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Redox-Active Cyclic Bis(cysteinyl)peptides as Catalysts for In Vitro Oxidative Protein Folding

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Summary

The active-site hexapeptides of glutaredoxin (Grx), thioredoxin (Trx), protein disulfide isomerase (PDI), and thioredoxin-reductase (Trr) containing the common motif Cys-Xaa-Yaa-Cys were conformationally restricted by backbone cyclization, and their redox potentials were found to increase in the rank order of Trr < Grx < Trx < PDI peptide, with E'_0 values ranging between −204 mV and −130 mV. In each peptide the thiol pK_a of one Cys residue was found to be lower than the other (e.g., 7.3 against 9.6 in the PDI peptide). Both the yield and rate of refolding of reduced RNase A in the presence of the bis(cysteinyl)peptides increased with the oxidizing character of the cyclic compounds. These results show that small peptides can function as adjuvants for the in vitro oxidative folding of proteins.

Introduction

Folding of nascent cysteine-containing proteins with concomitant formation of the correct disulfide connectivities is supported in vivo by a wide variety of molecular chaperones and folding catalysts. In the endoplasmic reticulum (ER) of eukaryotes, PDI acts as a molecular chaperone [1–3] and mediates the formation and reshuffling of disulfides [4], although more recently other enzymes have been identified that are possibly involved in this process. Such enzymes include the ER oxidoreductin 1 protein (Ero1p) from *S. cerevisiae* [5, 6] and the human counterpart Ero1-L [7]. In the periplasm of prokaryotes, the enzymes that catalyze the oxidation of nascent proteins belong to the Dsb (disulfide bond) family [8].

These enzymes and the other thiol/disulfide oxidoreductases Trx [9] and Grx [10] share the common redox-active motif Cys-Xaa-Yaa-Cys in an overall highly homologous 3D structure, the so-called Trx fold [11–15], where the tetrapeptide is located at the N terminus of an α helix. Also, Trr contains the same redox-active sequence, but the overall structure of this enzyme differs from the Trx fold [16]. Despite these homologies, the enzymes are characterized by redox potentials ranging from the highly reducing value of −270 mV for Trx [17] to the oxidizing values determined for PDI (−110 mV [18], −175 mV [19], and −145 mV for the isolated N-terminal domain [20]) or DsbA (−122 mV [21]). These different redox properties have mainly been attributed to the active-site sequence composition, as supported by mutagenesis studies [21–26]. However, synthetic fragments related to the active sites of Trx, Grx, Trr, and PDI were all found to exhibit redox potentials in the rather narrow range between −215 mV and −190 mV [27].

In order to investigate whether the artificial constraints imparted to synthetic active-site fragments of the oxidoreductases could mimic those present in the enzymes, we synthesized sequences 10–15 of Grx (**1**), 31–36 of Trx (**2**), 35–40 of PDI (**3**), and 134–139 of Trr (**4**) and subjected them to backbone cyclization (Figure 1). With the exception of the Trr peptide **4**, the Grx, Trx, and PDI peptides **1–3** were better oxidants than the related linear fragments [27], and a strong correlation between redox potentials and catalytic effects on rate and efficiency of RNase A refolding was observed, clearly supporting the usefulness of these small peptides as folding adjuvants. The results also suggest that the major foldase activity of PDI derives from its molecular-chaperone property.

Results

Synthesis of the Bicyclic Peptides 1–4

Based on the redox properties of synthetic linear octapeptides from the active sites of Grx, Trx, PDI, and Trr [27], four new peptides were designed by shortening the backbone length to six residues and by connecting the N and C termini to reduce the conformational freedom (Figure 1).

The linear hexapeptide precursors were synthesized on the chlorotrityl resin with Fmoc/tBu chemistry and cleaved in the side chain fully protected form. The N and C termini were condensed with HBTU/HOBt/DIEA under low peptide concentration in order to favor the intramolecular reaction. The S-*tert*-buthylthio (StBu) protecting groups of the Cys residues were selectively removed by reduction with phosphines [28], and the dithiol compounds were oxidized to the bicyclic form. The monocyclic Grx peptide was converted in high yields to the bicyclic compound **1** with di-*tert*-butylazodicarboxylate in DMF [29], whereas this procedure failed with the monocyclic Trx peptide. The NMR conformational analysis of this peptide in DMF revealed a well-defined planar conformation of the ring with the two thiols pointing outside into opposite directions, which would well explain the unfavorable formation of the disulfide bond in the organic solvent.

Air oxidation of the monocyclic Trx, PDI, and Trr peptides to the corresponding bicyclic compounds **2–4** was a slow process accompanied by formation of a minor fraction of oligomers.

Redox Potentials of the Bicyclic Peptides 1–4

The redox potentials were determined at pH 7 and 25°C by equilibration of the bicyclic peptides **1–4** with the reference redox pairs GSH/GSSG and cysteine/cystine. The oxidized and reduced forms of each reference redox pair were used in large excess over the peptides to allow

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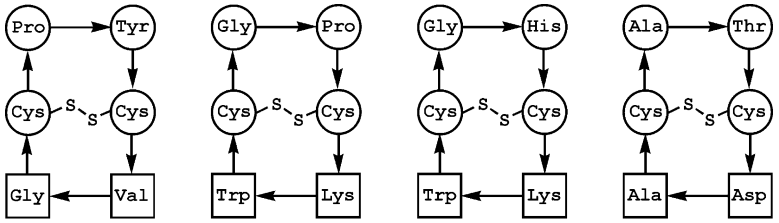

Figure 1. Amino Acid Sequences of the Bicyclic Peptides **1–4**

Circles indicate the active-site residues Cys-Xaa-Yaa-Cys. The arrows indicate the CO→NH amide bonds of the peptide backbone.

their concentrations at equilibrium to be considered unchanged. Multiple experiments were performed at different GSH/GSSG or cysteine/cystine ratios. All the expected redox species present at equilibrium (i.e., the reduced [P_{red}] and oxidized [P_{ox}] cyclic peptides, the two mixed mono-disulfide derivatives with glutathione [P-SG] or cysteine [P-Cys], and the mixed bis-disulfide derivatives with glutathione [P-$(SG)_2$] or cysteine [P-Cys_2], as shown in Figure 2 for compounds **1** and **2**) were identified by LC-ESI-MS. The product distribution at equilibrium was then quantified by HPLC, as previously reported for the linear octapeptides [27]. When compound **2** or **3** was treated with glutathione, the dominant component in the mixture was P-$(SG)_2$, whereas P_{ox} could not be detected, suggesting that formation of stable mixed disulfides was highly favored. The redox potentials for these two peptides were therefore determined only by using the cysteine redox system as a reference. For compounds **1** and **4**, both the glutathione and cysteine reference systems were used, and within the limits of experimental errors, identical redox potentials were derived from the E'_0 values of −240 mV for glutathione [30] and −223 mV for cysteine [31]. Interestingly, peptides **1** and **4** showed a lower tendency to form mixed disulfide bonds with cysteine than with glutathione. As listed in Table 1, the redox potentials of the four bicyclic peptides range from −204 mV for the Trr peptide **4** to −130 mV for the PDI peptide **3**, with the Grx (**1**) and Trx (**2**) peptides showing the intermediate E'_0 values of −178 mV and −152 mV, respectively. Compounds **1–3** were found to be considerably more oxidizing than the related linear octapeptides, whereas compound **4** and the related linear octapeptide had almost identical redox potentials, indicating either that backbone cyclization in the latter case did not induce relevant conformational features or that these could not significantly affect the redox property of the disulfide.

Thiol pK_a Values of the Monocyclic Peptides

pH titration of the thiol groups was followed by measuring the absorbance of the reduced peptides at 238 nm (Figure 3). The pH-dependent absorbance of the Grx peptide was corrected by subtracting the contribution of Tyr because the phenol group has a pK_a of about 10 and its extinction coefficient at 238 nm increases dramatically with the pH. For the Trx and PDI peptides, the contribution of Trp was taken into account, although its absorbance at 238 nm is only slightly dependent on pH. The titration curves of the Trx, PDI, and Trr peptides were fitted to a double titration model [32], which allowed extraction of the pK_a values of 7.7 and 9.8 for the Trx peptide, 7.3 and 9.6 for the PDI peptide, and 7.6 and 10.0 for the Trr peptide (Table 1). The Grx peptide was titrated only up to pH 9 to prevent complete ionization of the Tyr side chain, and a pK_a of 7.9 was derived from fitting the experimental data points to a single titration model [32]. By this procedure the deprotonation of the second thiol group was not experimentally observed, but it is supposed to occur at pH 9.5–10, as for the other peptides.

These pK_a values indicate that in the Cys-Xaa-Yaa-Cys motif of the cyclic peptides the two Cys residues are not equally strong nucleophiles and that one is even

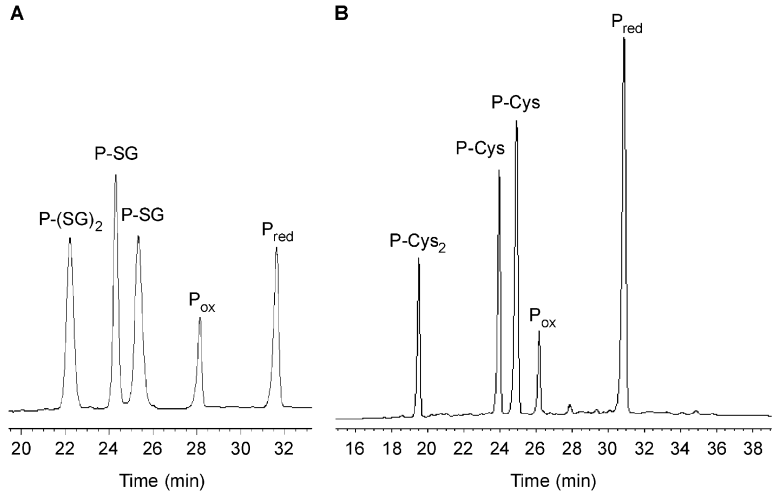

Figure 2. HPLC Profiles of the Thiol/Disulfide Exchange Reactions

(A) Compound **1** equilibrated with GSH/GSSG (1:1) at pH 7 and 25°C.

(B) Compound **2** equilibrated with cysteine/cystine (8.9:1) at pH 7 and 25°C.

Table 1. Thiol pK_a K_{ox} and E'_0 Values of Compounds **1–4**

Peptide	Thiol pK_a	K_{ox} [mM][b]	E'_0 [mV][c]	K_{ox} [mM][b]	E'_0 [mV][d]	E'_0 [mV][e]	E'_0 [mV]
		GSH/GSSG		Cysteine/Cystine		Octapeptide	Enxyme
Grx (**1**)	7.9 ± 0.1 n.d.[a]	9.4 ± 0.4	−179	33 ± 7	−178	−215	−233 [24]
Trx (**2**)	7.7 ± 0.1	n.d.	n.d.	4.2 ± 0.2	−152	−190	−270 [17]
	9.8 ± 0.1						
PDI (**3**)	7.3 ± 0.1	n.d.	n.d.	0.8 ± 0.2	−130	−205	−110 [18]
	9.6 ± 0.1						−145 [20]
							−175 [19]
Trr (**4**)	7.6 ± 0.1	52 ± 5	−201	230 ± 80	−204	−210	−254/−271 [39]
	10.0 ± 0.2						

For comparison, the redox properties of the linear active-site octapeptides Grx-(10–17), Trx-[His[37]]-(31–38), PDI-(35–42), and Trr-(134–141) and of the native enzymes are reported.
[a] Not determined.
[b] The K_{ox} values were extrapolated from the hyperbolic fitting with Equation 2.
[c] $E'_{0\,(GSH/GSSG)} = -240$ mV [30].
[d] $E'_{0\,(cysteine/cystine)} = -223$ mV (adapted from reference [31]).
[e] See reference [27]

a much stronger acid than usually expected for this amino acid (p$K_a \geq 8.4$).

For confirmation of the UV results, pH titration of the thiol groups of the Trx and PDI peptides was followed by NMR (Figure 4). From these experiments the pK_a values of 7.6 and 7.4 were attributed to Trx-Cys[32] and PDI-Cys[39], respectively. These values are in agreement with those determined by UV.

Refolding of Reduced and Denatured RNase A

Refolding of reduced and denatured RNase A was carried out at 30°C under optimal redox conditions (1 mM GSH/0.2 mM GSSG) at a GSH/GSSG/protein molar ratio of 40:8:1 [33], as well as under nonoptimal conditions (0.5 mM GSH/0.1 mM GSSG) at a GSH/GSSG/protein molar ratio of 20:4:1. Rates and yields of refolding were then compared with those determined upon addition of compounds **1–3** (Table 2 and Figure 5). Compound **4** was not investigated for catalytic activity in protein refolding because of its significantly more reducing character. Refolding experiments of RNase A with the corresponding linear octapeptides have been reported previously [34].

Under optimal refolding conditions, about 81% active enzyme was recovered after 23 hr. From a fitting of the experimental curve with a first-order kinetic equation, a maximal yield (A_{max}) of 83% was extrapolated, with an initial refolding rate (v_i) of 3.6 pmol/min. Upon addition of catalytic amounts of peptide, e.g., at a GSH/GSSG/peptide molar ratio of 40:8:0.8, A_{max} and v_i increased with the increasing oxidizing properties of the peptides. Accordingly, compound **1** led to 88% reactivation after 23 hr and to a 14% increment of v_i, whereas compounds **2** and **3** yielded 94% and 95% active RNase A, respectively, with 25% and 39% faster initial rates.

Under nonoptimal glutathione buffer concentrations,

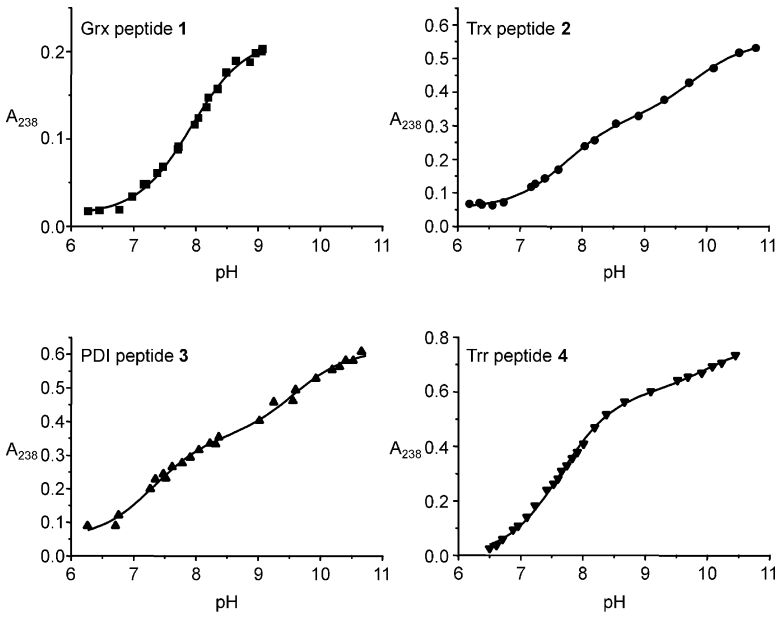

Figure 3. pH Titration of the Thiol Groups of the Reduced Compounds **1–4** at 25°C

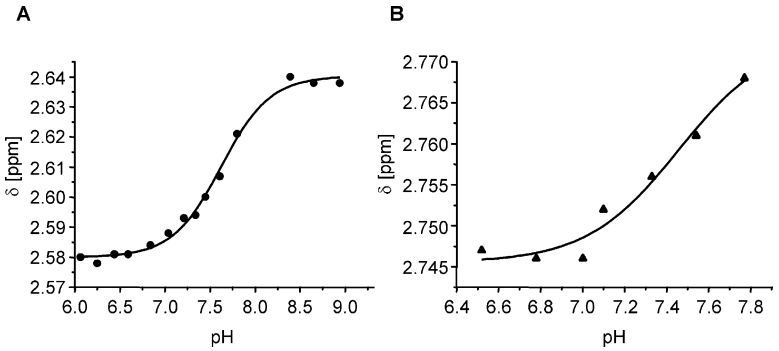

Figure 4. pH Dependence of the Chemical Shifts of the βCH_2 Protons of the Cys Residues

(A) Cys^{32} of the reduced compound **2**.
(B) Cys^{39} of the reduced compound **3**.

only 53% enzyme activity was recovered after 24 hr, and A_{max} and v_i were 64% and 1.1 pmol/min. The effects of the cyclic peptides on the refolding were investigated by replacement of half of the GSSG concentration with each peptide. Compounds **1** and **2** provided a significant recovery of activity (A_{max} about 80%), and the v_i values increased to 1.7 pmol/min and 2.2 pmol/min, respectively. In the presence of compound **3**, 83% active enzyme was recovered after 24 hr, and v_i was enhanced by a factor of 2.5.

These data confirm that both the efficacy and rate of refolding were significantly improved with the increasing oxidizing property of the cyclic peptides, with the PDI peptide **3** being the best oxidant as well as the best refolding adjuvant. Although adding catalytic amounts of the peptides to optimal GSH/GSSG concentrations afforded the best results, the nonoptimal conditions allowed for a clear differentiation of the catalytic activities of the cyclic peptides.

By quantitative replacement of GSSG with the peptides in the presence of GSH, i.e., at a GSH/peptide/protein molar ratio of 20:4:1, the initial rates were twice those obtained with GSSG/peptide ratios of 1:1. However, the yields of reactivated enzyme were significantly lowered; indeed, with compound **3** as the best catalyst only 64% active RNase A was recovered after 11 hr, despite the significantly enhanced initial rate of 6.3 pmol/min.

Finally, the redox activities of the peptides were investigated in the absence of glutathione buffer and compared to the redox property of GSSG at an oxidant/protein ratio of 4:1. After 24 hr, 42% activity was recovered with GSSG, with an A_{max} of 53% and a v_i of 0.9 pmol/min. In comparison, the peptides provided 72% up to 86% active RNase A within 24 hr, with a v_i increment of 2.5 up to four times.

Discussion

From findings of mutagenesis studies of T4 Trx [35], *E. coli* Trx [22, 36], human PDI [23, 37], and *E. coli* DsbA [21, 26], there is a general consensus that the redox properties of thiol/disulfide oxidoreductases are mainly dictated by the sequence composition of the common Cys-Xaa-Yaa-Cys motif in the spatial framework of their almost identical Trx fold. The Pro34His Trx mutant mimicking the active site of PDI provides one example. This mutant was 35 mV less reducing than the wild-type Trx [22], but although containing the PDI active site, it remained far from possessing the strongly oxidizing character of PDI. This would lead to the hypothesis that the amino acid sequence of the active site is not alone sufficient to determine the order of magnitude of the enzyme redox potentials, but additional conformational elements or even the whole 3D structure are likely to play a role as well. Another observation indicating the

Table 2. Kinetic Parameters of Refolding of 24 μM Reduced and Denatured RNase A at pH 7.4 and 30°C, in the Presence of Glutathione and upon Addition of Compounds **1–3**

Protein/GSH/GSSG/Peptide	A_{max}[a] (%)	k[a] (h^{-1})	v_i[b] (pmol/min)
1:40:8:-	83 ± 1	0.18 ± 0.01	3.6 ± 0.2
1:40:8:0.8 (**1**)	89 ± 1	0.19 ± 0.01	4.1 ± 0.2
1:40:8:0.8 (**2**)	93 ± 2	0.20 ± 0.01	4.5 ± 0.2
1:40:8:0.8 (**3**)	95 ± 1	0.22 ± 0.01	5.0 ± 0.2
1:20:4:-	64 ± 2	0.07 ± 0.01	1.1 ± 0.2
1:20:2:2 (**1**)	80 ± 2	0.09 ± 0.01	1.7 ± 0.2
1:20:2:2 (**2**)	77 ± 2	0.12 ± 0.01	2.2 ± 0.2
1:20:2:2 (**3**)	84 ± 2	0.14 ± 0.01	2.8 ± 0.2
1:20:-:4 (**1**)	58 ± 3	0.23 ± 0.03	3.2 ± 0.5
1:20:-:4 (**2**)	56 ± 1	0.37 ± 0.02	5.0 ± 0.3
1:20:-:4 (**3**)	66 ± 1	0.40 ± 0.02	6.3 ± 0.3
1:-:4:-	53 ± 2	0.07 ± 0.01	0.9 ± 0.1
1:-:-:4 (**1**)	75 ± 1	0.14 ± 0.01	2.5 ± 0.2
1:-:-:4 (**2**)	79 ± 1	0.19 ± 0.01	3.6 ± 0.2
1:-:-:4 (**3**)	85 ± 2	0.20 ± 0.02	4.1 ± 0.4

[a] A_{max} and k are the maximal yield and the first-order rate constant extrapolated from the fitting of the experimental curves with Equation 6.
[b] Initial rate obtained from the first derivative of Equation 6 for $t = 0$ and converted from %/h to pmol/min.

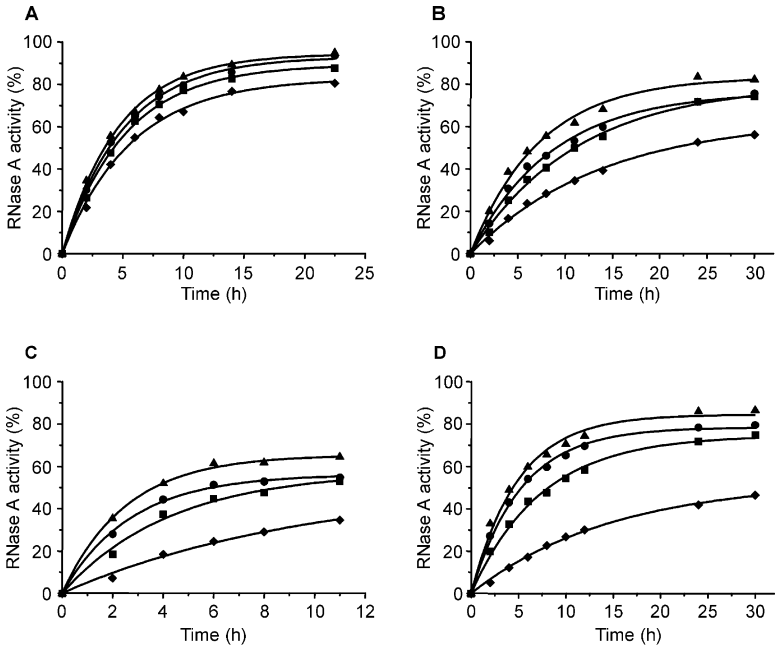

Figure 5. Reactivation of Reduced and Denatured RNase A (24 μM) in Different Redox Buffers at pH 7.4 and 30°C

(A) Forty equivalents of GSH with eight equivalents of GSSG (diamonds) in the presence of 0.8 equivalents of **1** (squares), **2** (circles), or **3** (triangles). The data points are the average of triplicate experiments and are fit to Equation 6.

(B) Either 20 equivalents of GSH with four equivalents of GSSG (diamonds) or 20 equivalents of GSH with two equivalents of GSSG and two equivalents of **1** (squares), **2** (circles), or **3** (triangles).

(C) Twenty equivalents of GSH with four equivalents of GSSG (diamonds), **1** (squares), **2** (circles), or **3** (triangles).

(D) Four equivalents of GSSG (diamonds), **1** (squares), **2** (circles), or **3** (triangles).

complexity of the redox system of oxidoreductases is that, although the β turn conformation adopted by the linear Trr octapeptide in the oxidized form was practically superimposable to the active site of the native protein [38], the redox potential of the synthetic fragment was not comparable to that of the protein [39].

The assumption that introducing an artificial conformational restriction into excised fragments of these enzymes might induce different redox properties was fully confirmed in the present study. Indeed, with the exception of the Trr motif, backbone cyclization conferred more oxidizing activity to the bis(cysteinyl) systems. Moreover, the redox potential range covered by the cyclic compounds **1–4** was almost three times broader than that covered by the related linear octapeptides, and this allowed us to markedly distinguish one sequence from the others.

Interestingly, with a redox potential of −130 mV, which is close to that of the bacterial enzyme DsbA [21], the cyclic motif of PDI was the best oxidant among those examined, just as the PDI enzyme is the strongest oxidant within the Trx family. Surprisingly, the E'_0 value of the cyclic Trx sequence was almost 120 mV higher than that of the native protein; however, it should be pointed out that, with the exception of the PDI-related linear fragment, all eight linear and cyclic synthetic fragments were more oxidizing than the natural proteins (Table 1). The striking difference between peptide and enzyme redox potentials would be in agreement with the previous hypothesis that the E'_0 values of the enzymes are the result of a combination of the amino acid composition of the redox site and the entire protein structure.

Of interest was the finding that in each of the peptide dithiols the pK_a value of one SH group was about two pK_a units lower than the other. This reflects what was also found in the native enzymes, in which the more N-terminal Cys residue is extraordinarily nucleophilic (e.g., pK_a of 3.3 for DsbA-Cys^{30} [21], 4.5 for PDI-Cys^{36} [23], 7.1–7.4 for Trx-Cys^{32} [40]), a property that has been attributed to the stabilization of the thiolate group by the active-site α helix dipole [41]. Moreover, a hydrogen bond between the N-terminal active-site Cys thiolate and the C-terminal active-site Cys thiol was suggested to be present in the NMR structure of reduced *E. coli* Trx [42], in the crystal structure of human Trx [43] and *E. coli* DsbA [44], and in the NMR structure of *E. coli* Grx-3 [45].

Although one of the two Cys residues within the Cys-Xaa-Yaa-Cys motifs of the cyclic peptides showed significantly different ionization properties compared to those of a normal Cys residue, its nucleophilic character was still much weaker than that found in the PDI and DsbA enzymes, and a sequence dependency of the pK_a value as observed in the case of mutants of the Dsb family [21] was not found in the cyclic active-site fragments. Again, these results suggest that the structural environment that surrounds the enzyme active site affects the nucleophilicity of the thiol groups.

The NMR experiments carried out on the reduced Trx and PDI peptides at different pH values revealed that Cys^{32} was the more acidic one in the Trx peptide, as in the enzyme, and Cys^{39} was the more acidic one in the PDI peptide, contrary to the situation in the native enzyme. The formation of the thiol anion of Cys^{39} instead of Cys^{36} in the PDI fragment may be partially favored by the presence of His^{38}, which accordingly showed an increase in its pK_a value from 6.0 to 6.5, as determined by NMR pH titration (data not shown).

The high tendency of the Trx and PDI peptides to form mixed disulfides with glutathione can be related to their high redox potentials, but also electrostatic interactions involving the negative net charge of glutathione and the lysine and/or histidine side chains on the two peptides [46] can favor the formation of mixed disulfides. In fact, mixed disulfides with cysteine lacking a net charge were formed in relatively smaller amounts.

In addition to its chaperone activity [1–3], which is still disputed [47], the main role of PDI relies on both the oxidation of cysteines and the isomerization of nonnative disulfide bonds. In glutathione buffer, folding intermediates between the reduced proteins and glutathione represent the PDI and Grx substrates that display synergistic activities on the catalysis of thiol/disulfide exchange reactions along the oxidative refolding pathway [48, 49]. Under optimal GSH/GSSG buffer conditions, addition of the PDI enzyme to reduced RNase A enhanced the steady-state rate of the in vitro refolding several times [33]. In comparison, catalytic amounts of the PDI peptide enhanced the initial rate only moderately, nevertheless of significance was the almost quantitative recovery of refolded RNase A. In the absence of glutathione buffer, and thus under conditions providing the required oxidizing equivalents by the cyclic peptides, i.e., at the 4:1 peptide/protein ratio, the peptides proved to be much more efficient than GSSG. Under these conditions, the folding pathway involves a direct transfer of the peptide disulfide to the reduced RNase A via covalent disulfide-bridged intermediates and then an escape of the peptide as a dithiol as a result of its low K_{ox} value. The dithiol form, in turn, initiates the isomerization process via its nucleophilic attack on nonnative disulfides, from which the peptide finally escapes in its reduced form.

The results obtained with the cyclic bis(cysteinyl)peptides confirm that, in addition to a highly oxidizing redox potential required for an efficient transfer of oxidizing equivalents to the protein substrates, a low pK_a of one Cys residue in the active site and particularly the chaperone activity of the protein disulfide isomerases PDI [1–3] and DsbC [50] are decisive for the catalytic efficiency of these foldases. The cyclic hexapeptides and especially the PDI peptide showed interesting redox and acidic properties and, despite the lack of chaperone activity, provided better protein refolding conditions than the glutathione redox buffer.

Significance

Since its introduction, the glutathione buffer has been routinely used for refolding cystine-containing proteins in vitro [51], and in order to bypass the absence of molecular chaperones, researchers have generally applied aggregation-preventing additives such as guanidine hydrochloride or related compounds to this process. Recently, the addition of dithiols has been shown to offer significant advantages [32]. In this context, low-mass cyclic bis(cysteinyl)peptides, which can be readily separated from the refolded proteins, may represent a major advance because members of this class of dithiol folding adjuvants guarantee efficient transfer of oxidizing equivalents to the substrate and retain the required disulfide reshuffling properties.

Experimental Procedures

Materials and Methods

All amino acid derivatives, resins, reagents, and solvents were of the highest quality commercially available. For peptide characterization, analytical HPLC was performed on Waters equipment (Eschborn, Germany) with Nucleosil C_8 (5 μm, 100 Å, 4 × 125 mm, Macherey-Nagel, Düren, Germany) and XTerra MS C_8 (5 μm, 3.9 × 150 mm, Waters) columns and a linear gradient from CH_3CN/2% H_3PO_4 (5:95) to CH_3CN/2% H_3PO_4 (90:10) in 15 min (flow rate, 1.5 ml/min; UV detection at 210 nm). Preparative HPLC was carried out on Abimed equipment (Langenfeld, Germany) with Nucleosil C_{18} (5 μm, 100 Å, 21 × 250 mm, Macherey-Nagel) or C_8 (5 μm, 300 Å, 21 × 250 mm, Macherey-Nagel) columns and the following gradients of 0.08% TFA in CH_3CN (A) and 0.1% TFA in H_2O (B) at a flow rate of 10 ml/min: 5%–55% (A) over 40 min for the Cys(StBu)-cyclopeptides; 15% (A) for 5 min to 50% (A) over 90 min for compound **1**; 2% (A) for 4 min to 50% (A) over 56 min for compound **2**; 2% (A) for 4 min to 50% (A) over 46 min for compound **3**; 2% (A) for 15 min to 40% (A) over 45 min for compound **4**.

ESI-MS spectra were recorded on a Perkin-Elmer SCIEX API 165 triple quadrupole spectrometer. LC-MS was carried out with a Nucleosil C_{18} column (5 μm, 100 Å, 1 × 250 mm, Macherey-Nagel) with linear gradients of 0.1% TFA in H_2O and 0.08% TFA in CH_3CN (flow rate: 30 μl/min; detection at 210 nm). Quantitative amino acid analysis of the acid hydrolysates (6 M HCl + 2.5% thioglycolic acid, 110°C, 48 hr) was performed on a Biotronik amino acid analyzer (model LC 6001), in order to confirm the amino acid composition as well as to determine the peptide content. UV measurements were recorded on a Perkin-Elmer spectrophotometer (model Lambda 19) equipped with a thermostated cuvette holder and a Peltier temperature programmer (DBS, Padova, Italy); quarz cuvettes with a light path of 1 cm were also used. The pH values were measured on a Metrohm (Herisau, Switzerland) pH meter (model E 632), freshly calibrated with pH 7.00, pH 8.00, pH 9.00, and pH 10.00 standard solutions from Merck.

Synthesis of the Monocyclic Grx, Trx, PDI, and Trr Hexapeptides

Peptide chain assembly was performed with an automated synthesizer (0.12 mmol scale) or by hand (0.3 mmol scale) on preloaded chlorotrityl resin (0.43–0.54 mmol/gr) from Pepchem (Tübingen, Germany) by the use of Fmoc amino acids (Alexis, Läufelfingen, Switzerland) with the following side-chain protecting groups: tBu for Asp, Thr, and Tyr; Boc for Lys and Trp; Trt for His; and StBu for Cys. Double couplings (2 × 45 min) were performed in NMP at Fmoc-Xaa-OH/HBTU/HOBt/DIEA molar ratios of 4:4:4:8, or 4:4:4:5 for Cys(StBu) to avoid racemization [52]. For Fmoc cleavage, double treatment with 20% piperidine in NMP (1 + 13 min) was applied. The side chain-protected linear peptides related to Trx, PDI, and Trr were cleaved from the resin with 1% TFA in CH_2Cl_2 (10 x 4 min). The filtrates were analyzed by TLC ($CHCl_3$/MeOH/H_2O, 8:3:1) and the peptide-containing filtrates were combined and poured into 10% pyridine in MeOH. The resulting solutions were concentrated to a minimum volume, and the peptides were precipitated from ice-cold water, filtered off, and dried. The Grx-related peptide was cleaved from the resin with TFA/triethylsilane (99:1) in order to simultaneously deprotect the Tyr side chain. After being stirred for 1 hr at room temperature, the mixture was concentrated to a small volume, and the product was precipitated from cold ether, isolated by centrifugation, and dried. The crude peptide was then purified by gel chromatography on Fractogel TSK/HW 40S (Merck AG, Darmstadt, Germany) under MPLC conditions with 2-propanol/*n*-butanol/0.1 M ammonium acetate (pH 5.8) (2:2:7) as eluent (detection at 214 nm). The linear hexapeptides were characterized by ESI-MS and analytical HPLC (homogeneity > 90%).

Head-to-tail cyclization was accomplished in freshly distilled DMF at 0.8 mM peptide concentration and 0°C–5°C in the presence of HOBt/HBTU (1.5 equivalents each) by the addition of DIEA (4 equivalents) portionwise over 2 hr. After completion of the reaction (generally within 3 hr) as monitored by HPLC, the solvent was removed, and the products were precipitated from AcOEt with *n*-hexane. The side chain-protected cyclic peptides were then exposed to TFA/triisopropylsilane/H_2O (92:4:4) for 2 hr at room temperature and successively concentrated to a small volume. The crude products were precipitated with cold ether, collected by centrifugation, lyophilized from *tert*-butanol/H_2O (4:1), and purified by preparative HPLC. The lyophilized peptides were analytically characterized by ESI-MS and HPLC (homogeneity > 95%).

Final deprotection of the cysteine thiol groups was achieved by

reduction at room temperature with excess tributylphosphine (20 equivalents) in TFE/H_2O (95:5) at 1 mM peptide concentration. After 3 hr, the reaction mixtures were taken to dryness, and the solid residues were dissolved in a small volume of MeOH and precipitated with cold ether. The precipitates were collected by centrifugation, washed several times with ether, and lyophilized from *tert*-butanol/H_2O (4:1). The dry peptides were found to be highly homogeneous at the HPLC (> 95%).

Reduced Grx peptide **1**: ESI-MS, 623.2 $[M + H]^+$; M_r = 622.22 (calculated for $C_{27}H_{38}N_6O_7S_2$).

Reduced Trx peptide **2**: ESI-MS, 675.4 $[M + H]^+$; M_r = 674.27 (calculated for $C_{30}H_{42}N_8O_6S_2$).

Reduced PDI peptide **3**: ESI-MS, 715.4 $[M + H]^+$; M_r = 714.27 (calculated for $C_{31}H_{42}N_{10}O_6S_2$).

Reduced Trr peptide **4**: ESI-MS, 565.2 $[M + H]^+$; M_r = 564.17 (calculated for $C_{20}H_{32}N_6O_9S_2$).

Synthesis of the Bicyclic Peptides 1–4

Oxidation of the monocyclic to the bicyclic peptides **1–4** was performed in organic solvent with di-*tert*-butyl azodicarboxylate or in aqueous solution with air oxygen. To the peptide solution (0.27 mM in DMF) di-*tert*-butyl azodicarboxylate (1 equivalent in DMF) was added portionwise over 20 min. The mixture was stirred for 3 hr at room temperature, then taken to dryness. The solid was dissolved in a small volume of MeOH and, after the addition of ether, the precipitate was centrifuged and lyophilized from *tert*-butanol/H_2O (4:1). This procedure was successful for the preparation of compound **1** and more or less successful for compounds **3** and **4**, but it failed in the case of compound **2**. Alternatively, the peptide solutions (0.8 mM in 0.1 M ammonium acetate [pH 7]) were gently shaken under air-oxygen at room temperature and analyzed at time intervals by HPLC. After 5 days the mixtures were acidified with acetic acid and lyophilized. The bicyclic products were purified by preparative HPLC and lyophilized.

Grx peptide **1**: ESI-MS, 621.4 $[M + H]^+$; M_r = 620.21 (calculated for $C_{27}H_{36}N_6O_7S_2$); HPLC, >98%; amino acid analysis, Gly 0.96 (1) Pro 1.00 (1) Cys 1.88 (2) Val 0.88 (1) Tyr 1.01 (1); peptide content, 85.5%.

Trx peptide **2**: ESI-MS, 673.4 $[M + H]^+$; M_r = 672.25 (calculated for $C_{30}H_{40}N_8O_6S_2$); HPLC, >95%; amino acid analysis, Gly 1.00 (1) Pro 1.00 (1) Cys 1.90 (2) Lys 0.91 (1) Trp 1.03 (1); peptide content, 76.7%.

PDI peptide **3**: ESI-MS, 713.4 $[M + H]^+$; M_r = 712.26 (calculated for $C_{31}H_{40}N_{10}O_6S_2$); HPLC, >98%; amino acid analysis, Gly 1.00 (1) Cys 1.81 (2) Lys 0.96 (1) His 0.98 (1) Trp 1.00 (1); peptide content, 68.0%.

Trr peptide **4**: ESI-MS, 563.2 $[M + H]^+$; M_r = 562.15 (calculated for $C_{20}H_{30}N_6O_9S_2$); HPLC, >95%.

Thiol/Disulfide Exchange Equilibria with GSH/GSSG

For equilibration with GSH/GSSG, compounds **1–4** were dissolved in argon-flushed phosphate buffer (0.1 M, pH 7.0) containing 0.1 M NaCl and 1 mM EDTA. GSH and GSSG were separately dissolved in phosphate buffer, and the pH of both solutions was adjusted to 7.0 with 1 M NaOH. In typical equilibration reactions the bicyclic peptides (0.20–0.27 mM) were incubated with 80- up to 200-fold excess glutathione at different [GSH]/[GSSG] molar ratios (0.31, 0.54, 0.80, 1.00, 1.07, 1.79, and 2.10 for compound **1**; 0.38, 0.80, 0.88, 1.07, and 2.14 for compound **2**; 0.30 and 1.05 for compound **3**; 0.50, 1.00, 1.50, 2.00, and 3.00 for compound **4**). The reaction mixtures were kept at 25.0 ± 0.1°C, and equilibration was monitored by HPLC on aliquots over 3 hr; generally, full equilibration was reached after 1 hr. The aliquots were immediately quenched with 1 M H_3PO_4 to pH 2 and analyzed by HPLC with linear gradients of either CH_3CN/2% H_3PO_4, 90:10 (A) and CH_3CN/2% H_3PO_4, 5:95 (B), or CH_3CN/ 2% H_3PO_4, 1:99 (B) for compound **4** (compound **1**, from 0 to 25% [A] over 20 min and to 35% [A] over 35 min; compound **2**, from 0 to 25% [A] over 20 min and to 50% [A] over 35 min; compound **3**, from 0 to 50% [A] over 40 min; compound **4**, from 0 to 20% [A] over 30 min and to 50% [A] over 30 min). Optimal HPLC conditions for baseline separation of all theoretically expected components were obtained for the cyclopeptides **1** and **4**, whereas in the case of peptides **2** and **3** the P_{ox} species could not be detected.

Assignment of each HPLC peak was achieved by LC-ESI-MS as well as by coelution with the authentic samples of P_{ox} and P_{red}.

Compound **1**: t_R(min)/$[M + H]^+$ 22.22/1233.6 (1232.8 calculated for P-$(SG)_2$), 24.30/928.4 and 25.33/928.4 (927.5 calculated for P-SG), 28.14/621.2 (620.2 calculated for P_{ox}), 31.63/623.2 (622.2 calculated for P_{red}).

Compound **4**: t_R(min)/$[M + H]^+$ 22.37/563.2 (562.2 calculated for P_{ox}), 24.84/1175.4 (1174.8 calculated for the P-$(SG)_2$), 25.97–26.12/870.4 (869.5 calculated for P-SG), 27.90/565.4 (564.2 calculated for P_{red}).

Thiol/Disulfide Exchange Equilibria with Cysteine/Cystine

Equilibration of compounds **1–4** with the cysteine/cystine redox pair was performed according to the same procedure as with glutathione. Because of the low solubility of cystine, lower peptide concentrations (5–16 μM) were used to maintain the reference redox pair in large excess over the peptides. The following [cysteine]/[cystine] molar ratios were used: 1.99, 3.95, 6.90, and 9.95 for compound **1**; 2.00, 3.95, 6.90, 8.86, and 9.96 for compound **2**; 2.00, 3.95, 6.90, and 9.96 for compound **3**; and 7.00, 9.99, 26.52, and 42.50 for compound **4**. The reaction mixtures were equilibrated at 25.0 ± 0.1°C for 4 hr, quenched with 1 M H_3PO_4, and analyzed by HPLC for their product distribution. Optimal elution conditions were achieved for all four systems with the linear gradients reported above for equilibration with glutathione.

Again, characterization of the product distribution was achieved by LC-ESI-MS and coelution with authentic samples of P_{ox} and P_{red}. The species P-Cys_2 and P-Cys of compounds **1** and **4** were present just at a minor extent and could be unambiguously assigned by LC-MS only for compound **1**.

Compound **1**: t_R(min)/$[M + H]^+$ 23.14/861.4 (860.5 calculated for P-Cys_2), 24.26/742.2 (741.4 calculated for P-Cys), 28.13/621.4 (620.2 calculated for P_{ox}), 31.68/623.6 (622.2 calculated for P_{red}).

Compound **2**: t_R(min)/$[M + H]^+$ 19.49/913.4 (912.6 calculated for P-Cys_2), 23.96/794.4 and 24.92/794.4 (793.4 calculated for P-Cys), 26.17/673.4 (672.3 calculated for P_{ox}), 30.89/675.4 (674.3 calculated for P_{red}).

Compound **3**: t_R(min)/$[M + H]^+$ 16.57/953.4 (952.6 calculated for P-Cys_2), 19.10/834.2 and 20.95/834.2 (833.4 calculated for P-Cys), 22.98/713.4 (712.3 calculated for P_{ox}), 24.20/715.4 (714.3 calculated for P_{red}).

Compound **4**: t_R(min)/$[M + H]^+$ 21.95/563.2 (562.2 calculated for P_{ox}), 27.98/565.2 (564.2 calculated for P_{red}). Two other peaks at 23.50 and 25.75 min could not be unambiguously assigned to the species P-Cys_2 and P-Cys by LC-MS. However, in analogy to the elution pattern of the corresponding glutathionylated derivatives, P-Cys_2 was assigned to the t_R(min) of 23.50 and, consequently, P-Cys to 25.75.

Redox Potentials of the Cyclic Peptides 1–4

Despite the multiple equilibria that are established in the thiol/disulfide exchange reactions [27], according to the "principle of detailed balancing" [53] each step can be considered as a separate one and treated as a two-state model. Assuming that in the HPLC of the equilibrated mixtures all species were quantitatively eluted and exhibited identical extinction coefficients at 210 nm, we correlated the concentration of each component at equilibrium to the area of the corresponding HPLC peak and used it to obtain the equilibrium constants K_{eq} relative to each redox reaction. The K_{eq} values were estimated as previously described by Siedler and coworkers [27] and were found to correlate well to each other, thus confirming the validity of the model. The two approaches applied for the determination of K_{eq} are reported more in the details of the calculation of K_{ox}, which describes the equilibrium between P_{red} and P_{ox} and is required to estimate the redox potential.

In the first method, the $[GSH]^2/[GSSG]$ or $[cysteine]^2/[cystine]$ value used for each experiment and the corresponding $[P_{ox}]/[P_{red}]$ ratio estimated from the HPLC were introduced into Equation 1:

$$K_{ox} = \frac{[P_{ox}]\,[GSH]^2}{[P_{red}]\,[GSSG]} \qquad (1)$$

The K_{ox} values that had been derived from multiple (four to seven) experiments carried out with each peptide were then averaged.

In the second method, the K_{ox} values were estimated from a rectangular hyperbolic fitting via Equation 2:

$$\frac{[P_{red}]}{[P_{ox}] + [P_{red}]} = \frac{[GSH]^2/[GSSG]}{K_{ox} + [GSH]^2/[GSSG]} \quad (2)$$

The K_{ox} values extrapolated from Equation 2 were found to be in agreement with those calculated from Equation 1.

Finally, the E'_0 values were obtained from the Nernst Equation:

$$E'_0 = E'_{0\,(GSH/GSSG)} - 0.03 \log K_{ox} \quad (3)$$

The redox potentials referring to GSH/GSSG were calculated with the $E'_{0\,(GSH/GSSG)}$ value of −240 mV [30]. Accordingly, the $E'_{0\,(cysteine/cystine)}$ value of −245 mV relative to the $E'_{0\,(GSH/GSSG)}$ value of −262 mV [31] was adjusted to −223 mV to calculate the redox potentials referring to cysteine/cystine.

Titration of the Thiol Groups of the Reduced Cyclic Peptides 1–4

The dithiol peptides were titrated spectrophotometrically at 238 nm [54, 55]. For this purpose solutions of the bicyclic peptides (300 μl, 0.38–0.55 μmol) in argon-flushed phosphate buffer (0.1 M, pH 7.0) were treated with tris-(2-carboxyethyl)phosphine hydrochloride, TCEP (31.2 μl of a 50 mM solution in phosphate buffer, 1.56 μmol). After 1 hr at room temperature, an aliquot was analyzed by HPLC and ESI-MS to confirm total reduction of the disulfide bond. Samples (30 μl) of these solutions were diluted with phosphate buffer (1030 μl) to a final concentration of 32–47 μM and titrated with 1.7 M H_3PO_4 and 2 M NaOH directly in the cuvette (the dilution factor on the peptide concentration was neglected because the overall volume change was <5%). The absorbance at 238 nm was measured against phosphate buffer as the blank. Titration of GSSG, reduced with TCEP under identical conditions as the peptides, was also performed to assess the confidence of the protocol. All UV measurements were recorded at 25°C. To take into account the UV contribution of the Tyr and Trp residues at 238 nm, we pH titrated solutions of these amino acids at the same molar concentration as that used for the cyclic peptides.

After subtraction of the absorbance due to the aromatic residues, when present, the data points relative to the monocyclic Trx, PDI, and Trr peptides were fit to a double titration model (equation 4) [32], whereas for glutathione a single titration model was used (Equation 5) and gave a pK_a value of 9.1, which is in agreement with the value reported in the literature. For the monocyclic Grx peptid, only data points below pH 9, before Tyr deprotonation, were fit to Equation 5.

$$A_{238} = C_0 \left(\frac{\epsilon^{S-}_{S-}\, 10^{(pH - pK_{a2})} + \epsilon^{S-}_{SH} + \epsilon^{SH}_{SH}\, 10^{(pK_{a1} - pH)}}{10^{(pH - pK_{a2})} + 1 + 10^{(pK_{a1} - pH)}} \right) \quad (4)$$

$$A_{238} = C_0 \left(\frac{\epsilon_{S-}\, 10^{(pH - pK_a)} + \epsilon_{SH}}{1 + 10^{(pH - pK_a)}} \right) \quad (5)$$

The total thiol concentration, C_0, was given as a fixed parameter prior to the fitting, whereas the extinction coefficients of the fully protonated, singly deprotonated, and fully deprotonated species as well as the pK_a values were extrapolated from the curve fitting.

For the titration experiments performed by NMR spectroscopy, solutions of reduced Trx and PDI peptides at 0.8–1.0 mM concentration in phosphate buffer (0.1 M, pH 6) were treated with small aliquots of 1.5 M NaO^2H. The pH value was measured directly before the acquisition of each 1D NMR spectrum (25°C) and was not corrected for deuterium isotope effects. The chemical shifts of the βCH_2 protons of Trx-Cys^{32} and PDI-Cys^{39} were calibrated on the sodium 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)propionate NMR resonance. The data points were fit to a sigmoidal function.

Refolding of Reduced and Denatured RNase A

RNase A type III-A from bovine pancreas (Sigma-Aldrich) was reduced and denatured according to known procedures [56]. For this purpose RNase A (14 mg) was incubated overnight in 2 ml argon-flushed Tris·HCl buffer (0.1 M, pH 8.6) containing 6 M guanidine hydrochloride and 62 mM DTT. The reduced, denatured enzyme was chromatographically separated from salt and DTT by gel filtration on Sephadex G-25 (Pharmacia) with 0.01 M HCl as eluent (UV detection at 275 nm). Fractions corresponding to the central part of the elution peak were collected, and the protein concentration was determined spectrophotometrically [57]. The free thiol concentration was determined by UV measurements with (2,2′-dithio)dipyridine [58]. The reduced protein solution was used immediately or stored under argon at −20°C overnight.

Refolding experiments were performed in triplicate at 30.0 ± 0.1°C in argon-flushed Tris·HCl buffer (0.1 M, pH 7.4) containing 1.2 mM EDTA. Stock solutions of compounds **1–3**, GSH, and GSSG were prepared by weight and controlled by UV spectroscopy at 248 nm for GSSG [59], at 275 and 280 nm for the Tyr- and Trp-containing peptides, respectively, and by the Grassetti test for GSH [58]. The following refolding mixtures were used: GSH/GSSG/protein (40:8:1 or 20:4:1), GSH/GSSG/peptide/protein (40:8:0.8:1 or 20:2:2:1), GSH/peptide/protein (20:4:1), and peptide/protein (4:1). The assays were initiated by addition of the reduced and denatured RNase A to the redox buffers to a final protein concentration of 24 μM. At time intervals, aliquots (60 μl) of the refolding mixtures were taken and added to 800 μl of 465 μM cCMP in MOPS buffer (0.1 M, pH 7), and substrate hydrolysis was monitored at 284 nm against a reference substrate solution [60] over 3 min at 25°C. The concentration of the refolded enzyme was extrapolated from the slope of the approximately linear initial UV traces with a calibration curve derived from known concentrations of native RNase A [61]. Enzyme reactivation (average of triplicate experiments) was plotted against time, and the experimental curves were fit with Equation 6 to obtain the extrapolated maximal yield of the reactivation process (A_{max}) and the first-order rate constant (k).

$$\%\ \text{RNase A activity} = A_{max}\,(1 - e^{-kt}) \quad (6)$$

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References

1. Wang, C.C., and Tsou, C.L. (1993). Protein disulfide isomerase is both an enzyme and a chaperone. FASEB J. *7*, 1515–1517.
2. Cai, H., Wang, C.C., and Tsou, C.L. (1994). Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds. J. Biol. Chem. *269*, 24550–24552.
3. Noiva, R. (1994). Enzymatic catalysis of disulfide formation. Protein Expr. Purif. *5*, 1–13.
4. Freedman, R.B., Hirst, T.R., and Tuite, M.F. (1994). Protein disulphide isomerase: building bridges in protein folding. Trends Biochem. Sci. *19*, 331–336.
5. Frand, A.R., and Kaiser, C.A. (1998). The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol. Cell *1*, 161–170.
6. Pollard, M.G., Travers, K.J., and Weissman, J.S. (1998). Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. Mol. Cell *1*, 171–182.
7. Cabibbo, A., Pagani, M., Fabbri, M., Rocchi, M., Farmery, M.R., Bulleid, N.J., and Sitia, R. (2000). ERO1-L, a human protein that favors disulfide bond formation in the endoplasmic reticulum. J. Biol. Chem. *275*, 4827–4833.
8. Missiakas, D., and Raina, S. (1997). Protein folding in the bacterial periplasm. J. Bacteriol. *179*, 2465–2471.
9. Holmgren, A. (1985). Thioredoxin. Annu. Rev. Biochem. *54*, 237–271.
10. Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. J. Biol. Chem. *264*, 13963–13966.

11. Holmgren, A. (1995). Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure *3*, 239–243.
12. Katti, S.K., LeMaster, D.M., and Eklund, H. (1990). Crystal structure of thioredoxin from *Escherichia coli* at 1.68 Å resolution. J. Mol. Biol. *212*, 167–184.
13. Sodano, P., Xia, T.H., Bushweller, J.H., Björnberg, O., Holmgren, A., Billeter, M., and Wüthrich, K. (1991). Sequence-specific ^{1}H n.m.r. assignments and determination of the three-dimensional structure of reduced *Escherichia coli* glutaredoxin. J. Mol. Biol. *221*, 1311–1324.
14. Kemmink, J., Darby, N.J., Dijkstra, K., Scheek, R.M., and Creighton, T.E. (1995). Nuclear magnetic resonance characterization of the N-terminal thioredoxin-like domain of protein disulfide isomerase. Protein Sci. *4*, 2587–2593.
15. Martin, J.L., Bardwell, J.C.A., and Kuriyan, J. (1993). Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*. Nature *365*, 464–468.
16. Waksman, G., Krishna, T.S., Williams, C.H., Jr., and Kuriyan, J. (1994). Crystal structure of *Escherichia coli* thioredoxin reductase refined at 2 Å resolution. Implications for a large conformational change during catalysis. J. Mol. Biol. *236*, 800–816.
17. Lin, T.Y., and Kim, P.S. (1989). Urea dependence of thiol-disulfide equilibria in thioredoxin: confirmation of the linkage relationship and a sensitive assay for structure. Biochemistry *28*, 5282–5287.
18. Hawkins, H.C., de Nardi, M., and Freedman, R.B. (1991). Redox properties and cross-linking of the dithiol/disulphide active sites of mammalian protein disulphide-isomerase. Biochem. J. *275*, 341–348.
19. Lundström, J., and Holmgren, A. (1993). Determination of the reduction-oxidation potential of the thioredoxin-like domains of protein disulfide-isomerase from the equilibrium with glutathione and thioredoxin. Biochemistry *32*, 6649–6655.
20. Darby, N.J., and Creighton, T.E. (1995). Characterization of the active site cysteine residues of the thioredoxin-like domains of protein disulfide isomerase. Biochemistry *34*, 16770–16780.
21. Huber-Wunderlich, M., and Glockshuber, R. (1998). A single dipeptide sequence modulates the redox properties of a whole enzyme family. Fold. Des. *3*, 161–171.
22. Krause, G., Lundström, J., Barea, J.L., Pueyo de la Cuesta, C., and Holmgren, A. (1991). Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in *Escherichia coli* thioredoxin. J. Biol. Chem. *266*, 9494–9500.
23. Kortemme, T., Darby, N.J., and Creighton, T.E. (1996). Electrostatic interactions in the active site of the N-terminal thioredoxin-like domain of protein disulfide isomerase. Biochemistry *35*, 14503–14511.
24. Åslund, F., Berndt, K.D., and Holmgren, A. (1997). Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. J. Biol. Chem. *272*, 30780–30786.
25. Yang, Y., and Wells, W.W. (1991). Identification and characterization of the functional amino acids at the active center of pig liver thioltransferase by site-directed mutagenesis. J. Biol. Chem. *266*, 12759–12765.
26. Grauschopf, U., Winther, J.R., Korber, P., Zander, T., Dallinger, P., and Bardwell, J.C. (1995). Why is DsbA such an oxidizing disulfide catalyst? Cell *83*, 947–955.
27. Siedler, F., Rudolph-Böhner, S., Doi, M., Musiol, H.J., and Moroder, L. (1993). Redox potentials of active-site bis(cysteinyl) fragments of thiol-protein oxidoreductases. Biochemistry *32*, 7488–7495.
28. Moroder, L., Gemeiner, M., Göhring, W., Jäger, E., Thamm, P., and Wünsch, E. (1981). New synthesis of somatostatin according to the S-tert-butylthiocysteine procedure. Biopolymers *20*, 17–37.
29. Mukaiyama, T., and Takahashi, K. (1968). A convenient method for the preparation of unsymmetrical disulfides by the use of diethyl azodicarboxylate. Tetrahedron Lett. *9*, 5907–5908.
30. Rost, J., and Rapoport, S. (1964). Reduction-potential of glutathione. Nature (London) *201*, 185.
31. Millis, K.K., Weaver, K.H., and Rabenstein, D.L. (1993). Oxidation/reduction potential of glutathione. J. Org. Chem. *58*, 4144–4146.
32. Woycechowsky, K.J., Wittrup, K.D., and Raines, R.T. (1999). A small-molecule catalyst of protein folding *in vitro* and *in vivo*. Chem. Biol. *6*, 871–879.
33. Lyles, M.M., and Gilbert, H.F. (1991). Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. Biochemistry *30*, 613–619.
34. Moroder, L., Besse, D., Musiol, H.J., Rudolph-Böhner, S., and Siedler, F. (1996). Oxidative folding of cystine-rich peptides vs regioselective cysteine pairing strategies. Biopolymers *40*, 207–234.
35. Joelson, T., Sjöberg, B.M., and Eklund, H. (1990). Modifications of the active center of T4 thioredoxin by site-directed mutagenesis. J. Biol. Chem. *265*, 3183–3188.
36. Chivers, P.T., Laboissière, M.C.A., and Raines, R.T. (1996). The CXXC motif: imperatives for the formation of native disulfide bonds in the cell. EMBO J. *15*, 2659–2667.
37. Holst, B., Tachibana, C., and Winther, J.R. (1997). Active site mutations in yeast protein disulfide isomerase cause dithiothreitol sensitivity and a reduced rate of protein folding in the endoplasmic reticulum. J. Cell Biol. *138*, 1229–1238.
38. Renner, C., Behrendt, R., Heim, N., and Moroder, L. (2002). Photomodulation of conformational states. III. Water-soluble bis-cysteinyl-peptides with (4-aminomethyl)phenylazobenzoic acid as backbone constituent. Biopolymers *63*, 382–393.
39. O'Donnell, M.E., and Williams, C.H., Jr. (1983). Proton stoichiometry in the reduction of the FAD and disulfide of Escherichia coli thioredoxin reductase. Evidence for a base at the active site. J. Biol. Chem. *258*, 13795–13805.
40. Mössner, E., Huber-Wunderlich, M., and Glockshuber, R. (1998). Characterization of Escherichia coli thioredoxin variants mimicking the active-sites of other thiol/disulfide oxidoreductases. Protein Sci. *7*, 1233–1244.
41. Kortemme, T., and Creighton, T.E. (1995). Ionisation of cysteine residues at the termini of model α-helical peptides. Relevance to unusual thiol pK_a values in proteins of the thioredoxin family. J. Mol. Biol. *253*, 799–812.
42. Jeng, M.F., Holmgren, A., and Dyson, H.J. (1995). Proton sharing between cysteine thiols in *Escherichia coli* thioredoxin: implications for the mechanism of protein disulfide reduction. Biochemistry *34*, 10101–10105.
43. Weichsel, A., Gasdaska, J.R., Powis, G., and Montfort, W.R. (1996). Crystal structures of reduced, oxidized, and mutated human thioredoxins: evidence for a regulatory homodimer. Structure *4*, 735–751.
44. Guddat, L.W., Bardwell, J.C.A., and Martin, J.L. (1998). Crystal structures of reduced and oxidized DsbA: investigation of domain motion and thiolate stabilization. Structure *6*, 757–767.
45. Nordstrand, K., Åslund, F., Meunier, S., Holmgren, A., Otting, G., and Berndt, K.D. (1999). Direct NMR observation of the Cys-14 thiol proton of reduced *Escherichia coli* glutaredoxin-3 supports the presence of an active site thiol-thiolate hydrogen bond. FEBS Lett. *449*, 196–200.
46. Bulaj, G., Kortemme, T., and Goldenberg, D.P. (1998). Ionization-reactivity relationships for cysteine thiols in polypeptides. Biochemistry *37*, 8965–8972.
47. Woycechowsky, K.J., and Raines, R.T. (2000). Native disulfide bond formation in proteins. Curr. Opin. Chem. Biol. *4*, 533–539.
48. Lundström-Ljung, J., Vlamis-Gardikas, A., Åslund, F., and Holmgren, A. (1999). Reactivity of glutaredoxins 1, 2 and 3 from Escherichia coli and protein disulfide isomerase towards glutathionyl-mixed disulfides in ribonuclease A. FEBS Lett. *443*, 85–88.
49. Ruoppolo, M., and Freedman, R.B. (1995). Refolding by disulfide isomerization: the mixed disulfide between ribonuclease T1 and glutathione as a model refolding substrate. Biochemistry *34*, 9380–9388.
50. Chen, J., Song, J.L., Zhang, S., Wang, Y., Cui, D.F., and Wang, C.C. (1999). Chaperone activity of DsbC. J. Biol. Chem. *274*, 19601–19605.
51. Saxena, V.P., and Wetlaufer, D.B. (1970). Formation of three-dimensional structure in proteins. I. Rapid nonenzymic reactivation of reduced lysozyme. Biochemistry *9*, 5015–5023.

52. Behrendt, R., Schenk, M., Musiol, H.J., and Moroder, L. (1999). Photomodulation of conformational states. Synthesis of cyclic peptides with backbone-azobenzene moieties. J. Pept. Sci. *5*, 519–529.
53. Weston, R.E., and Schwarz, H.A. (1972). Chemical Kinetics. (Englewood Cliffs, NJ: Prentice-Hall).
54. Benesch, R.E., and Benesch, R. (1955). The acid strength of the –SH group in cysteine and related compounds. J. Am. Chem. Soc. *77*, 5877–5881.
55. Polgár, L. (1974). Spectrophotometric determination of mercaptide ion, an activated form of SH-group in thiol enzymes. FEBS Lett. *38*, 187–190.
56. Pigiet, V.P., and Schuster, B.J. (1986). Thioredoxin-catalyzed refolding of disulfide-containing proteins. Proc. Natl. Acad. Sci. USA *83*, 7643–7647.
57. Anfinsen, C.B., Haber, E., Sela, M., and White, F.H., Jr. (1961). The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain. Proc. Natl. Acad. Sci. USA *47*, 1309–1314.
58. Grassetti, D.R., and Murray, J.F., Jr. (1967). Determination of sulfhydryl groups with 2,2′- or 4,4′-dithiodipyridine. Arch. Biochem. Biophys. *119*, 41–49.
59. Chau, M.-H., and Nelson, J.W. (1991). Direct measurement of the equilibrium between glutathione and dithiothreitol by high performance liquid chromatography. FEBS Lett. *291*, 296–298.
60. Crook, E.M., Mathias, A.P., and Rabin, B.R. (1960). Spectrophotometric assay of bovine pancreatic ribonuclease by the use of cytidine 2′:3′-phosphate. Biochem. J. *74*, 234–238.
61. Hantgan, R.R., Hammes, G.G., and Scheraga, H.A. (1974). Pathways of folding of reduced bovine pancreatic ribonuclease. Biochemistry *13*, 3421–3431.