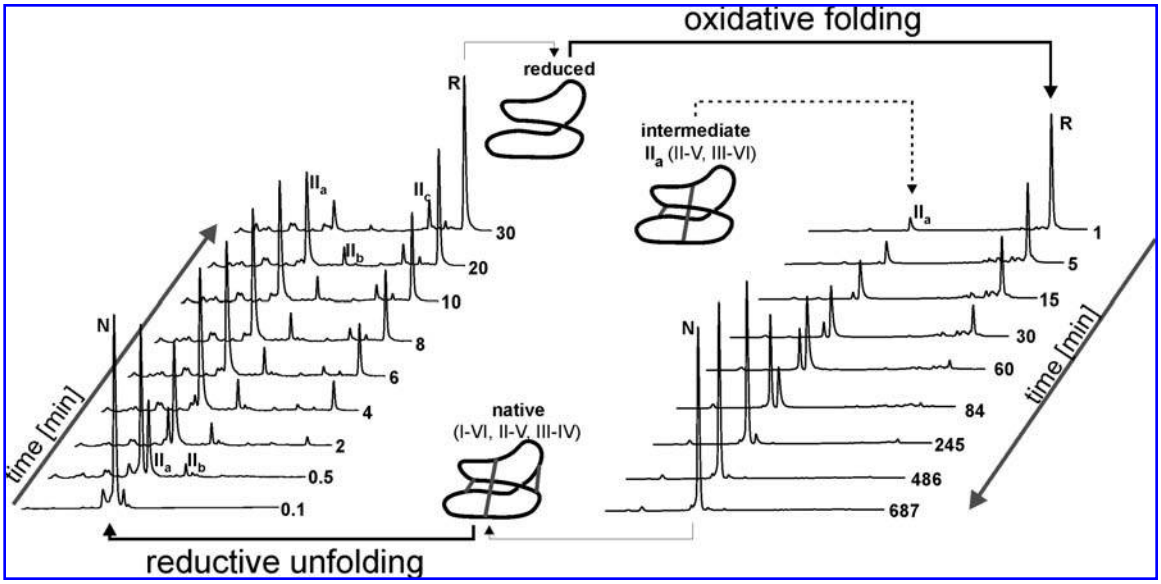

FIG. 4. Oxidative folding and reductive unfolding of MCoTI-II. RP–HPLC chromatograms for both the reductive unfolding and the oxidative folding pathways for MCoTI-II peptide are shown. As can be seen from the *top part* of the figure, when completely reduced peptide is subjected to oxidative folding conditions, it first forms the two disulfide intermediate II_a, which is the direct precursor of the native state. When the native species is subjected to reductive unfolding conditions (*bottom of the figure*), it also forms several partially reduced intermediate species, among them the two-disulfide intermediate II_a.

when no other species were present. This finding points to the fact that II_a is the direct precursor of the native species (3).

The conversion of the II_a to the native species seems to be the rate-determining step despite the fact that it is a direct conversion. This intermediate is stabilized to an extent that it prevails over the formation of other intermediate species, especially any non-native three-disulfide intermediate species, which have been seen to be present on the oxidative folding pathways of some linear cystine knots (4–7). It is clear that II_a has a low free energy and high stability as judged by its relative abundance. At the same time, the energy barrier for the conversion to the native state must be sufficiently high for the intermediate to be kinetically detected along with the native state, but also sufficiently low to allow for the direct conversion of the three-dimensional conformation from the intermediate to the native state (3). The oxidative folding process is summarized schematically in Fig. 5.

Reductive unfolding pathway of MCoTI-II

The reductive unfolding of MCoTI-II is pH dependent. MCoTI-II is highly resistant to reduction at pH 3 with 20 m*M* TCEP, and under these conditions did not produce a significant amount of any refolding intermediates or reduced peptide over 8 h. However, at pH 8, reduction with DTT easily produced >90% of fully reduced species within 30 min. Similarly to the process of oxidative folding, the reductive unfolding pathway features the native (N), fully reduced (R), and the intermediate species detected previously (II_a) and a small number of other intermediate species (Fig. 4). It is therefore clear that MCoTI-II

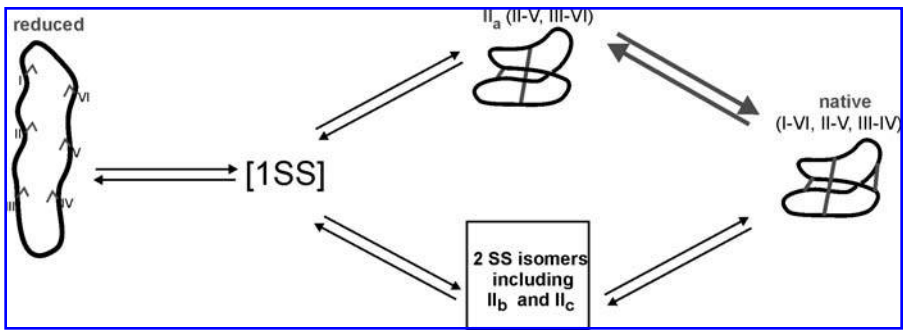

FIG. 5. Summary of oxidative folding process for MCoTI-II. This schematic representation shows a summary of the oxidative folding process for a representative of the trypsin inhibitory family of cyclotides, MCoTI-II. From the completely reduced species, several one and two-disulfide species are formed, the most abundant being the native two-disulfide intermediate species II_a, which is the direct precursor of the native species. This intermediate defines the major pathway in the formation of the native McoTI-II peptide.

is not only oxidized via this intermediate species, but is also reduced via it. Two other minor intermediates, II_b and II_c, appearing on the reductive unfolding pathway were found to be two-disulfide species.

As mentioned above, the reductive unfolding mechanism of a protein depends on how the disulfides are mutually stabilized. The study of the reductive unfolding of MCoTI-II showed that the disulfide bonds are not stabilized in an interdependent concerted fashion, as has been seen above for kalata B1 and some other cystine knot proteins (5, 7). Rather, reductive unfolding involves several intermediate species, among them the one observed in oxidative folding (II_a). This means that the disulfides in this cyclotide are independently stabilized and have different degrees of solvent accessibilities. A similar observation is made when II_a is subjected to reductive unfolding: some minor intermediates are observed that have been previously seen on the reductive unfolding pathway of native MCoTI-II (3). Overall, II_a has a similar mechanism of reductive unfolding as the native species.

STRUCTURES OF OXIDATIVE FOLDING INTERMEDIATES IN CYCLIC CYSTINE KNOTS

The ultimate characterization of folding intermediates requires their 3D structure determination, a task that is not easy given their transient nature. Nevertheless, both of the representatives of the cyclic cystine knot family described above have had the structures of their intermediates elucidated.

Kalata B1 intermediate and its analogue

The major oxidative folding intermediate of kalata B1 with two native disulfide bridges was analysed by NMR, and despite its transient nature, there was sufficient information gained from the TOCSY/NOESY spectra to assign the peaks to individual protons. Analysis of its H_α chemical shifts showed that II_a has a native-like structure and that the two disulfides remaining are II–V and III–VI. The instability of II_a over time prevented a complete structural characterization of this intermediate and so an alanine double mutant was synthesized, replacing the two cysteines that are reduced in the intermediate (Cys I and Cys IV) with alanines. The three-dimensional structure of this modified peptide is very similar to the native peptide, as seen from Fig. 6.

Structural analysis of this double alanine mutant of the major folding intermediate provides a possible explanation why II_a accumulates as a kinetic trap and needs to undergo reduction to one-disulfide species to later reoxidize to the native three disulfides. An analysis of the surface of the double alanine mutant reveals that the side chain of Ala1 replacing Cys1 is almost completely (97.5%) solvent exposed and not in close proximity to Ala15 (20). This unfavorable orientation of the side chain suggests that that the Cys1 side chain in II_a may be distant from that of Cys15, thereby preventing the oxidation of the final disulfide bond. In the native molecule the side chains of the cysteine residues point towards the core of the molecule, which is practically made up of the three disulfide bonds and the side chains of the six cysteine residues.

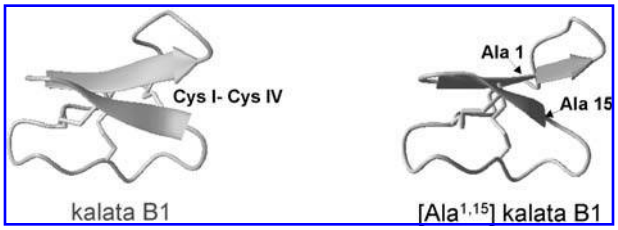

FIG. 6. The three-dimensional structure of a double alanine mutant of kalata B1. A comparison of the three-dimensional structures of kalata B1 and its double alanine mutant Ala(1,15) is shown (20). It can be clearly seen that both structures have an anti-parallel β-sheet and the same connectivity of two disulfide bonds.

The intermediate II_a is a native-like species, so we can expect that the free energy difference between the native and II_a species is small. However, there must be a large energy barrier between these two species since we do not observe direct conversion of II_a to the native state. As noted earlier, there is another two-disulfide intermediate species, which is a direct precursor of the native state. This intermediate must be higher in free energy (if its structure is less native-like), but it apparently has a smaller energy barrier for the conversion to the native state.

This is a clear example of how a highly native-like two-disulfide intermediate species with a low free energy can be a kinetic trap on the oxidative folding process. The intermediate has to be partially (or possibly completely) reduced for it to be able to undergo a rearrangement into a higher energy state (with possible less native-like structure), which has a smaller energy barrier and can rapidly convert to the native state (20).

MCoTI-II intermediate species

The structure of the two-disulfide intermediate II_a for MCoTI-II was determined by solution NMR, and it was noted that it is indeed highly native-like (unpublished data). The native and the intermediate appear similar in most regions, with the major differences occurring between Cys^1–Ile^4 and Ala^{17}–Ile^{19}, where the two cysteines that eventually make up the missing disulfide bridge (Cys^1–Cys^{18}) are located (3) (Fig. 7). One interesting feature of the structure of II_a is the disorder in loops 1 and 6, which are the regions that differ most between the native and intermediate species. It has been suggested that in the native protein the array of different conformations for loops 1 and 6 reflects flexibility rather than just a lack of structural restraints (24).

COMPARISON OF OXIDATIVE FOLDING PATHWAYS IN CYCLIC AND LINEAR CYSTINE KNOTS

The squash trypsin inhibitor EETI-II is structurally very similar to the cyclotides, but does not contain a cyclic peptide backbone. It also has a native two-disulfide intermediate that is detectable during oxidative folding (41). Similarly to MCoTI-II of the trypsin inhibitory subfamily of cyclotides, and in contrast to

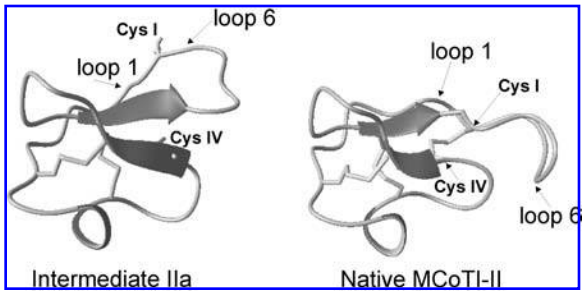

FIG. 7. Structure of the intermediate and native species of MCoTI-II. This representation of the two-disulfide intermediate form II_a and the native species of MCoTI-II shows the similarities and differences between their three-dimensional structures. Specifically, the final disulfide bond appears to influence the orientation of loops 1 and 6 relative to the rest of the structure.

the Möbius subfamily representative kalata B1, the EETI-II intermediate also accumulated on the reductive unfolding pathway and appears to be a true on-pathway intermediate and direct precursor of the native state. Overall, it is clear that there are differences in how disulfides are stabilized in these peptides. For EETI-II and MCoTI-II, both oxidative folding and reductive unfolding pathway show independence of disulfide bridges, which can be reduced or oxidized separately. Kalata B1 by contrast shows an interdependence of its disulfide bridges.

The major differences between these peptides are the size of the cystine knot and the presence or absence of the cyclic backbone. Given that the folding pathways and indeed the sequences of MCoTI-II and EETI-II are more similar to each other than to kalata B1, it appears that it is not the cyclic backbone that is responsible for the differences in the folding pathways. Rather, the size of the embedded ring of cystine knot appears to be crucial for determining the way the disulfide bonds are stabilized. Both EETI-II and MCoTI-II have 11 residues embedded in the ring that forms the core of the cystine knot, whereas kalata B1 has only eight residues due to a shorter loop 1. The larger cystine knot ring for MCoTI-II and EETI-II means that the disulfide bonds are more separated in space and hence can adopt a more independent character in their oxidation or reduction.

OXIDATIVE FOLDING OF CYCLIC CYSTINE KNOT PROTEINS USING PROTEIN DISULFIDE ISOMERASE

All of the discussion so far has related to *in vitro* folding studies on cyclic cystine knot proteins, but there have also been recent developments concerning how folding may occur *in planta*. Protein disulfide isomerases (PDIs) are oxidoreductase

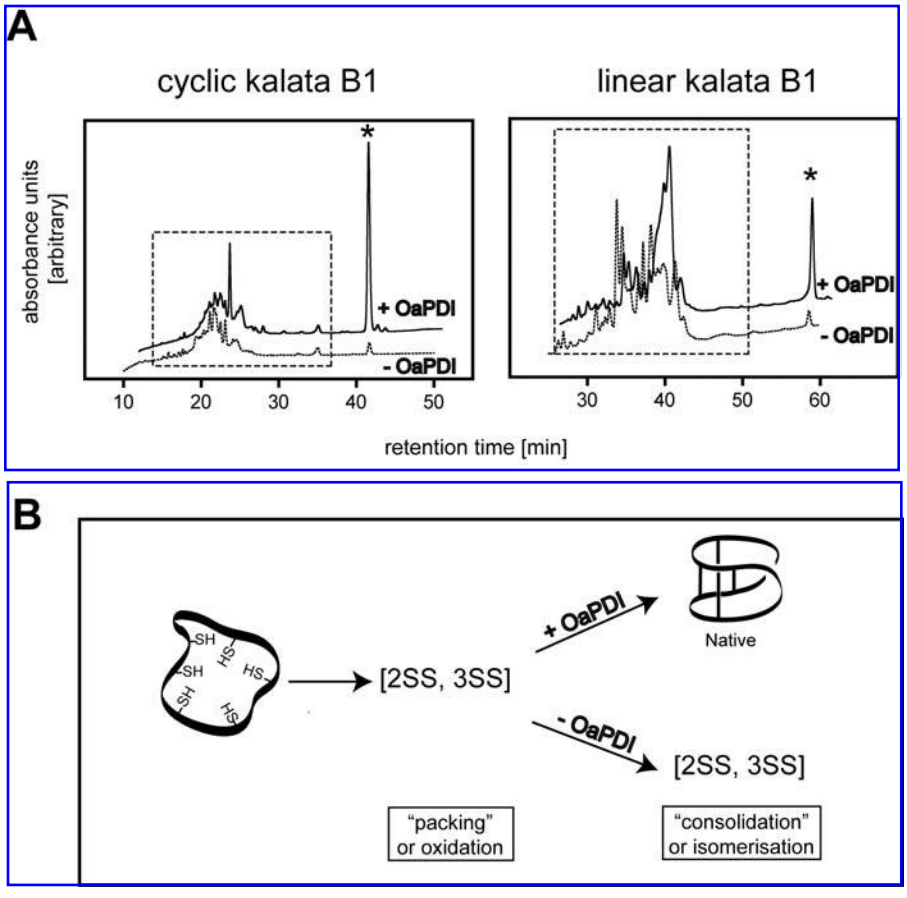

FIG. 8. Oxidative folding of cyclic cystine knot proteins using PDI. (**A**) HPLC spectra of linear and cyclic kalata B1 folded for 24 h at pH 7.5 with *O. affinis* PDI (OaPDI, *solid lines*) or without the addition of enzyme (*dotted lines*). The oxidoreductase OaPDI significantly increases the yield of correctly folded peptide (indicated with an *asterisk*) after 24 h of folding under physiological conditions (31). (**B**) Analysis of folding intermediates of both linear and cyclic kalata B1 without OaPDI (*dashed box* in A) showed mainly three-disulfide misfolded (3SS) and two-disulfide partially folded (2SS) cystine knot peptides. Regardless of the presence or absence of the enzyme, the peptides undergo thiol oxidation, so called packing, to form disulfide bonds. However, the second step, in which non-native disulfide bonds shuffle into their native connectivity (referred to as the consolidation step), is dramatically compromised without OaPDI. Hence, OaPDI appears to have an important role in the isomerization of disulfide bonds during the folding of cystine knot peptides.

enzymes that belong to the thioredoxin superfamily (23, 32). Their major function is the oxidative folding of polypeptides in the endoplasmic reticulum (ER) of eukaryotic cells and they are important ER chaperones (55). We recently isolated a protein disulfide isomerase from *O. affinis* (OaPDI) and characterized it biochemically, confirming it to be a functional oxidoreductase. We further showed that OaPDI is able to correctly fold cyclotides to yield functional insecticidal molecules, suggesting that it may play an important role in folding cyclotides in plants (31).

The mechanism of enzyme-assisted folding of cyclotides was investigated by comparing the folding of linear and cyclic kalata B1 derivatives in the presence and absence of OaPDI. OaPDI dramatically enhances the correct oxidation and folding of kalata B1 at physiological pH (Fig. 8). Folding without PDI under these conditions did not yield any significant amount of correctly oxidized peptides, but rather led to the accumulation of mostly non-native 3SS species and some partially folded 2SS species (31). The presence of OaPDI similarly assisted the folding of a linear version of kalata B1 (Fig. 8). For either linear or cyclic reduced substrate, it appears that oxidation ("packing") proceeds without the enzyme, but the shuffling ("consolidation") of misfolded or partial folded intermediates into their native disulfide connectivity is significantly reduced. This reinforces the notion that isomerization is a major function of the PDI isolated from *O. affinis*.

CONCLUSIONS

With estimates that the cyclotides may grow to contain thousands of members (13), cyclic cystine knot proteins are a potentially large family of molecules. Their topological complexity belies an elegant simplicity of their molecular engineering: interlocking cross-braces are a long-established engineering principle on the macro-scale that nature has produced on the nano-scale in these proteins. The folding studies carried out so far have shown some surprises in that different subfamily members have different direct two-disulfide precursors to the native peptides, although they share a similar stable intermediate. But broadly speaking, the important message to emerge from the folding studies is that these topologically complex molecules fold with remarkable efficiency and specificity. This is good news for the application of these molecules as pharmaceutical templates in peptide-based drug design (12, 14). At the other end of the complexity scale amongst tri-disulfide cyclic peptides is the laddered arrangement of disulfide bonds seen in molecules such as RTD-1 (51, 54) and retrocyclin (42). No systematic folding studies have been reported for these molecules so far, but it will interesting to see if their simpler topology of disulfide bonding leads to different oxidative folding pathways.

ACKNOWLEDGMENTS

Work in our laboratory on cyclotides is supported by grants from the Australian Research Council (ARC) and the National Health and Medical Research Council. Maša Čemažar is an ARC Postdoctoral Fellow, David Craik is an ARC Professorial Fellow, and Christian Gruber is grateful for the support of a University of Queensland Confirmation scholarship.

ABBREVIATIONS

DTT, dithiothreitol; EETI, *Ecballium elataterium* trypsin inhibitor; ER, endoplasmic reticulum; MCoTI, *Momordica cochinchinensis* trypsin inhibitor; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PDI, protein disulfide isomerase; RP-HPLC, reverse-phase high performance liquid chromatography; RTD-1, Rhesus theta defensin-1; TCEP, Tris (2-carboxyethyl)phosphine; TOCSY: total correlation spectroscopy.

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Date of first submission to ARS Central, July 23, 2007; date of acceptance, July 31, 2007.

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