The disulfide-coupled folding pathway of apamin as derived from diselenide-quenched analogs and intermediates

STEFANO PEGORARO, STELLA FIORI, JÖRG CRAMER, SABINE RUDOLPH-BÖHNER,
AND LUIS MORODER

Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany

(RECEIVED January 22, 1999; ACCEPTED April 14, 1999)

Abstract

The sequence of apamin, an 18 residue bee venom toxin, encloses all the information required for the correct disulfide-coupled folding into the cystine-stabilized α -helical motif. Three apamin analogs, each containing a pair of selenocysteine residues replacing the related cysteines, were synthesized to mimic the three possible apamin isomers with two crossed, parallel, or consecutive disulfides, respectively. Refolding experiments clearly revealed that the redox potential of selenocysteine prevails over the sequence encoded structural information for proper folding of apamin. Thus, selenocysteine can be used as a new device to generate productive and nonproductive folding intermediates of peptides and proteins. In fact, disulfides are selectively reduced in presence of the diselenide and the conformational features derived from these intermediates as well as from the three-dimensional (3D) structures of the selenocysteine-containing analogs with their nonnatural networks of diselenide/disulfide bridges allowed to gain further insight into the subtle driving forces for the correct folding of apamin that mainly derive from local conformational preferences.

Keywords: apamin; disulfide bridges; folding; secondary structure; selenocysteine

For an understanding of the complex process of protein folding, the cooperativity between individual interactions required for generation of the energetically most favored, and thus most stable 3D structure has to be identified. With Anfinsen's classic *in vitro* experiments on the refolding of RNase A, the central principle of self-assembly of proteins has been established (Anfinsen, 1973). Correspondingly, proteins do not fold randomly, and thus nonrandom pathways are essential. Delineation of these pathways, however, is intrinsically difficult since transient intermediates of the fast and highly cooperative folding processes are poorly populated.

But in disulfide-coupled folding processes trapping of such transient intermediates becomes possible, and their conformation can be characterized and their kinetic roles determined (for recent reviews see Creighton et al., 1996; Creighton, 1997). The intermediates are generally trapped by alkylating cysteine residues as originally developed by Creighton (1986) for studies of the folding of BPTI and further exploited with some modifications for the refolding of RNase A (Rothwarf & Scheraga, 1993; Rothwarf et al., 1998). More recently, synthetic peptide models were used to mimic folding intermediates (Oas & Kim, 1988; Staley & Kim, 1990) or site-directed mutagenesis with cysteine residues replaced by alanines (Staley & Kim, 1992), or more appropriately, by the isosteric serines was applied for such purpose (van Mierlo et al., 1991a, 1991b, 1994). With latter approaches, the disadvantages of microenvironmental changes introduced by alkylation of cysteine residues is largely bypassed.

In the present communication, we propose an alternative procedure that relies on the peculiar redox potential of selenocysteine (Sec) as isomorphous amino acid analog of cysteine (Besse et al., 1997a). Using apamin, an 18-membered bee venom toxin containing four cysteine residues as model peptide, rational pairwise replacement of cysteines with selenocysteines allowed to produce all three possible diselenide-quenched isomers and related intermediates, and to characterize their structural features for a more precise delineation of their kinetic roles in the folding pathway of this peptide.

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

Abbreviations: Sec, selenocysteine; G-APA, [Sec1,11]-apamin; R-APA, [Sec3,11]-apamin; B-APA, [Sec1,3]-apamin; UV, ultraviolet; CD, circular dichroism; NMR, nuclear magnetic resonance; 1D, 2D, one- and two-dimensional, respectively; DQF-COSY, 2D double-quantum filtered correlation spectroscopy; TOCSY, 2D total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; WATERGATE, water suppression by gradient-tailored excitation; TPPI, time proportional phase incrementation; HPLC, high-performance liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione; *t*Bu, *tert*-butyl; Mob, 4-methoxybenzyl; Trt, trityl; TFA, trifluoroacetic acid; TRIS-HCl, tris-(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediamine-tetraacetic acid; DTT, dithiothreitol; Nle, norleucine = 2-amino-hexanoic acid; MWCO, molecular weight cut-off.

The selenocysteine approach

In a previous study, we have determined the redox properties of bis-cysteinyl-peptides related to the active site portion [10–17] of glutaredoxin with one or both Cys residues replaced by selenocysteine (Besse et al., 1997a; Besse & Moroder, 1997). The redox potential of the bis-Sec-peptide (−381 mV) was found to be significantly more reducing than that of the mixed Sec/Cys-peptide (−326 mV), DDT (−323 mV), glutathione (−205 mV) (Szajewsky & Whiteside, 1980), and the parent Cys,Cys-peptide (−180 mV) (Siedler et al., 1993). These values of the redox potentials clearly suggested that formation of a diselenide bridge is highly more favored than that of a mixed selenide/sulfide bridge. With the synthetic [Sec3,Sec11,Nle7]-endothelin-1 analog, this assumption was fully confirmed, since oxidative refolding of the fully reduced peptide led to the quantitative production of the diselenide and disulfide bridged endothelin in the correct natural isomeric form (Pegoraro et al., 1998). Furthermore, the solution structure of this endothelin-1 analog, as determined by NMR conformational analysis, as well as its bioactivities confirmed the isomorphous character of a Cys → Sec mutation even in absence of strong conformational restraints as present in larger globular proteins. Correspondingly, pairwise replacement of Cys residues with Sec in multiple-cysteine peptides or proteins could open an interesting new approach for the design of productive or nonproductive intermediates of disulfide-coupled folding pathways, since diselenide bridging should occur independently of the presence of additional Cys residues.

To prove the efficiency of such new approach apamin was selected as multiple-cysteine peptide (Callewaert et al., 1968) (Fig. 1). The solution structure of this venom toxin is well established (Bystrov et al., 1978; Miroshnikov et al., 1978; Bystrov et al., 1980; Wemmer & Kallenbach, 1983; Pease & Wemmer, 1988), and it consists of a C-terminal two-turn α -helix cross-linked with two disulfide bonds to the N-terminus itself folded into two reverse-turn structures, thus representing a classical example of the cystine-stabilized α -helix motif (CSH) (Kobayashi et al., 1991; Tamaoki et al., 1998). The native structure of apamin is very stable over a broad range of pH, temperature, and denaturants (Miroshnikov et al., 1978; Chau & Nelson, 1992), and deletion of the C-terminal tripeptide is without any effect on the overall fold and stability of this molecule (Ramalingam & Snyder, 1993). Oxidative refolding of reduced apamin leads to quantitative recovery of the correct natural isomer (Chau & Nelson, 1992; Huyghues-Despointes & Nelson, 1992; Ramalingam & Snyder, 1993). This process is highly cooperative with transient formation of mono-disulfide intermediates with a K_{ox} of ~ 0.025 M (Huyghues-Despointes & Nelson, 1992), followed by a very fast step where the second disulfide bridge ($K_{ox} = 17$ M) leads to the native cysteine connectivities with stabilization of the overall structure (Chau & Nelson, 1992). The one-disulfide intermediates are poorly populated when apamin is equilibrated in glutathione or DDT redox buffers (Chau & Nelson, 1992), and their structures have not yet been determined. Consequently, the folding pathway of apamin is still uncertain in its details.

Results and discussion

Peptide design and synthesis

Random oxidation of peptides containing four cysteine residues leads to a maximum of three isomers named the globule isomer with two crossed, the ribbon isomer with two parallel and the

Fig. 1. Amino acid sequence of apamin and schematic representation of the three synthetic Sec-apamin analogs obtained with regioselective pairing procedures. The selenocysteine residue is represented with a black ball, while the rest of the sequence, represented with grey balls, is identical to the native apamin.

beads isomer with two consecutive disulfide bridges (nomenclature according to Zhang & Snyder, 1991). Of these isomers exclusively, the natural globule isomer is formed in oxidative refolding of apamin, suggesting imperative structural information being encoded in the remaining 14 amino acid residues. To possibly uncode this information, three apamin analogs corresponding to the three possible isomers were synthesized each containing a pair of Sec residues in place of a pair of Cys residues as shown in Figure 1. The substitution pattern was based on the working assumption that formation of the diselenide bridge in oxidative refolding should occur independently of the residual two cysteine residues, thus producing the three possible structural isomers of apamin unless the apparently very strong conformational preferences of this toxin would prevail over the highly differentiated redox potentials of the diselenide and disulfide group. The three Sec-containing apamin analogs are named G-APA as globule, R-APA as ribbon, and B-APA as beads “isomer.” The Sec-containing analogs were synthesized by regioselective diselenide and disulfide bridge pairing procedures as described for the synthesis of the endothelin-1 analog (Pegoraro et al., 1998).

Refolding of the apamin analogs

To prove the ability of selenocysteine to act as an internal “chaperone” and thus, to dictate the folding pathway, the product distribution of oxidative refolding of the fully reduced apamin analogs was analyzed and compared to the starting products. Generation of the natural isomer with crosslinks between residues 1 and 11, and

3 and 15, respectively, from the reduced R-APA or B-APA analogs would mean formation of two mixed selenide/sulfide bridges. The three Sec-apamin analogs were exposed to a large excess of NaBH_4 (pH 7.0) or DDT (pH 8.1) to assure quantitative reduction of the diselenide group (Besse et al., 1997b). Upon dialysis of the reductant (DTT) or its consumption by aqueous buffer (NaBH_4), random oxidative refolding was allowed to proceed and the resulting product distribution was analyzed by CD (Fig. 2) and HPLC. As expected, oxidation of the reduced [Sec1,Sec11]-apamin produced quantitatively the natural G-APA isomer (Fig. 2A) as shown by the superimposable CD spectra and confirmed by the coelution on HPLC of the reoxidation product with the parent peptide. Even for the reduced R-APA and B-APA analogs, almost quantitative formation of the starting compounds, i.e., of the nonnatural isomers, was observed. Therefore the tendency to form the diselenide bridge, as dictated by its redox potential, fully compensates the gain in free energy coupled to the natural fold of apamin. In this context, the quantitative formation of the beads isomer is of particular interest (Fig. 2C), since it contains a disulfide bridge between two Cys residues separated by a tripeptide sequence, and a diselenide bridge between two Sec residues with one intervening amino acid residue. Although such intramolecular cyclic structures are occasionally found in proteins, it is known that the efficient concentrations (C_{eff}) (Creighton, 1983; Lin & Kim, 1989) of these disulfide bridges, as index of how close the cysteine residues are constrained to be, are low (Zhang & Snyder, 1989). HPLC analysis of the oxidation mixture of the R-APA analog revealed a predominant formation of the starting compound with a minor component in a ratio of approximately 9:1. The nature of this second product, which shows the identical mass and tryptic cleavage profile as the R-APA ana-

log, could not be identified. However, the CD spectrum of the reaction mixture is practically identical to the CD spectrum of the R-APA analog (Fig. 2B).

The three possible apamin isomers, i.e., the globule, ribbon, and beads isomer, have previously been synthesized as des-(16–18)-apamin analogs by regioselective disulfide-bridging methods (Ramalingam & Snyder, 1993). Their equilibration in the GSH/GSSG redox buffer at pH 7.4 was found to reshuffle the nonnatural isomers exclusively into the natural globule fold with a K_{ox} of 0.25 M^2 , a value that is only slightly lower than that determined by Chau and Nelson (1992) for refolding of the full length apamin (0.42 M^2) despite the C-terminally truncated sequence.

Equilibration of the Sec-apamin analogs in the GSH/GSSG redox buffer should involve only the disulfide group and thus allow for direct measurement of the C_{eff} of the cysteine pairs in the three diselenide-quenched potential folding intermediates. The K_{ox} of the Cys3,Cys15-pair for formation of the natural globule fold is $\gg 7.1 \text{ M}$, a value that compares well to that estimated for this cysteine pair (17 M) in the highly cooperative refolding of apamin (Chau & Nelson, 1992; Huyghues-Despointes & Nelson, 1992). But high K_{ox} values were obtained also for the Cys1,Cys15-pair in the R-APA analog ($K_{\text{ox}} = 2.5 \text{ M}$) and even more surprisingly for the Cys11,Cys15-pair in the B-APA analog ($K_{\text{ox}} = 7.1 \text{ M}$) where disulfide bridging of the Cys-(Xaa)₃-Cys portion is by orders of magnitude more favored than in model peptides (Zhang & Snyder, 1989), and even three times more favored than the Cys1,Cys15 disulfide of the ribbon analog. These findings suggest a strong link between disulfide-bond stability and stabilization of secondary structure elements (Creighton, 1983), which could yield interesting hints for the folding pathway of apamin.

Fig. 2. Oxidative refolding experiments of (A) G-APA, (B) R-APA, and (C) B-APA. The peptides ($5 \times 10^{-5} \text{ M}$) were dissolved in 10 mM phosphate buffer containing 1 mM EDTA (pH 7.0). CD spectra of the solutions were measured at 20°C (●). A large excess of NaBH_4 was added and after a initial gas formation (30 min), the CD spectra showed the characteristic minimum of random coil structures (◆). The solution was allowed to stand at 20°C and after complete consumption of NaBH_4 by the aqueous buffer the spectra of the refolded peptides were obtained (○).

Solution structures of the apamin analogs and their mono-diselenide intermediates

The CD spectrum of wt-apamin is characteristic of an α -helical peptide with the minima centered at 208 and 222 nm (Miroshnikov et al., 1978; Huyghues-Despointes & Nelson, 1992; Ramalingam & Snyder, 1993). A very similar CD spectrum was obtained for the Sec-containing G-APA analog (Fig. 2A) confirming the isomorphous character of the sulfur/selenium replacement as previously reported for the [Sec3,Sec11,Nle7]-endothelin-1 analog (Pegoraro et al., 1998). The CD spectrum of the ribbon analog R-APA is characterized by a more intense negative band at 206 nm, while the minimum at 222 nm is of reduced intensity (Fig. 2B). It strongly reminds the CD spectrum of [Sec3,Sec11,Nle7]-endothelin-1, which is also folded into the CSH motif, but with cysteine connectivities of the ribbon-type isomer (Pegoraro et al., 1998). The CD spectrum of R-APA is also very similar to that of the truncated des-(16–18)-apamin in the ribbon isomeric form, although it exhibits higher dichroic intensities (Ramalingam & Snyder, 1993). Unexpected was the finding that even the CD spectrum of the B-APA isomer is very similar to that of wt-apamin (Fig. 2C) with large negative bands at 208 and 222 nm, respectively, thus reflecting the presence of a high content of α -helical conformation, whereas for the truncated des-(16–18)-apamin as beads isomer a mainly random coil spectrum was reported (Ramalingam & Snyder, 1993). This might be attributed to destabili-

zation of the α -helix by the C-terminal truncation. However, in 6 M guanidinium hydrochloride where the α -helix should be similarly destabilized, no effect on the dichroic properties of the B-APA analog was observed (data not shown).

Since CD spectra offer only a crude picture of the overall fold, NMR structural analysis was performed on wt-apamin and the three Sec-apamin analogs. A common feature of all the peptides was the *cis/trans*-isomerization of the Ala5–Pro6 peptide bond at different ratios depending upon the compound. However, only for the R-APA analog, which contained the highest amount of *cis*-Pro6-isomer (>20%), a complete conformational analysis was possible, whereas for the G-APA and B-APA molecules in the *cis*-Pro6 isomeric form, the full assignment could not be achieved.

NOEs-derived distance constraints were then used to generate the solution structures of wt-apamin and of its Sec-containing analogs from distance geometry refined by molecular dynamics-simulated annealing calculations. In agreement with the CD spectra, the NMR structures of wt-apamin and Sec1,Sec11-apamin are practically identical, thus fully confirming the isomorphous character of the Cys $\rightarrow$ Sec replacements (Fig. 3A,B). Most unexpected, but already suggested by the CD spectra, was the observation that the three isomers differed in their spatial structures to lower extents than predicted. The overall fold of the three analogs consists of a C-terminal α -helix with a chain reversal in the middle portion of the molecule that brings the N-terminus of the molecule into close contact with the C-terminal α -helix. The wt-apamin and its G-APA

Fig. 3. Solution structures of the (A) native apamin and of the Sec-containing analogs; (B) G-APA, (C) B-APA, and (D) R-APA *trans*-Ala5-Pro6 as derived from 2D NMR spectroscopy. For the single peptides one of the structures with the lowest distance violations is drawn with the program MOLMOL (Koradi et al., 1996). Sequence positions of the Sec and Cys residues are labeled, and the diselenide and disulfide bridges are represented in magenta and yellow ball and stick, respectively. The heavy atoms of Pro6 side chain are also represented in red ball and stick.

analog exhibit the best defined α -helix, which spans the sequence 9 to 16 with a display of the amino acid side chains into the same conformational space. The second turn of the α -helix in the Sec1,Sec11-analog is slightly larger, and this could well be a consequence of the spatial requirements of the selenium atoms. In agreement with previous conformational studies of wt-apamin (Pease & Wemmer, 1988), clear evidence was found for a type-1 β -turn in the N-terminal Asn2 $\rightarrow$ Ala5 sequence part, which orients the backbone axis perpendicular to the α -helix, thus favoring the natural crossed disulfide network between Cys1,Cys11 and Cys3,Cys15.

In the B-APA analog, the C-terminal α -helix is retained although partially distorted and compressed by the intrahelical Cys11,Cys15 disulfide bond (Fig. 3C). Again a hairpin conformation brings the N- and C-termini into proximity despite the absence of a disulfide or diselenide constraint. This well-expressed preference for a bended structure has to be attributed to the strong tendency of such molecules to bury a disulfide, and expectedly, in even more pronounced manner the more hydrophobic diselenide into an hydrophobic environment (Staley & Kim, 1990, 1992). Thereby the side chain of Pro6 is positioned in proximity of the disulfide group as in the natural globule fold. The hydrogen bond between Thr8CO and the Cys11NH found in both wt-apamin and G-APA is still present and contributes to stabilization of the hairpin conformation. A series of bends can be recognized in the N-terminal region of the peptide up to Asp7 with a "nascent" β -turn structure, although the Sec1,Sec3 diselenide bond prevents its full onset. Due to the lack of covalent cross-links, the N-terminus is more flexible than in the other isomers.

As mentioned above, for the R-APA analog complete sequence-specific assignments were achieved for both the *trans*-Pro6 and *cis*-Pro6 isomer. The two isomers were unambiguously identified by the $d_{\alpha\alpha}$ (Ala5,Pro6) and $d_{\alpha\delta}$ (Ala5,Pro6) NOEs derived from the NOESY spectrum of the mixture at conformational equilibrium. No exchange cross peaks were detected in the ROESY spectrum, indicating that the rate of *cis/trans* isomerization of the Ala5-Pro6 peptide bond is slow relative to the 300 ms mixing time applied in

the NOESY experiment. For the *trans*-Pro6 conformer, the parallel ribbon type arrangement of the disulfide and diselenide bridges does not produce radical structural changes (Fig. 3D). In fact, the overall structure of this molecule is very similar to that of the natural globule apamin with a slightly distorted C-terminal α -helix. The N-terminal portion of the molecule is also aligned in perpendicular orientation to the α -helix as in wt-apamin, but the presence of the parallel cross bridges prevents formation of the β -turn. A significantly different spatial array is observed in the N-terminus of the *cis*-Pro6 conformer, where the β -turn structure is definitely disrupted. This N-terminus adopts an extended conformation with the peptide chain aligned in parallel orientation to the α -helix in a mode very similar to that observed in endothelin-1 with its ribbon-type cystine network, as shown in Figure 4 (Tamaoki et al., 1991; Pegoraro et al., 1998). Furthermore, the side chain of Pro6 is moved from the interior core of the molecule to the bulk solvent, whereas no effect is exerted on the C-terminal α -helix.

The folding pathway of apamin

The CD spectra of wt-apamin and of its three Sec-containing analogs, when fully reduced, are very similar in shape and dichroic intensities and are consistent with mainly unfolded structures (Fig. 2). They also remind the CD spectrum of [Ser1,3,11,15]-apamin as mimic of the fully reduced wt-apamin (Huyghues-Despointes & Nelson, 1992). NMR conformational analysis of [Ser1,3,11,15]-apamin analog revealed the absence of detectable medium-range NOEs. However, weak NH-NH NOEs from Glu7 to Arg13 were obtained that were attributed to a nascent α -helical conformation in this portion of the molecule (Xu & Nelson, 1994). The diselenide-quenched Sec1,Sec3-apamin obtained by selective reduction (1.5 equiv. DTT) of the Cys11,Cys15 disulfide bond of the B-APA analog exhibits a CD spectrum with a negative band at 203–204 nm and a large shoulder in the 215–225 nm range of relatively strong dichroic intensities (Fig. 5). This pattern should reflect the presence of α -helical conformation in significant amounts

Fig. 4. Comparison between the solution structure of (A) R-APA *cis*-Ala5-Pro6 (one of the structures with the lowest distance violations is drawn with the program MOLMOL; Koradi et al., 1996) and (B) endothelin-1 (Tamaoki et al., 1991). Sequence positions of the Sec and Cys residues are labeled, and the diselenide and disulfide bridges are represented in magenta and yellow ball and stick, respectively. The heavy atoms of Pro6 side chain in R-APA *cis*-Ala5-Pro6 are represented in red ball and stick.

Fig. 5. CD spectra of the three diselenide-quenched Sec-apamins obtained by selective reduction of the corresponding disulfide bonds with 1.5 equivalent DTT. Cys11,Cys15 in B-APA (◆); Cys1,Cys15 in R-APA (□), and Cys3,Cys15 in G-APA (●).

despite the absence of cross links between the N- and C-terminus of the molecule. Similar CD spectra were obtained also for the diselenide-quenched Sec3,Sec11- and Sec1,Sec11-apamin analogs resulting from reduction of the Cys1,Cys15- and Cys3,Cys15-disulfide bond of the R-APA and G-APA, respectively (Fig. 5). Shape and dichroic intensities of these CD spectra compare well with those reported for the mono-disulfide apamin intermediates trapped by replacing pairwise Cys1,Cys11 or Cys3,Cys15 with alanines (Huyghues-Despointes & Nelson, 1992). NMR conformational analysis performed on these two mimetics of potential apamin folding intermediates confirmed the presence of the C-terminal α -helix in both compounds with a more native-like overall conformation for the [Ala1,Ala11]-apamin analog with the Cys3,Cys15 disulfide bridge, where the NOE pattern from Asn2 to Ala5 was indicative of a type-1 reverse turn in the N-terminus (Xu & Nelson, 1994). From this NMR structural analysis and from the equilibrium constants determined for formation of the mono-disulfide intermediates as well as of wt-apamin, a highly cooperative folding was proposed for this peptide. It consists in a first step of the Cys3,Cys15-disulfide bridging of the N- to the C-terminus of the molecule with induction of considerable native-like structure, which is then stabilized in a second fast step by formation of the Cys1,Cys11-disulfide (Chau & Nelson, 1992; Xu & Nelson, 1994). Thereby the interactions responsible for the stabilization of the Cys3,Cys15-disulfide folding intermediate could not be identified.

In this context, the structural data collected for the nonnatural isomers and the diselenide-quenched intermediates add further pre-

cious information. If the nascent α -helix observed in [Ser1,3,11,15]-apamin (Xu & Nelson, 1994) represents the promoter of the refolding process, the surprisingly high equilibrium constant ($K_{ox} = 7.1$ M) obtained for the Cys11,Cys15 disulfide bridging in the C-terminal α -helix of the B-APA analog may partly result from the tendency to bury the hydrophobic Sec1,Sec3 diselenide group. Nevertheless, it represents a strong indication for a favored formation of this disulfide due to the sequence proximity of the two Cys residues. On the other hand, disulfide bridged Cys residues in positions i and $i + 4$ are not compatible with the classical α -helix. In fact, in the B-APA analog a partly distorted and compressed α -helix was found that should result in significant tension on the disulfide bond, and thus "activate" it for thiol/disulfide exchange reactions. Burying of disulfides is the driving force for the bended structure observed in B-APA, which seems to be ulteriorly favored by the *trans*-Pro6 with his side chain directed toward the inner core of the hairpin. This bended structure brings the thiol groups of both Cys1 and Cys3 into proximity for the nucleophilic attack on the "activated" Cys11,Cys15 disulfide to produce the first link between the N- and C-terminus. The NMR structural analysis of the *cis*-Pro6- and *trans*-Pro6-R-APA molecules clearly revealed that the *cis* configuration favors the endothelin-like extended structure of the N-terminus with parallel alignment to the α -helix, whereas the *trans* configuration, although directing the peptide chain perpendicular to the helix, does not favor the formation of a well-defined β -turn. Turn-type structures have been recognized as nucleating sites of protein folding that form early in the process and direct other folding events (Zimmerman & Scheraga, 1977). Since in the [Ala1,Ala11]-apamin, disulfide bridged between Cys3 and Cys15, a type-1 β -turn was found to be well expressed (Xu & Nelson, 1994), a nucleophilic attack of the Cys3 thiol group on the disulfide-linked Cys15 sulfur should represent the second productive event in the folding pathway (Fig. 6). The final disulfide bridging of Cys1 with Cys11 should then follow with high C_{eff} as proposed by Chau and Nelson (1992) and Huyghues-Despointes and Nelson (1992). That the tendency of the C-terminal portion of the molecule to fold into an α -helical structure is the real promoter of the folding process of apamin is well documented by the study on N-terminal apamin/C-terminal S-peptide hybrids where the helix propensity of the S-peptide and the turn propensity of the N-terminus of apamin with Pro in position 6 were exploited to induce the apamin-type CSH fold (Pease et al., 1990).

Conclusion

In the present study, we have fully confirmed that with selenocysteine replacing cysteine residues in peptides productive and non-productive folding intermediates can be trapped as diselenides in presence of cysteines. This new approach could facilitate studies of disulfide-coupled folding pathways, but more importantly constitute a promising new device for enhancing rate and yields of correct folding of peptides and proteins. How nature encodes for this 21st natural amino acid it is well established (Böck et al., 1991).

Materials and methods

Peptide synthesis

The wt-apamin and the three selenocysteine-containing analogs were synthesized on an ABI 431A automatic synthesizer using

Fig. 6. Schematic drawing of the productive steps in a possible folding pathway of apamin. The off-pathway mechanisms (i.e., *cis-trans* isomerism of Ala5–Pro6 amide bond) are not shown.

TentaGel S RAM (Rink linker) as solid support. For the Sec-containing peptides, the optimized protocols elaborated in previous syntheses (Besse & Moroder, 1997; Pegoraro et al., 1998) were applied and regioselective pairing of Sec and Cys residues was achieved using the Cys(STBu) and Sec(Mob) derivatives as described for the [Sec3,Sec11,Nle7]-endothelin-1 analog (Pegoraro et al., 1998). The wt-apamin was synthesized with standard Fmoc/tBu protocols in combination with the Cys(STrt) protection and air oxidation of the resulting crude product. The oxidized peptides were purified by preparative HPLC using Nucleosil C¹⁸ columns (220 × 12.5 mm) (Machery and Nagel, Dürren, Germany) with linear gradients of 0.1% aqueous TFA and 0.08% TFA in CH₃CN at flow rates of 10 mL min⁻¹ and UV monitoring at 210 nm. Analytical HPLC was performed on Waters equipments with a photodiode array detector using Nucleosil C⁸ columns (250 × 4 mm) by elution with linear gradients of 2% H₃PO₄/CH₃CN (95:5) to 2% H₃PO₄/CH₃CN (20:80) in 30 min at flow rates of 1 mL min⁻¹.

Oxidative refolding experiments

The peptides (5 × 10⁻⁵ M) were dissolved in 10 mM phosphate buffer containing 1 mM EDTA (pH 7.0). CD spectra of the solutions were measured at 20 °C. A large excess of NaBH₄ was added to assure complete reduction of the diselenide bridge. After an initial gas formation (30 min), CD spectra were measured. The solution was allowed to stand at 20 °C and after complete consumption of NaBH₄ by the aqueous buffer, the CD spectra of the refolded peptides were obtained.

In a different experiment, the peptides were dissolved in 0.1 M TRIS·HCl buffer containing 1 mM EDTA (pH 8.1) at a concen-

tration of 10⁻⁵ M. A 1,000-fold excess of DTT was added and the solutions were allowed to react for 90 min, then the reducing agent was removed by dialysis against the TRIS·HCl buffer using benzoylated dialysis tubes with MWCO~2000 (Sigma, München, Germany). The product distribution was determined using analytical HPLC.

Determination of the K_{ox} of disulfide exchange reactions

The peptides (0.1 mM) were dissolved in 50 mM phosphate buffer containing 1 mM EDTA and 0.2 M KCl at pH 7.0 and were equilibrated with GSH/GSSG mixtures at different ratios (total concentration of GSH/GSSG = 50 mM) under exclusion of air. Aliquots of the solution were taken at different intervals, quenched with HCl 6 N and analyzed by HPLC. At equilibrium, peaks areas of HPLC elution profiles served for quantification of the product distribution. The mono-reduced peaks were identified by comparison with the chromatograms obtained for the peptides upon reduction with 1.5 equivalent of DTT. The K_{ox} were determined using the following equation:

$$K_{ox} = \frac{[ox][GSH]^2}{[mono-red][GSSG]}$$

CD spectroscopy

CD spectra were recorded on a Yobin-Yvon dichrograph Mark IV. All spectra are averages of 10 scans and were recorded at 20 °C using quartz cells of 0.1 cm path length. The CD spectra are reported in terms of ellipticity units per mole of peptide residue ($[\theta]_R$).

NMR spectroscopy

NMR spectra of wt-apamin and the Sec-analogs ($2 \div 4$ mM in H_2O containing 10% CD_3COOD) were recorded at 300 K on a Bruker AMX500 spectrometer equipped with a Z-gradient unit for pulsed field gradient spectroscopy. In the 1D proton spectra and in the DQF-COSY experiments, the water peak was suppressed by continuous low power irradiations during the relaxation delays (typically 2 and 1.5 s, respectively), while in the 2D TOCSY, NOESY, and ROESY experiments, the WATERGATE selective single echo scheme (Piotto et al., 1992; Sklenár et al., 1993) was used to remove the water trace. The $\text{NH}-\alpha$ coupling constants were derived from the 1D spectra. Hydrogen-deuterium exchange spectroscopy was performed for all analogs dissolving the peptides in 10% deuterated acetic acid, and 90% D_2O , and measuring 1D spectra at time intervals. All 2D spectra were acquired in phase-sensitive mode using the States et al. (1982) or States-TPPI (Marion et al., 1989) methods. In the TOCSY experiments, a trim pulse of 1.5 ms and a mixing time of $75 \div 82$ ms for the MLEV-17 sequence were implemented and in the NOESY mixing times of 300 ms were applied. Data were zero-filled in the second dimension to yield frequency-domain matrices of $2,048 \times 1,024$ real data points, giving a digital resolution of 2.6 Hz/point in F2 and 5.2 Hz/point in F1. The spectra were multiplied by Gaussian or shifted square sine-bell window functions in both dimensions and Fourier transformed using the XWINNMR 2.0 software on a Silicon Graphics O2 workstation.

Structure calculations

All DG and MD calculations were performed on Silicon Graphics O2 R5000 and Origin 200 workstations using the MSI program package containing InsightII, Discover, and NMRchitect/NMRefine, including the DG-II program. The calculations used a modified consistent valence force field implemented with additional selenium parameters.

Distance geometry (DG) calculations were performed in vacuo. One hundred structures were generated by DG for every peptide using 19 chiral restrictions, the NOEs, 4 or 5 hydrogen bonds, and 11 to 14 dihedral restraints generated from analysis of NOESY and ROESY experiments. NOE intensities were translated into interproton distances by applying (2.0 ± 0.3) Å for very strong, (2.5 ± 0.3) Å for strong, (3.0 ± 0.4) Å for medium, (3.5 ± 0.5) Å for weak, and (4.0 ± 0.8) Å for very weak NOEs. $^3J_{\alpha\text{N}}$ coupling constants were converted to dihedral angles by the Karplus equation with $A = 6.7$, $B = 1.3$, $C = 1.5$. The hydrogen bond patterns used in the calculations were derived from H-D amide exchange experiments, and the carbonyls were identified from NOE connectivities and coupling constants. The force constant used for distance restraints was $50.0 \text{ kcal mol}^{-1}$ and was raised up to $100 \text{ kcal mol}^{-1}$ for hydrogen bonds during calculation. No constraints were included concerning the diselenide or disulfide bonds. The structures were first generated in four dimensions, optimized using the distance-driven dynamics method (Kaptein et al., 1988), reduced to three dimensions using the EMBED algorithm and then optimized using a simulated annealing step in vacuo, and 250 steps minimization with the conjugate gradient algorithm maintaining the distance constraints. Simulated annealing was performed determining first the initial energy and then using distance-driven dynamics (10^4 steps; time step 2 fs) for each structure.

One structure belonging to the DG derived family of most convergent and least violated structures was taken for each apamin

isomer to perform combined molecular dynamic-simulated annealing (MD-SA) calculations with dielectric constant = 80 to obtain correct dihedral angles of disulfide and diselenide bridges. A conjugate gradient algorithm minimization (10^3 steps; time step 1 fs) at 10 K was first run to obtain planar amide bonds. NOE restraints were then introduced, and a $250 \text{ kcal mol}^{-1}$ force was applied on the peptide bonds to keep them planar throughout the simulation. After 1 ps at 10 K the temperature was increased exponentially to a target temperature of 900 K during a 24 ps restraint molecular dynamics simulation. The time constant used was 2 ps. During the following 1,000 ps 500 high temperature structures were saved (one every 2 ps). Subsequent to this, each structure was cooled down to 0 K during 9.5 ps using again a decreasing exponential curve with a time constant of 1 ps.

Acknowledgment

S.P. is supported by a Marie Curie Research Training Grant of the EU.

References

- Anfinsen CB. 1973. Principles that govern the folding of protein chains. *Science* 181:223–230.
- Besse D, Moroder L. 1997. Synthesis of selenocysteine-peptides and their oxidation to diselenide-bridged compounds. *J Peptide Sci* 3:442–453.
- Besse D, Budisa N, Karnbrock W, Minks C, Musiol H-J, Pegoraro S, Siedler F, Weyher E, Moroder L. 1997a. Chalcogen-analogs of amino acids. Their use in X-ray crystallographic and folding studies of peptides and proteins. *Biol Chem* 378:211–218.
- Besse D, Siedler F, Diercks T, Kessler H, Moroder L. 1997b. The redox potential of selenocysteine in unconstrained cyclic peptides. *Angew Chem Int Ed* 36:883–885.
- Böck A, Forchhammer K, Heider J, Baron C. 1991. Selenoprotein synthesis: An expansion of the genetic code. *TIBS* 16:463–467.
- Bystrov VF, Arseniev AS, Gavrilov YD. 1978. NMR spectroscopy of large peptides and small proteins. *J Magn Res* 30:151–184.
- Bystrov VF, Okhanov VV, Miroshnikov AI, Ovchinnikov YA. 1980. Solution spatial structure of apamin as derived from NMR study. *FEBS Lett* 119:113–117.
- Callewaert GL, Shipolini R, Vernon CA. 1968. The disulphide bridges of apamin. *FEBS Lett* 1:111–113.
- Chau MH, Nelson JW. 1992. Cooperative disulfide bond formation in apamin. *Biochemistry* 31:4445–4450.
- Creighton TE. 1983. An empirical approach to protein conformation stability and flexibility. *Biopolymers* 22:49–58.
- Creighton TE. 1986. Disulphide bonds as probes of protein folding pathways. *Methods Enzymol* 131:83–106.
- Creighton TE. 1997. Protein folding coupled to disulphide bond formation. *Biol Chem* 378:731–744.
- Creighton T, Darby NJ, Kemmink J. 1996. The roles of partly folded intermediates in protein folding. *FASEB J* 10:110–118.
- Huyghues-Despointes BM, Nelson JW. 1992. Stabilities of disulfide bond intermediates in the folding of apamin. *Biochemistry* 31:1476–1483.
- Kaptein R, Boelens R, Scheek RH, van Gunsteren WF. 1988. Protein structure from NMR. *Biochemistry* 27:5389–5395.
- Kobayashi Y, Takashima H, Tamaoki H, Kyogoku Y, Lambert P, Kuroda H, Chino N, Watanabe T, Kimura T, Sakakibara S, Moroder L. 1991. The cystine stabilized α -helix. A common structural motif of ion channel blocking neurotoxic peptides. *Biopolymers* 31:1213–1220.
- Koradi R, Billeter M, Wüthrich K. 1996. MOLMOL: A program for display and analysis of macromolecular structures. *J Mol Graphics* 14:51–55.
- Lin TY, Kim PS. 1989. Urea dependence of thiol-disulfide equilibria in thio-redoxin: Confirmation of the linkage relationship and a sensitive assay for structure. *Biochemistry* 28:5282–5287.
- Marion D, Ikura M, Tschudin R, Bax A. 1989. Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J Magn Reson* 85:393–399.
- Miroshnikov AI, Elyakova EG, Kudelin AB, Senyavina LB. 1978. A study of the physicochemical characteristics of the neurotoxin apamin from the venom of the honeybee *Apis mellifica*. *Sov J Bioorg Chem (engl transl)* 4:746–752.
- Oas TG, Kim PS. 1988. A peptide model of a protein folding intermediate. *Nature* 336:42–48.

- Pease JH, Storrs RW, Wemmer DE. 1990. Folding and activity of hybrid sequence, disulfide-stabilized peptides. *Proc Natl Acad Sci USA* 87:5643–5647.
- Pease JH, Wemmer DE. 1988. Solution structure of apamin determined by nuclear magnetic resonance and distance geometry. *Biochemistry* 27:8491–8498.
- Pegoraro S, Fiori S, Rudolph-Böhner S, Watanabe TX, Moroder L. 1998. Isomorphous replacement of cysteine with selenocysteine in endothelin: Oxidative refolding, biological and conformational properties of [Sec³, Sec¹¹, Nle⁷]-endothelin-1. *J Mol Biol* 284:779–792.
- Piotto M, Saudek V, Sklenár V. 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J Biomol NMR* 2:661–665.
- Ramalingam K, Snyder GH. 1993. Selective disulfide formation in truncated apamin and sarafotoxin. *Biochemistry* 32:1155–1161.
- Rothwarf DM, Li Y-J, Scheraga HA. 1998. Regeneration of bovine pancreatic ribonuclease A: Identification of two nativelike three-disulfide intermediates involved in separate pathways. *Biochemistry* 37:3760–3766.
- Rothwarf DM, Scheraga HA. 1993. Regeneration of bovine pancreatic ribonuclease A. I. Steady-state distribution. *Biochemistry* 32:2671–2679.
- Siedler F, Rudolph-Böhner S, Doi M, Musiol H-J, Moroder L. 1993. Redox potentials of active site bis-cysteiny fragments of thiol-protein oxidoreductases. *Biochemistry* 32:7488–7495.
- Sklenár V, Piotto M, Leppik R, Saudek V. 1993. Gradient-tailored water suppression for ¹H-¹⁵N HSQC experiments optimized to retain full sensitivity. *J Magn Reson Series A* 102:241–245.
- Staley JP, Kim PS. 1990. Role of a subdomain in the folding of bovine pancreatic trypsin inhibitor. *Nature* 344:685–688.
- Staley JP, Kim PS. 1992. Complete folding of bovine pancreatic trypsin inhibitor with only a single disulfide bond. *Proc Natl Acad Sci USA* 89:1519–1523.
- States DJ, Haberkorn RA, Ruben DJ. 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J Magn Reson* 48:286–292.
- Szajewski RP, Whiteside GM. 1980. Rate constants and equilibrium constants for thiol-disulfide interchange reactions involving oxidized glutathione. *J Am Chem Soc* 102:2011–2026.
- Tamaoki H, Kobayashi Y, Nishimura N, Ohkubo T, Kyogoku Y, Nakajima K, Kumagaye S, Kimura T, Sakakibara S. 1991. Solution conformation of endothelin determined by means of ¹H-NMR spectroscopy and distance geometry calculations. *Protein Eng* 4:509–518.
- Tamaoki H, Miura R, Kusunoki M, Kyogoku Y, Kobayashi Y, Moroder L. 1998. Folding motifs induced and stabilized by distinct cystine frameworks. *Protein Eng* 11:649–659.
- van Mierlo CP, Darby NJ, Neuhaus D, Creighton TE. 1991a. (14–38, 30–51) Double-disulphide intermediate in folding of bovine pancreatic trypsin inhibitor: A two-dimensional 1H nuclear magnetic resonance study. *J Mol Biol* 222:353–371.
- van Mierlo CP, Darby NJ, Neuhaus D, Creighton TE. 1991b. Two-dimensional 1H nuclear magnetic resonance study of the (5–55) single-disulphide folding intermediate of bovine pancreatic trypsin inhibitor. *J Mol Biol* 222:373–390.
- van Mierlo CP, Kemmink J, Neuhaus D, Darby NJ, Creighton TE. 1994. 1H NMR analysis of the partly-folded non-native two-disulphide intermediates (30–51,5–14) and (30–51,5–38) in the folding pathway of bovine pancreatic trypsin inhibitor. *J Mol Biol* 235:1044–1061.
- Wemmer D, Kallenbach NR. 1983. Structure of apamin in solution: A two-dimensional nuclear magnetic resonance study. *Biochemistry* 22:1901–1906.
- Xu X, Nelson JW. 1994. One-disulfide intermediates of apamin exhibit native-like structure. *Biochemistry* 33:5253–5261.
- Zhang RM, Snyder GH. 1989. Dependence of formation of small disulfide loops in two-cysteine peptides on the number and types of intervening amino acids. *J Biol Chem* 264:18472–18479.
- Zhang R, Snyder GH. 1991. Factors governing selective formation of specific disulfides in synthetic variants of α -conotoxin. *Biochemistry* 30:11343–11348.
- Zimmerman SS, Scheraga HA. 1977. Local interactions in bends of proteins. *Proc Natl Acad Sci USA* 74:4126–4129.