Peptide models of four possible insulin folding intermediates with two disulfides

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Abstract

The single-chain insulin (PIP) can spontaneously fold into native structure through preferred kinetic intermediates. During refolding, pairing of the first disulfide A20–B19 is highly specific, whereas pairing of the second disulfide is likely random because two two-disulfide intermediates have been trapped. To get more details of pairing property of the second disulfide, four model peptides of possible folding intermediates with two disulfides were prepared by protein engineering, and their properties were analyzed. The four model peptides were named [A20–B19, A7–B7]PIP, [A20–B19, A6–B7]PIP, [A20–B19, A6–A11]PIP, and [A20–B19, A7–A11]PIP according to their remaining disulfides. The four model peptides all adopt partially folded structure with moderate conformational differences. In redox buffer, the disulfides of the model peptides are more easily reduced than those of the wild-type PIP. During in vitro refolding, the reduced model peptides share similar relative folding rates but different folding yields: The refolding efficiency of the reduced [A20–B19, A7–A11]PIP is about threefold lower than that of the other three peptides. The present results indicate that the folding intermediates corresponding to the present model peptides all adopt partially folded conformation, and can be formed during PIP refolding, but the chance of forming the intermediate with disulfide [A20–B19, A7–A11] is much lower than that of forming the other three intermediates.

Keywords: Insulin; folding; intermediate; disulfide bonds; kinetics

Since Anfinsen and coworkers first demonstrated that the three-dimensional structure of a globular protein is uniquely determined by its amino acid sequence in the 1960s (Anfinsen 1973), advances have been made in the understand-

ing of protein folding through experimental and theoretical approaches. Studies on the disulfide-coupled folding of some small globular proteins, such as bovine pancreatic trypsin inhibitor (BPTI), RNaseA, and epidermal growth factor (EGF), have revealed a sequence of preferred kinetic intermediates, which define a folding pathway (Weissman and Kim 1991; Creighton et al. 1996; Wu et al. 1998; Wedemeyer et al. 2000). Disulfide bonds introduce nonlocal topological constraints in the folding of polypeptide chain, and their formation provides a convenient assay to monitor kinetic stages of oxidative folding. By stopping refolding at different times, kinetic intermediates with different disulfide linkages can be trapped.

Insulin is a structurally and functionally well-characterized small globular protein containing A- and B-chains linked by three disulfides (one intrachain bond, A6–A11; two interchain bonds, A7–B7 and A20–B19). Its three-di-

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Abbreviations: PIP, recombinant single-chain insulin in which the C terminus of porcine insulin B-chain and the N terminus of porcine insulin A-chain were linked together by a dipeptide, Ala-Lys; IGF-1, insulin-like growth factor 1; BPTI, bovine pancreatic trypsin inhibitor; EGF, epidermal growth factor; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; NMR, nuclear magnetic resonance.

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mensional structure has been well solved by X-ray crystallography (Baker et al. 1988) and nuclear magnetic resonance (NMR; Roy et al. 1990; Weiss et al. 1991). Although the separate A- and B-chains of insulin can be recombined successfully in vitro (Wang and Tsou 1991), a single-chain polypeptide (preproinsulin) is synthesized in vivo. When B29Lys and A1Gly are linked together by a peptide bond directly, the mini-proinsulin still retains the three-dimensional structure identical to that of insulin (Derewenda et al. 1991; Hua et al. 1998). Our laboratory has constructed a single-chain insulin (PIP) that can fold correctly and can be secreted efficiently from transformed yeast cells (Zhang et al. 1996). It can be reasonably presumed that the three-dimensional structure of PIP is identical or very similar to that of insulin/mini-proinsulin.

Insulin-like growth factor 1 (IGF-1) is a 70-residue single-chain globular protein composed of B-, C-, A-, and D-domains from N terminus to C terminus (Humbel 1990). Its B- and A-domains are homologous to the B- and A-chains of insulin, respectively. IGF-1 adopts an insulin-like structure (Cooke et al. 1991; Vajdos et al. 2001; Brzozowski et al. 2002) with three disulfides (18–61, 6–48, 47–52) identical to those of insulin (A20–B19, A7–B7, A6–A11). During refolding of IGF-1, formation of the first disulfide 18–61 (corresponding to the disulfide A20–B19 of insulin) is highly specific, whereas pairing of the second disulfide is random; native and nonnative disulfides can be formed (Rosenfeld et al. 1997; Milner et al. 1999; Yang et al. 1999). Finally, these two-disulfide intermediates are converted into two disulfide isomers with similar thermodynamic stability (Hober et al. 1992, 1994, 1997, 1999; Miller et al. 1993).

In vitro PIP can spontaneously fold into native structure through preferred kinetic intermediates (Qiao et al. 2001). During refolding, formation of the first disulfide A20–B19 is also highly specific and coupled with formation of a partially folded intermediate (Yan et al. 2003). However, pairing of the second disulfide is still elusive. We have trapped two two-disulfide intermediates, but this cannot exclude the formation of other two-disulfide intermediates that play important roles during refolding. It is known that when the remaining folding process is very quick, the percentage of the intermediate is very low and cannot be trapped. To get a more detailed view of the PIP folding pathway at the two-disulfide intermediate stage, four model peptides corresponding to the possible two-disulfide intermediates were prepared by protein engineering. The four model peptides were named [A20–B19, A6–A11]PIP, [A20–B19, A7–A11]PIP, [A20–B19, A7–B7]PIP, and [A20–B19, A6–B7]PIP according to their remaining disulfides. In the four model peptides, A6Cys or A11Cys was replaced by Ala residue, whereas A7Cys or B7Cys was replaced by Ser residue. Here, we report the properties of the four model peptides and the possible role of their corresponding intermediates during PIP refolding.

Results

Analyses of the structural changes of the model peptides with two disulfides

The molecular masses of the purified model peptides were measured by electrospray mass spectrometry: [A20–B19, A7–B7]PIP, 5899.0; [A20–B19, A6–B7]PIP, 5912.0; [A20–B19, A7–A11]PIP, 5913.0. All of the measured values are consistent with the expected values, indicating that the sequences of the model peptides are correct. During purification, we found that all of the secretion yields of the four model peptides are decreased significantly compared with that of the wild-type PIP: ~500 to 1000 µg of purified model peptides can be obtained from 8 L of fermentation supernatant, whereas usually ~100 mg of purified wild-type PIP can be obtained from the same volume of fermentation supernatant. Therefore, the secretion yields of the four model peptides are decreased ~100-fold compared with that of the wild-type PIP.

The disulfides are critical for maintaining the intact structure of insulin/PIP. Deletion of the disulfide A6–A11 or A7–B7 of insulin leads to formation of a partially folded structure (Hua et al. 1996, 2001; Weiss et al. 2000). Here, the conformational changes of the four model peptides were analyzed by native polyacrylamide gel electrophoresis (PAGE; at pH 8.3), reverse-phase high-performance liquid chromatography (HPLC), and circular dichroism (CD), respectively.

The four model peptides were first analyzed by native PAGE (at pH 8.3) as shown in Figure 1. In general, all of the model peptides run more slowly than wild-type PIP. The two model peptides with two interchain disulfides have almost identical mobility rates, so do the two model peptides with one interchain disulfide. However, the model peptides with two interchain disulfides run a little faster than the peptides with one interchain disulfide. Because the mutations do not introduce charge residues into the model peptides, the decrease of their mobility rates must be caused by their conformational changes. This result indicates that the

Figure 1. Native PAGE (at pH 8.3) analysis of the model peptides with two disulfides. Lanes 1–5 represent wild-type PIP, [A20–B19, A6–A11]PIP, [A20–B19, A7–B7]PIP, [A20–B19, A7–A11]PIP, and [A20–B19, A6–B7]PIP, respectively. In each lane, 2 µg of purified sample was loaded. The gel was stained by Coomassie brilliant blue R250.

interchain disulfide is more important than the intrachain disulfide for maintaining the native structure of insulin/PIP.

The four model peptides were also analyzed by C4 reverse-phase HPLC as shown in Figure 2. Here, we need to point out that in this analysis, acetonitrile and trichloroacetic acid (TFA) are used in the elution buffer, and so, the model peptides probably have been denatured in this analysis. However, wild-type PIP is likely still in native state because it is very robust and needs ~7 M guanidine chloride to be fully denatured (Guo et al. 2003). In this analysis, all of the model peptides have longer retention times than wild-type PIP. Among the model peptides, the peptides with two interchain disulfides are eluted earlier than the peptides with one intrachain disulfide. Moreover, the model peptide with two native disulfides is eluted earlier than the model peptide with one native disulfide when the peptides with two interchain disulfides or the peptides with one intrachain disulfide are compared. The different elution times are probably caused by two reasons. First, some hydrophobic residues

buried in wild-type PIP are already exposed in the non-denatured state of the four model peptides, and the different disulfide linkages cause different exposure of the hydrophobic residues. Second, the model peptides have been partially denatured in the elution buffer, more hydrophobic residues are exposed, and the model peptides with different disulfides have different resistance to the denaturation effect of the elution buffer.

The conformational changes of the four model peptides were analyzed by CD, too. Together with previous published results of the insulin analogs with disulfide deleted, it is possible for us to deduce the conformation of the present model peptides. As shown in Figure 3, in the far-UV region, the spectra of the four model peptides are different from that of the wild-type PIP significantly, whereas the differences among the four model peptides are relatively small. Their α -helix contents estimated from the CD spectra are listed as follows: wild-type PIP, 47.5%; [A20-B19, A7-B7]PIP, 17.6%; [A20-B19, A6-B7]PIP, 15%; [A20-B19, A6-A11]PIP, 15%; and [A20-B19, A7-A11]PIP, 13%. Therefore, the α -helix contents of the model peptides are decreased significantly compared with that of wild-type PIP, which indicates that the secondary structures of the model peptides are disturbed significantly by the loss of one disulfide. The near-UV spectra of the four model peptides are also significantly different from that of wild-type PIP, which indicates that the tertiary structures of the model peptides are also disturbed significantly. In previous reports, the NMR results showed that the α -helix in the N-terminal of A-chain (A2-A8) is unfolded, whereas the other two α -helical segments (B9-B19 and A13-A19) are still retained when the intra A-chain disulfide A6-A11 of insulin is deleted (replaced by Ser or Ala residue; Hua et al. 1996; Weiss et al. 2000). Based on this result, we deduce that the conformation of the model peptide with the intra A-chain disulfide A6-A11 deleted, [A20-B19, A7-B7]PIP, is very similar or identical to that of its corresponding insulin mutant: One α -helix (A2-A8) is unfolded, whereas the other two α -helical segments (A13-A19 and B9-B19) are still retained. The NMR results showed that the α -helix in the N terminus (A2-A8) and part of the α -helix in the C terminus (A13-A19) are unfolded, but the B-chain retains native-like supersecondary structure when the interchain disulfide A7-B7 of insulin is deleted (Hua et al. 2001). Therefore, we deduce that the conformation of the model peptide with interchain disulfide A7-B7 deleted, [A20-B19, A6-A11]PIP, is very similar or identical to that of the insulin mutant: Most of the A-chain (α -helix A2-A8 and part of α -helix A13-A19) is unfolded, whereas the B-chain is still folded. As the model peptide with one mispaired interchain disulfide, [A20-B19, A6-B7]PIP, is concerned, we deduce its conformation is between [A20-B19, A7-B7]PIP and [A20-B19, A6-A11]PIP: Most of the A-chain (α -helix A2-A8 and part of α -helix A13-A19) is unfolded, whereas the

Figure 2. C4 reverse-phase HPLC analysis of the model peptides with two disulfides. In each analysis, 100 μ L of sample (5 μ g) was loaded onto a C4 column and eluted by using the elution gradient listed in Materials and Methods.

Figure 3. CD analysis in far-UV region (A) and near-UV region (B). The filled circles indicate wild-type PIP; open circles, [A20-B19, A7-B7]PIP; filled triangles, [A20-B19, A6-A11]PIP; open triangles, [A20-B19, A6-B7]PIP; and filled squares, [A20-B19, A7-A11]PIP.

B-chain still remains folded. For the model peptide with one mispaired intrachain disulfide, [A20-B19, A7-A11]PIP, its structural disturbance is a little more serious than that of [A20-B19, A6-A11]: Probably all of the A-chain is unfolded, but the disturbance on the B-chain is limited because the model peptide with the single disulfide A20-B19 retains some native structure (Yan et al. 2003).

Based on the above analysis, we deduce that all of four model peptides adopt partially folded conformations with part or full of the A-chain unfolded but the B-chain still folded. As deduced above, deletion of one disulfide in the model peptides causes some extent unfolding of the ordered

structure that exists in native insulin/PIP; it probably does not lead to the formation of some ordered nonnative structure. According to the contents of their retained folded structure, the four model peptides can be ranked as follows: [A20-B19, A7-B7]PIP > [A20-B19, A6-B7]PIP > [A20-B19, A6-A11]PIP > [A20-B19, A7-A11]PIP.

Disulfide stability of the model peptides with two disulfides

The intact structure of insulin/PIP must be stabilized by its three disulfides. In turn, the structure has effect on the disulfide stability. Here, the disulfide stability of the four model peptides was analyzed. As shown in Figure 4, on each gel the upper band is the native species with intact disulfides (labeled with native), and the lowest band is the species with disulfides fully reduced (labeled with reduced). For each sample, the disulfides of some species will be reduced after incubation in the redox buffer, and these reduced species will run faster on the native PAGE after modification of their free thiol groups with sodium iodoacetate. On each gel, the different lanes represent different

Figure 4. Disulfide stability analysis of the model peptides with two disulfides. Lanes 1-9 represent that in redox buffer, the ratio of GSH/GSSG (mM/mM) was 0 : 0, 1 : 10, 1 : 5, 5 : 5, 5 : 1, 7 : 1, 10 : 1, 20 : 1, and 30 : 1, respectively. The gel was stained by Coomassie brilliant blue R250.

redox potentials (reduced glutathione [GSH]/ oxidized glutathione [GSSG]) in which the sample is incubated. From lane 1 to lane 9, the ratio of GSH to GSSG is increased gradually; therefore, more and more native species are converted into reduced species. The more stable the disulfides are, the higher the ratio of GSH to GSSG is needed in order to convert the same percentage of native species into fully reduced species. As shown in Figure 4, for wild-type PIP, when 50% of native species is converted into reduced species, the ratio of GSH to GSSG is between 20 : 1 and 30 : 1; whereas for the model peptides, the ratio is between 5 : 1 and 10 : 1. Therefore, the disulfides of the model peptides are more easily reduced than those of wild-type PIP, whereas the differences among the four model peptides are not significant. This indicates that the three disulfides of PIP are mutually stabilized: When one disulfide is deleted, the remaining disulfides are more easily reduced.

In vitro refolding of the model peptides with two disulfides

During PIP refolding, preferred kinetic intermediates are formed. To demonstrate the probability of forming the intermediates corresponding to the four model peptides, *in vitro* refolding of the reduced model peptides was carried out as shown in Figure 5. From the disulfide stability analysis, it is known that the disulfides of the four model peptides are more easily reduced than those of wild-type PIP, so more oxidative redox potential (2.5 mM GSSG, 0.6 mM DTT) is used in present refolding experiments. The high pH value is essential for the efficient refolding of PIP and its analogs (Qiao et al. 2001; Guo et al. 2003); therefore, refolding solution with high pH is used. Although high pH buffer is used, the refolding is still dominated by the redox potential but not by air-oxidation because when the fully reduced PIP (the DTT is removed by desalting) is incubated in the refolding buffer without redox potential for 15 min at 16°C, >90% is still the fully reduced species (data not shown), whereas in the buffer with the redox potential, the refolding is almost complete as shown in Figure 6. The purified model peptides and wild-type PIP are first reduced by 6 mM DTT. The native PAGE (at pH 8.3) analysis indicates that 6 mM DTT is enough to reduce the disulfides of the model peptides and PIP (data not shown). During refolding, the reduced [A20-B19, A6-A11]PIP, [A20-B19, A7-B7]PIP, and [A20-B19, A6-B7] can reform their disulfides with high efficiency; whereas the refolding of [A20-B19, A7-A11]PIP is inefficient; its final refolding yield is approximately threefold lower than that of the other three model peptides. Under more oxidative redox potential (10 mM GSSG, 0.6 mM DTT), this problem cannot be overcome. The refolding curves of the four model peptides and wild-type PIP are shown in Figure 6. The relative refolding rates of the four model peptides are similar, whereas

Figure 5. HPLC profiles of the refolding of the four model peptides with two disulfides. At the indicated reaction time, 100 μ L of reaction mixture was removed, acidified with TFA, and then loaded onto the C4 reverse-phase column and eluted by using the elution gradient listed in Materials and Methods.

the refolding rate of wild-type PIP is a little lower. Therefore, we deduce that the intermediates corresponding to the four model peptides all can be formed during PIP refolding

Figure 6. The actual (A) and normalized (B) refolding curves of the four model peptides with two disulfides. Filled circles indicate [A20–B19, A6–A11]PIP; open circles, [A20–B19, A7–A11]PIP; filled triangles, [A20–B19, A7–B7]PIP; open triangles, [A20–B19, A6–B7]PIP; and filled squares, wild-type PIP. The actual refolding yield was calculated from the peak area of HPLC profiles; the normalized refolding yield is the ratio of actual refolding yield to the highest refolding yield of each model peptide.

because they share similar folding rates. However, because the four model peptides have different folding efficiency, the chance of forming the intermediate with disulfides [A20–B19, A7–A11] is much lower than that of forming the intermediates corresponding to the other three model peptides.

Discussion

The PIP refolding has been investigated previously by trapping disulfide intermediates, and a refolding pathway has been proposed (Qiao et al. 2001). Thereafter, a more de-

tailed view of the refolding at one-disulfide stage has been obtained by constructing the model peptide with the single disulfide A20–B19 (Yan et al. 2003). Although two two-disulfide intermediates have been trapped previously, it is still unknown if other two-disulfide intermediates can be formed during refolding because the intermediates cannot be trapped if the remaining folding step is very quick. Here, four model peptides corresponding to the possible folding intermediates have been prepared in order to get a more detailed view of the PIP refolding at two-disulfide stage. Based on the present low-resolution results (CD analysis) and the previously reported high-resolution structure of the corresponding insulin analogs (Hua et al. 1996; Weiss et al. 2000; Hua et al. 2001), models of structure of the model peptides have been proposed: The B-chain retains full or most of the native supersecondary structure, the structural disturbance is mainly focused on the A-chain, and the α -helix (A2–A8) is fully unfolded. The α -helix (A13–A19) has different extent of unfolding in the four model peptides. However, high-resolution analysis is still necessary in the future to support the present models. In the refolding analysis, the refolding rates of the four model peptides are similar, but their refolding yields are different. This indicates that the two-disulfide intermediates corresponding to the model peptides all can be formed during PIP refolding, but their probabilities are different: The chance of forming the intermediate with disulfides [A20–B19, A7–A11] is much lower than that of forming the other three two-disulfide intermediates.

During PIP and IGF-1 refolding, formation of the first disulfide A20–B19/18–61 is highly specific and coupled with the formation of a compact partially folded conformation (Narhi et al. 1993; Yan et al. 2003). However, pairing of the second disulfide is likely random: Intermediates with native and nonnative disulfides can be formed on the basis of the first disulfide A20–B19/18–61. This indicates that the activation energy of forming the different second disulfide is similar. For IGF-1, these two-disulfide intermediates are finally converted into two disulfide isomers with similar thermodynamic stability; for PIP, these two-disulfide intermediates are finally converted into a unique structure. Therefore, in the refolding of PIP and IGF-1, probably also including other member of insulin superfamily, pairing of the second disulfide is likely a random process: Several intermediates with native or nonnative disulfides can be formed based on the first disulfide A20–B19, but the chance of forming the different intermediates is probably different, just as shown by the present four model peptides. The present four model peptides are only part of all possible two-disulfide intermediates based on the first disulfide A20–B19; there are still two possible two-disulfide intermediates, [A20–B19, B7–A11]PIP and [A20–B19, A6–A7]PIP. In later work, their model peptides will be prepared, and more details of the PIP folding pathway will be revealed.

Materials and methods

Materials

The *E. coli* strains used were DH12S and RZ1032 (*dut*⁻, *ung*⁻). *Saccharomyces cerevisiae* XV700-6B (*leu2*, *ura3*, *pep4*) and helper phage R408 were kindly provided by Michael Smith (University of British Columbia, Vancouver, Canada). The expression vectors of wild-type PIP (pVT102-U/ α MFL-PIP; Zhang et al. 1996) and [A20-B19, A6-A11]PIP (pVT102-U/ α MFL-[A7Ser, B7Ser]PIP; Guo and Feng 2001) were constructed previously. The mutagenesis oligonucleotide primers were chemically synthesized. The chemical reagents used in the experiments were of analytical grade. The Pharmacia Biotech reverse-phase column (Sephasil Peptide C4 5 μ m ST 4.6/250), Gilson 306 HPLC system, and Gilson 115 UV detector were used. In HPLC analysis, a gradient elution was used. Solvent A was 0.15% aqueous TFA; solvent B was 60% acetonitrile containing 0.125% TFA. The elution gradient was as follows: 0 min, 0% solvent B; 1 min, 0% solvent B; 5 min, 45% solvent B; 32 min, 90% solvent B; 33 min, 100% solvent B; 35 min, 100% solvent B; 38 min, 0% solvent B; and 42 min, 0% solvent B. During analysis, the flow rate is 0.5 mL/min, and the detection wavelength is 230 nm.

DNA manipulation

The expression vector encoding the three model peptides was constructed by using a gapped duplex DNA approach for site-directed mutagenesis (Kramer et al. 1984). For the expression vectors of [A20-B19, A7-B7]PIP and [A20-B19, A6-B7]PIP, the plasmid pVT102-U/ α MFL-PIP was used as mutagenesis template; for the expression vector of [A20-B19, A7-A11]PIP, the plasmid pVT102-U/ α MFL-[A7Ser, B7Ser]PIP was used as template. The expected mutations were confirmed by DNA sequencing.

Expression and purification of the model peptides with two disulfides

The expression vectors encoding the model peptides were transformed into *S. cerevisiae* XV700-6B (*leu2*, *ura3*, *pep4*). The transformed yeast cells were cultured in a 16-L fermenter, and the model peptide was purified from the media supernatant according to previously described procedures (Zhang et al. 1996) with some modifications. First, the model peptide was precipitated from the media supernatant by trichloroacetic acid. Second, the precipitate was dissolved with 1 M acetic acid and applied to a Sephadex-G50 column. Third, the product was purified by the DEAE ion-exchange chromatography. Fourth, the eluted model peptide from the ion-exchange column was lyophilized and then dissolved with 2 to 3 mL water, acidified to pH 2–3 with TFA, and then centrifuged. Fifth, the pellet containing the model peptide was purified by C4 reverse-phase HPLC. The purity of the model peptide was analyzed by native PAGE (at pH 8.3) and analytical C4 reverse-phase HPLC.

CD analysis

The model peptides and wild-type PIP were dissolved with 1 mM HCl. The protein concentration was determined by UV absorbance at 276 nm. CD measurements were performed on a Jasco-715 CD spectropolarimeter. The spectra were recorded at room temperature, and the final protein concentration was adjusted to 0.2 mg/

mL. The near-UV spectra were scanned from 300 to 245 nm by using a cell with 1.0-cm path length; the far-UV spectra were scanned from 250 to 190 nm by using a cell with 0.1-cm path length. The data were expressed as molar ellipticity. The software J-700 for Windows secondary structure estimation, version 1.10.00, was used for secondary structural content estimation from CD spectra.

Disulfide stability of the model peptides in redox buffer

The model peptides were dissolved in the buffer (0.1 M Tris-HCl, 1 mM EDTA at pH 8.7) containing different redox potential (GSH/GSSG) at the final concentration of 0.15 mg/mL, respectively. The total volume of each reaction was 30 μ L. At the same time, a negative control (the sample was dissolved in the buffer not containing redox potential) was carried out. The reaction was carried out overnight at 0°C. After incubation, 1 : 5 v/v of freshly prepared 0.5 M sodium iodoacetate solution was added to modify the free thiol groups. The carboxymethylation reaction was carried out for 5 min at room temperature. Then the modified mixture was analyzed by native PAGE (pH 8.3).

In vitro refolding of the model peptides with two disulfides

The samples were dissolved in the buffer (0.5 M Arg-HCl, 1 mM EDTA at pH 9.5) containing 6 mM DTT at the final concentration of 0.5 mg/mL. After incubation for 30 min at 16°C, 6 μ L of reaction mixture was removed and immediately mixed with 3 μ L of 0.5 M sodium iodoacetate solution to carboxymethylate the free thiol groups. Then, the modified samples were analyzed by native PAGE to determine if the samples were fully reduced. Then, the fully reduced sample was 10-fold diluted into the refolding buffer; the final composition of the refolding solution was 0.5 M Arg-HCl, 1 mM EDTA (pH 9.5), 2.5 mM GSSG, 0.6 mM DTT, and 0.05 mg/mL reduced sample. The refolding reaction was carried out at 16°C. At the indicated reaction time, 100 μ L of refolding mixture was removed and acidified to pH 2.0 with TFA and then analyzed by using C4 reverse-phase HPLC. The refolding yield was calculated from the peak area of the refolded samples.

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