Photomodulation of the Redox and Folding Adjuvant Properties of Bis(cysteinyl) Peptides[‡]

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The octapeptide [134–141] related to the active-site fragment of thioredoxin reductase, in which three residues outside the characteristic Cys-Xaa-Yaa-Cys motif of thiol/disulfide oxidoreductases were replaced by lysines, was head-to-tail-cyclized by using the suitably functionalized (4-aminomethyl)-phenylazobenzoic acid (AMPB). The resulting monocyclic and disulfide-bridged bicyclic compounds underwent light-induced *cis*/*trans* isomerization in a fully reversible manner, with well-defined conformational transitions as a result of the strong differences in the molecular geometries of the *trans*- and *cis*-azobenzene units. Correspondingly, the *trans* and *cis* forms of the cyclic bis(cysteinyl)-AMPB peptide were characterized by significantly differentiated redox potentials, which were exploited to catalyze the oxidative refolding of reduced RNase A with distinct efficiencies. The experimental results showed that the incorporation of the azobenzene moiety into conformationally restricted bis(cysteinyl) peptide systems provided folding adjuvants that photocontrolled the rates of oxidative protein folding.

Introduction

In previous studies we have shown that the incorporation of an azobenzene unit such as (4-amino)phenylazobenzoic (APB) or (4-aminomethyl)phenylazobenzoic (AMPB) acid into the backbone of monocyclic or disulfide-linked bicyclic peptides allows for photocontrol of the preferred conformational states of such molecules, by exploiting the geometrical changes of the azobenzene upon its light-triggered *cis*/*trans* isomerization.[2] While following up investigations on the effect of the amino acid composition of the active-site consensus motif Cys-Xaa-Yaa-Cys of thiol/disulfide oxidoreductases on the redox potential of related bis(cysteinyl) peptides,[3] we recently succeeded in amplifying the sequence-intrinsic free energy of formation of the disulfide-bridged Cys-Xaa-Yaa-Cys motif by constraining its conformational space in cyclic bis(cysteinyl) peptides, thus successfully modulating the redox potentials of the peptides as resulting from the structural restraints imparted by the backbone cyclization.[4] It was thus compelling for us to combine both approaches for the design of photoresponsive redox systems based on cyclic bis(cysteinyl) peptides containing an azobenzene unit as light switch, possibly to allow for photocontrol of their catalytic activities in oxidative refolding of proteins.

For this purpose, the Cys-Ala-Thr-Cys active-site sequence of *E. coli* thioredoxin reductase was selected, since its intervening dipeptide composition only marginally affects the redox potential of the cysteine residues,[3] and this should allow conformational effects to be exploited in a more pronounced manner. To extend this tetrapeptide to the octapeptide size required for cyclization by the AMPB moiety, three residues outside the cysteine motif in the native sequence H-Ala-Cys-Ala-Thr-Cys-Asp-Gly-Phe-OH [134−141] of thioredoxin reductase were replaced with lysines to assure water solubility (Figure 1). The resulting *cyclo*-[Lys-AMPB-Lys-Cys-Ala-Thr-Cys-Asp-Lys], compound **1**, was found fully to retain its photoresponsive properties in water, with the *trans* → *cis* isomerization of the monocyclic and oxidized bicyclic peptide being accompanied by significant transitions from well-defined and rigid conformations as *trans*-azo isomers to ensembles of less constrained conformations in the *cis*-azo configuration.[1,5]

Because of these conformational transitions, distinct redox potentials were expected for the cyclic bis(cysteinyl)

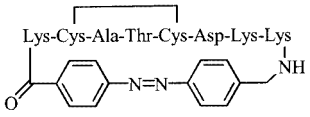

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Figure 1. Structure of the AMPB peptide **1** containing the modified active site fragment [134−141] Lys-Cys-Ala-Thr-Cys-Asp-Lys-Lys from the thioredoxin reductase and the azobenzene moiety, AMPB

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peptide in the two isomeric states. In this study, the redox properties of the cyclic bis(cysteinyl)-AMPB peptide were determined and the ability of this compound to photomodulate the catalysis of oxidative protein folding was investigated by use of reduced RNase A as model protein.

Results and Discussion

Redox Properties of the Bis(cysteinyl)-AMPB Peptide

The complex network of reactions taking place in the thiol/disulfide exchange reaction of the bis(cysteinyl)-AMPB peptide in the GSH/GSSG redox buffer is shown in Figure 2.

In addition to the two glutathione components, the oxidized and reduced peptide species **1** and **2**, the two monoglutathione-peptide mixed disulfides **3** and **4**, the bis(glutathione)-peptide mixed disulfide **5**, and even peptide dimers, oligomers, and polymers could be formed in this reaction. However, HPLC analysis of the equilibrated reaction mixtures in the peptide concentration range from 0.16 to 0.39 mM did not reveal formation of oligomers to any detectable extent. Since reduction of the azobenzene group was observed to occur with strongly reducing agents such as DTT,[6] the equilibration mixtures were carefully monitored by HPLC, and degradation products were detected to a minor extent only upon incubation for longer than 24 h. As shown representatively for one redox experiment in Figure 3, the single peaks of the HPLC chromatograms (five for the *trans* species and five for the *cis* species) were baseline-separated under optimized conditions, thus al-

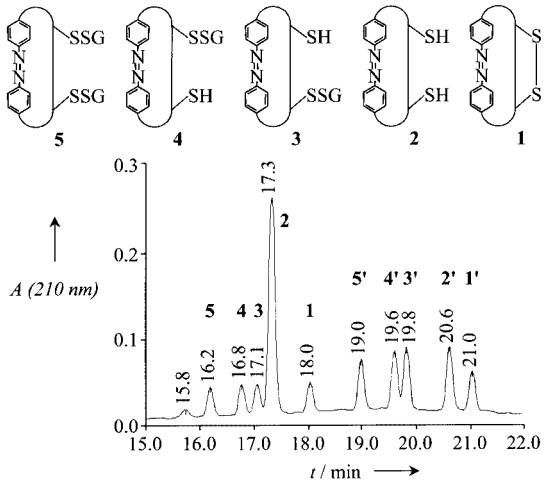

Figure 3. HPLC profile of the mixture of the *cis* and *trans* isomers of the AMPB peptide **1** equilibrated with GSH/GSSG at pH = 7 and 25 °C; the species related to the *trans* isomer are indicated with ′

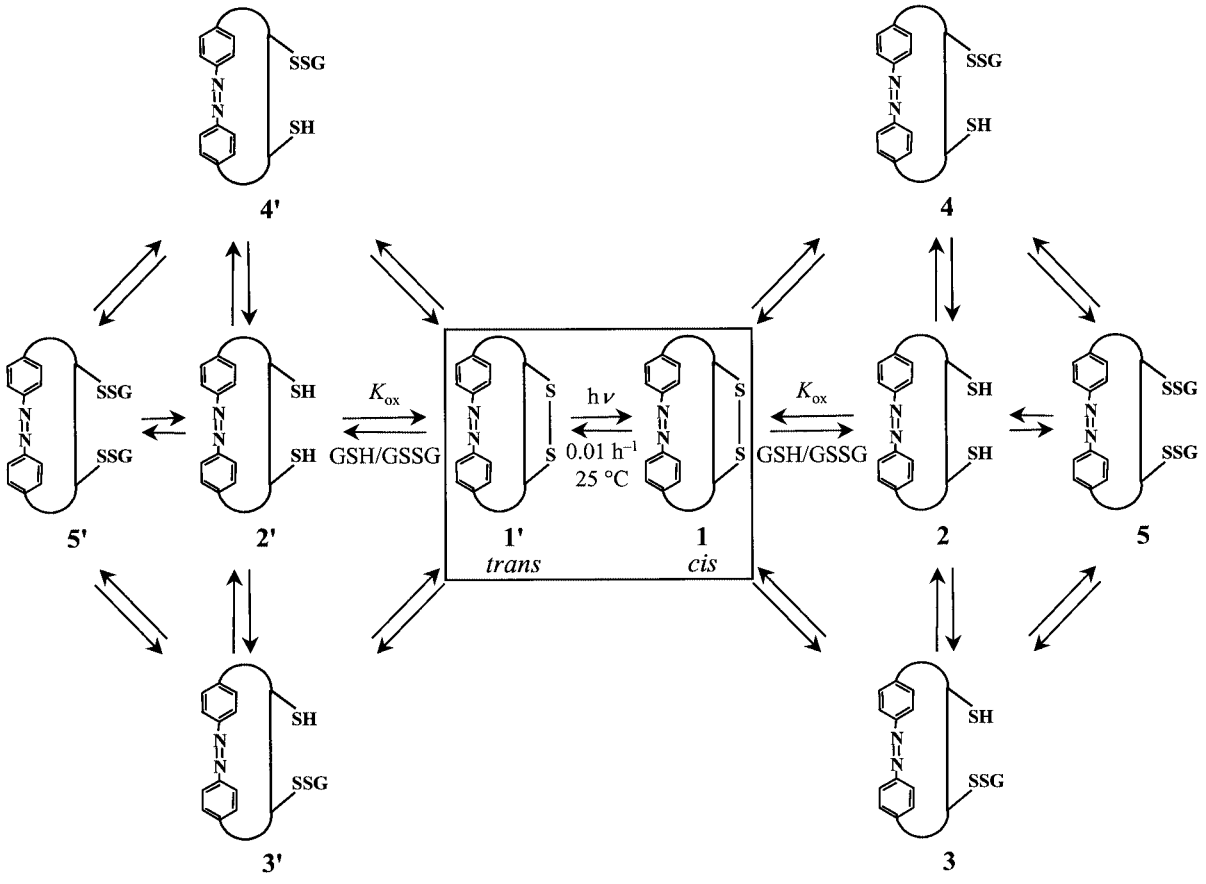

Figure 2. Thiol/disulfide exchange equilibria of the AMPB peptide **1** in the *cis*- and *trans*-azo configuration with the glutathione redox buffer; the species related to the *trans* isomer are indicated with ′

lowing quantitative analysis of the product distribution. Since equilibration was performed at 25 °C with GSH/GSSG at different ratios, but with the total glutathione concentration kept much higher than that of the peptide, the redox potential of the system was dictated by glutathione.

The K_{ox} values determined for the *trans* and *cis* isomers as the averages of ten and five separate experiments, respectively, are reported in Table 1. The relative redox potentials were calculated by using an E_0' value of −240 mV for glutathione[7] as the reference redox pair.

Table 1. Equilibrium constants of the thiol/disulfide exchange reactions between the AMPB peptide **1** in the *cis*- and *trans*-azo configuration and glutathione; the corresponding redox potentials were calculated with the value of −240 mV for glutathione,[7] at pH = 7 and 25 °C

Azobenzene configuration	K_{ox} [mM]	E_0' [mV]
trans	49 ± 2	−201 ± 1
cis	0.80 ± 0.05	−147 ± 1

In comparison to the *trans* isomer, with its high K_{ox} value (49 mM), the *cis* isomer (K_{ox} = 0.80 mM) showed a 60-fold higher tendency to exist in its reduced form. This fact was reflected in the apparent redox potential of the *cis* isomer (E_0' = −147 mV), which was significantly more oxidizing than that of the *trans* isomer. In fact, the redox properties of the *trans* isomer (E_0' = −201 mV) were very similar to those of the linear active-site octapeptide fragment of thioredoxin reductase (E_0' = −210 mV)[3] − that is, of a flexible bis(cysteinyl) peptide − in terms of the tendency to form the 14-membered disulfide loop.

On analysis of the different redox potentials of the *trans* and *cis* isomers of the cyclic AMPB peptide from a thermodynamic point of view, the K_{ox} value of the *trans* isomer (49 mM) for forming the 14-membered ring was about five times higher than the values determined for unstructured peptides with disulfide loops of identical size.[8] However, this value was lower than that measured for the linear active-site octapeptide of thioredoxin reductase (K_{ox} = 0.123 M),[3a] probably due to some strain on the disulfide bond. On comparison of the preferred conformation of the disulfide-bridged linear octapeptide with that of the bicyclic AMPB peptide as determined by NMR analysis,[5] the linear peptide exhibited a loop structure almost identical to that of the active site in the backbone-cyclized parent peptide (Figure 4). This would support the hypothesis of an intrinsically highly favored structure for the Cys-Ala-Thr-Cys loop and, correspondingly, a free energy of formation of the 14-membered ring that would also favor oxidation. The cyclic AMPB peptide in the *trans*-azo configuration is characterized by a more stretched and rigid structure that remains almost unchanged in the bis(seryl) analog, mimicking its reduced state.[1] In this case the free energy of formation of the 14-membered ring should mainly be dictated by the entropic contributions associated with the juxtaposition

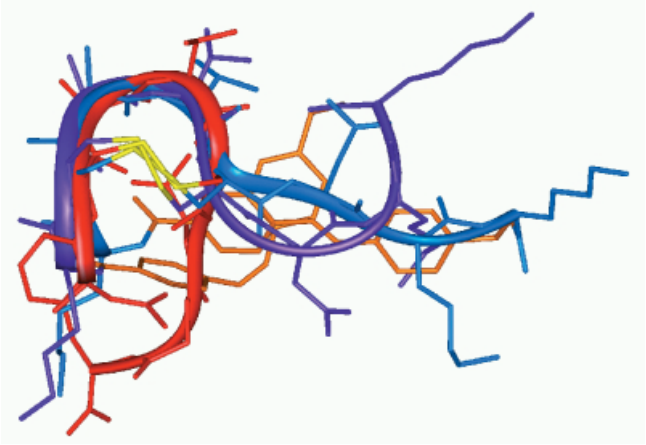

Figure 4. Preferred conformations of the AMPB peptide **1** as *trans* (blue) and *cis* isomer (violet) and of the linear active-site fragment [134−141] (red) of thioredoxin reductase; the three structures are superimposed on the backbone atoms of the Cys-Ala-Thr-Cys motif

of the cysteine side chains, but the slightly lower K_{ox} could as well derive from some conformational strain on the disulfide derived from the stretched and rigid conformation of the peptide backbone. Conversely, the preferred conformation of the *cis* isomer differs significantly in the oxidized and reduced forms. The entropic penalty resulting from the conformational rearrangement of the peptide backbone most probably represents the major factor that governs the free energy of disulfide ring formation.

Unfortunately, because of the strong absorbance of the azobenzene moiety in the UV region in which the thiolate group is observed (about 240 nm), determination of the thiol pK_a values was prevented, and thus it could not be established whether the cluster of positively charged lysine residues contributed to the acidity of one or both thiols as well as to the highly oxidizing redox properties of the *cis* isomer. In general, the presence of positive charges should favor the formation of the thiolate, and, consequently, the thiol/disulfide exchange. However, the thiol pK_a values of model peptides containing a single cysteine could not always be correlated with the presence of charged side chains,[9] but in some cases they were strongly dependent on the conformation.[10] For the redox-active sequence Cys-Ala-Thr-Cys in thioredoxin reductase, comparable E_0' values were found both in the linear native active-site octapeptide and in the *trans* isomer of the AMPB peptide containing three nonnative lysines, suggesting that there was no effect of the basic residues on the redox activity of the two thiol groups in the cyclic compound. On the other hand, favorable electrostatic interactions between the cysteine and lysine side chains in the structure of the *cis* isomer might contribute to disulfide destabilization.

Catalysis of Oxidative Refolding of RNase A

Since Anfinsen's first classical experiments on oxidative refolding of RNase A,[11] this enzyme has been the most widely studied model protein, as the formation of the four native disulfide bonds thermodynamically coupled with

Table 2. Effect of the AMPB peptide **1** azo configuration and concentration on the refolding of reduced and denatured RNase A (24 μM) at pH = 7.4 and 30 °C; the concentration of GSH was 960 μM in Entries 1−5, and 480 μM in Entries 6−12; the concentration of GSSG was 192 μM in Entries 1−5, 96 μM in Entry 6, 72 μM in Entries 7 and 10, 48 μM in Entries 8 and 11

Entry	RNase A/GSH/GSSG/1	A_{max} [a] [%]	k_{app} [a] [h^{-1}]	Initial rate[b] [pmol min^{-1}]
1	1:40:8:−	77 ± 4	0.14 ± 0.02	2.6 ± 0.4
2	1:40:8:0.5 *trans*	75 ± 2	0.16 ± 0.01	2.9 ± 0.2
3	1:40:8:1 *trans*	85 ± 6	0.15 ± 0.02	2.9 ± 0.4
4	1:40:8:0.5 *cis*	75 ± 5	0.18 ± 0.02	3.2 ± 0.4
5	1:40:8:1 *cis*	86 ± 5	0.16 ± 0.02	3.3 ± 0.5
6	1:20:4:−	64 ± 2	0.072 ± 0.004	1.1 ± 0.1
7	1:20:3:1 *trans*	83 ± 3	0.054 ± 0.003	1.1 ± 0.1
8	1:20:2:2 *trans*	101 ± 4	0.046 ± 0.003	1.1 ± 0.1
9	1:20:−:4 *trans*	50 ± 2	0.13 ± 0.01	1.6 ± 0.1
10	1:20:3:1 *cis*	94 ± 3	0.064 ± 0.004	1.4 ± 0.1
11	1:20:2:2 *cis*	98 ± 4	0.072 ± 0.005	1.7 ± 0.1
12	1:20:−:4 *cis*	72 ± 6	0.12 ± 0.02	2.1 ± 0.4

[a] A_{max} and k_{app} represent the extrapolated maximal activity and the "apparent rate constant" (k_{app}) obtained from the fitting of the experimental curves with the following Equation: % RNase A activity = A_{max} $(1 - e^{-k_{app}t})$. [b] The initial rate was estimated from the first derivative at $t = 0$ of the reactivation curves.

folding into the native structure is directly correlated with its enzymatic activity (for a recent review, see ref.[12]). For such oxidative refolding, the glutathione redox system (GSH/GSSG) is routinely used as the auxiliary agent[13] to mimic the natural environment in the endoplasmic reticulum.[14] However, optimized concentrations and GSH/GSSG ratios are required to avoid trapping of mixed disulfide intermediates.[15]

To analyze the effect of the different redox potentials of the cyclic AMPB peptide as *trans* and *cis* isomer on rates and efficiency of oxidative refolding of reduced RNase A, optimal and nonoptimal GSH/GSSG concentrations were used. As shown in Table 2, under optimal redox buffer conditions (1 mM GSH and 0.2 mM GSSG) the effect of catalytic amounts of AMPB peptide was minimal in both configurations, with only a slight increase in the initial folding rates, a fact moderately more pronounced for the *cis* than for the *trans* isomer.

From comparison of the rates and folding efficiency of nonoptimal glutathione concentration (i.e., 0.5 mM GSH and 0.1 mM GSSG) with those when GSSG was replaced with increasing amounts of the AMPB peptide, the rates of refolding were significantly more affected by the *cis* isomer than by the *trans* isomer, as would be expected from its higher and thus more oxidizing redox potential, whereas both isomers proved to be efficient additives in terms of recovered active enzyme (Table 2). In fact, the mixtures of GSSG/*trans* isomer at the 3:1 and 2:2 ratios (overall 4 disulfide equiv. per RNase) resulted in extrapolated final yields of 83% and 100%, respectively, although the initial rates were not improved. On the other hand, the reactivation process with the GSSG/*cis* isomer mixture was already characterized by an extrapolated maximum yield of 94% at the 3:1 ratio, and the initial reaction was accelerated approximately 1.5-fold by use of GSSG/*cis* isomer at the 2:2 ratio. These results reflect a remarkable disulfide reshuffling activity of the bis(cysteinyl) peptide with respect to the glutathione. Moreover, the positive effect on the initial rate observed with the *cis* isomer, but not with the *trans*, may be attributed to the higher oxidation activity of the *cis* form, resulting in a more efficient buildup of mixed disulfides of RNase either with the peptide itself or with GSH.[15] These complex intermediates involving the enzyme and the redox agents are formed in the initial phase of the RNase folding and are successively subjected to disulfide rearrangements.[16] Unlike the *cis* isomer, the *trans* isomer lacks the ability to affect the initial rate of the refolding positively, but it maintains the reshuffling activity, which is more pronounced with increasing concentration. In Figure 5 the effect of the *cis* isomer is compared to that of the *trans* isomer when GSSG was quantitatively replaced by the cyclic peptide. Under these conditions, both the *cis* and *trans* isomers accelerated the initial oxidation of RNase twofold and 1.5-fold, respectively, relative to GSSG. However, the incomplete reactivation indicated the formation and accumulation of trapped mixed disulfide intermediates, a side effect that is generally more pronounced with monothiols. Therefore, optimal con-

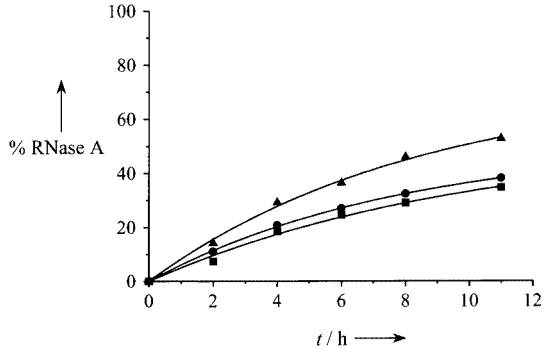

Figure 5. Effect of the substitution of GSSG (black squares) by the *trans* (black circles) and *cis* isomers (black triangles) of AMPB peptide **1** on the refolding of reduced and denatured RNase A (24 μM) at pH = 7.4 and 30 °C; the refolding mixture contained RNase/GSH/oxidant at a 1:20:4 ratio

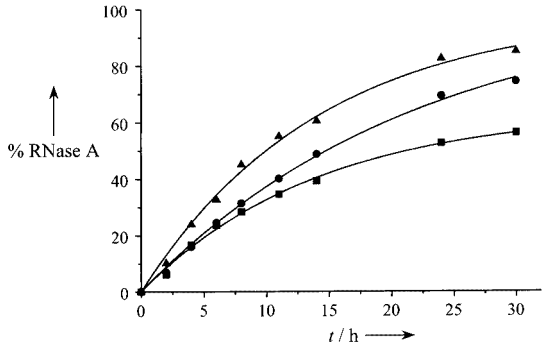

Figure 6. Effect of AMPB peptide **1** on the glutathione-dependent refolding of reduced and denatured RNase A (24 μM) at pH = 7.4 and 30 °C; the following refolding conditions were used: RNase/GSH/GSSG (black squares) 1:20:4; RNase/GSH/GSSG/*trans* isomer (black circles) 1:20:2:2; RNase/GSH/GSSG/*cis* isomer (black triangles) 1:20:2:2

ditions were obtained by only partial replacement of GSSG with the peptide, as shown in Figure 6, with well-differentiated catalytic effects being obtainable with the cyclic AMPB peptide as *cis* and *trans* isomer.

Conclusion

Thiol/disulfide oxidoreductases that are involved in oxidative protein folding, such as PDI (protein disulfide isomerase) in eukaryotes[17] and Dsb (disulfide bond) proteins in prokaryotes,[18] are characterized by high redox potentials (e.g., $E_0' = -110$ mV for PDI[19] and -122 mV for DsbA[20]) and low pK_a values of the *N*-terminal cysteine residue, which is generally associated with the dipole moment of the helix spanning the active site.[10,21] These features give rise to superior catalytic activities of PDI[22] and Dsb proteins compared to other oxidoreductases such as glutaredoxins and thioredoxin, which have markedly lower redox potentials, ranging between -200 mV and -270 mV.[23]

The redox potential of the cyclic AMPB peptide in the *cis* form is close to that of the protein disulfide isomerases, but the related catalytic efficiency in the oxidative protein refolding remains moderate. In this context it is worth saying that PDI acts not only as a redox enzyme but also as a chaperone,[24] a property that may play a role in the oxidative refolding process and that is missing in the AMPB peptide. Anyway, as a small-size peptide it is able to provide appreciable catalytic conditions for the oxidative refolding of a reduced and denatured protein.

Of importance, particularly in view of the goal of this study, was the finding that the two isomers differed significantly in their catalysis of oxidative refolding in vitro. Therefore, insertion of a photoswitchable group into the cyclic bis(cysteinyl) peptide proved to be an efficient approach to photocontrol of biochemical processes such as the protein folding.

Experimental Section

Materials and Methods: All reagents and solvents used in this study were of the highest grade commercially available. RNase A type III-A from bovine pancreas, reduced (GSH) and oxidized (GSSG) glutathione, DL-dithiothreitol (DTT), and guanidine hydrochloride were purchased from Sigma-Aldrich (Schnelldorf, Germany). Cytidine 2′,3′-cyclic monophosphate (cCMP), phosphoric acid, and 2,2′-dithiodipyridine were from Fluka (Taufkirchen, Germany). Sodium hydrogen phosphate, sodium dihydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris), and 3-morpholinopropanesulfonic acid (MOPS) were from Merck (Darmstadt, Germany). GSH and GSSG were analyzed for purity by ESI-MS, and the peptide content was determined by quantitative amino acid analysis of the acid hydrolysates (6 M HCl, 24 h, 110 °C). GSH and GSSG were used without further purification. Mass spectra were recorded with a PE SCIEX API 165 triple quadrupole LC-ESI-MS system. The spectra were collected in the 200−1200 amu range, with an ion source high voltage of 4.7 kV, an orifice voltage of 10 V, a dwell time of 0.6 ms per scan, and a step size of 0.2 amu. UV measurements were recorded with a Perkin−Elmer spectrophotometer (model Lambda 19) equipped with cuvette holders thermostatted at 25 °C with circulating water, by using quartz cuvettes with a light path of 1 cm. The pH values were measured with a Metrohm (Herisau, Switzerland) pH-meter (model E 632) freshly calibrated with pH = 7.00 and 8.00 standard solutions from Merck. For irradiation of the peptide samples at 360 nm a xenon lamp (450 XBO, Osram, München) and filter (Itos, Mainz) were used with a light intensity of ca. 15 mW. After irradiation, the maximum *cis*/*trans* isomer ratio at the photostationary state was ca. 80:20, with a *cis* → *trans* relaxation rate of 0.01 h^{-1} at 25 °C.[5]

Thiol/Disulfide Exchange Equilibria: The synthesis and spectroscopic characterization of *cyclo*-[Lys-AMPB-Lys-Cys-Ala-Thr-Cys-Asp-Lys] peptide are reported elsewhere.[5] The exchange reactions were carried out in brown plastic vials to protect the samples from daylight. The desired equilibration temperature was maintained with a jacketed bath connected to a thermostat with temperature regulation of ± 0.2 °C. Stock solutions of the cyclic AMPB peptide, GSH, and GSSG were prepared by dissolving weighed samples in degassed and argon-flushed phosphate buffer (0.1 M, pH = 7.0) containing NaCl (0.1 M) and EDTA (1 mM). Each solution was checked before equilibration for a final pH value of 7.0 and, if required, pH adjustments were carried out with NaOH. Stock solutions of the peptide were left in the dark for several days to allow for complete relaxation to the *trans* isomer, and the concentrations were monitored by UV measurements ($\varepsilon_{335} = 21.04 \times 10^3$ M^{-1} cm^{-1}).[5] GSSG and GSH concentrations determined by weight were confirmed by UV absorbance at 248 nm for GSSG ($\varepsilon_{248} = 382$ M^{-1} cm^{-1})[25] and spectrophotometrically with 2,2′-dithiodipyridine for GSH.[26] Irradiation of the all-*trans* isomer solutions for 1 h at 360 nm was carried out to allow for partial *trans* → *cis* isomerization, in order to obtain simultaneous exposure of both the *trans* and *cis* isomers to the equilibration with the reference redox pair. All reaction components were prepared in freshly argon-flushed buffers and mixed just prior to incubation.

Quenching: Before HPLC analysis of the product distribution in the equilibration mixtures, aliquots were quenched with phosphoric acid as follows: samples (70 μL) of the reaction mixtures were taken under argon and treated with phosphoric acid (1 M, 30 μL) to lower the pH immediately to a value of 2; the samples were stored at 5 °C and HPLC analyses were performed within a few hours.

Chromatographic Separation and Quantification of the Reaction Products: All equilibration mixtures were analyzed by HPLC on a C-18 Nucleosil 300 column (4 mm × 300 mm, 5 μm; Macherey & Nagel, Düren, Germany) by using binary linear gradients of acetonitrile/H_3PO_4 (2%) as eluents, at a flow rate of 1 mL min^{-1}. UV absorbance was monitored at 210 nm and 330 nm. The elution conditions were optimized to achieve baseline separation of all the components of the thiol/disulfide exchange reaction mixture. The retention times of GSSG, GSH, and of the oxidized bicyclic peptide as *trans* and *cis* isomer were determined with authentic samples. Regarding the mixed disulfide and the reduced monocyclic peptide species, their identification was achieved by LC-ESI-MS analysis on a MB C-18 Nucleosil 100 column (1 mm × 250 mm, 5 μm; Macherey & Nagel) using binary linear gradients of 0.1% TFA in water (A) and 0.08% TFA in acetonitrile (B) as eluents, at a flow rate of 30 μL min^{-1} and UV detection at 210 nm. Optimized gradients were developed for LC-ESI-MS analysis of equilibration mixtures containing either only the *trans* isomer species (20% B to 40% B in 30 min, to 60% B in 5 min and to 95% B in 5 min), or mixtures of both *trans* and *cis* isomers (5% B to 25% B in 30 min, to 60% B in 10 min and to 95% B in 10 min). Chromatographic isolation of the glutathione-peptide mixed disulfides for a more detailed characterization was not carried out. Assuming quantitative elution of the reaction components, concentrations of the reduced and oxidized species at equilibrium were correlated to the HPLC peak areas, since the reduced and oxidized peptide species as the *trans* and *cis* isomers were found to exhibit identical extinction coefficients at 210 nm, within the limit of error of the spectroscopic measurements.

Equilibration Constants: For equilibration, the oxidized peptide as *trans* isomer or as a mixture of the *trans* and *cis* isomers at different ratios and at different concentrations (from 0.16 to 0.39 mM in the above-mentioned buffer) was exposed to the thiol/disulfide exchange reaction in the presence of a large excess of glutathione (80 to 100 times) at varying GSH/GSSG ratios (0.85, 2.0, 2.8, 3.0, and 4.6 for the all-*trans* isomer; 0.15, 0.32, 0.65, 0.97, and 1.45 for the *cis*/*trans* isomer mixture). The redox reactions were left to equilibrate under argon in a thermostatted bath (25 °C). The equilibration conditions of the samples were assessed by analyzing quenched aliquots (50 to 100 μL) at increasing time intervals by HPLC until the product distribution remained unchanged. Generally, 2−3 h were sufficient to reach equilibration, and an additional HPLC analysis was performed after 24 h to confirm the acquired data. Although the rates of *cis* → *trans* thermal relaxation of the monocyclic and bicyclic peptides differed to some extent,[5] it was assumed that the product distribution was not significantly affected by these slow processes in the time course of equilibration. This was also confirmed by the reproducible K_{ox} values obtained at different *cis*/*trans* isomer ratios.

Data Analysis: In the network of equilibria given in Figure 2 for the *trans* and *cis* isomers, the single steps can be considered as separate entities according to the "principle of detailed balancing".[27] The equilibrium constant K_{ox} is therefore given by following Equation (1).

$$K_{ox} = \frac{[\text{Peptide}_{ox}]\ [\text{GSH}]^2}{[\text{Peptide}_{red}]\ [\text{GSSG}]} \quad (1)$$

From the K_{ox} values, the apparent redox potentials of the bis(cysteinyl)-AMPB peptide in the *trans*- and *cis*-azo configurations were calculated by applying the Nernst Equation (2).

$$E_0' = E_0'\ (\text{glutathione}) - 0.03 \log K_{ox} \quad (2)$$

A standard redox potential of −240 mV[7] for glutathione was chosen to maintain congruence with the E_0' values reported in the literature for the natural redox proteins.

Refolding of Reduced and Denatured RNase A: For denaturation and refolding of RNase A, essentially known procedures were applied.[15] RNase A (14 mg) was incubated overnight in argon-flushed Tris buffer (0.1 M, pH = 8.6, 2 mL) containing guanidine hydrochloride (6 M) and DTT (62 mM). The reduced, denatured enzyme was chromatographically separated from the salt and DTT on Sephadex G-25, with HCl (0.01 M) as eluent, with monitoring of the elution pattern by UV absorbance at 275 nm. Fractions containing reduced RNase A were collected, and protein concentration in the eluate was estimated by using an ε_{275} value of 9200 M^{-1} cm^{-1},[11a] and, additionally, by determination of the thiol content with 2,2′-dithiodipyridine.[26] The RNase A solution was used immediately or stored under argon at −20 °C overnight. Refolding experiments were performed in triplicate in argon-flushed Tris buffer (0.1 M, pH = 7.4) containing EDTA (1.2 mM), and the temperature was maintained at 30 °C (± 0.1 °C) with a water bath connected to a thermostat. A solution of the oxidized AMPB peptide as all-*trans* isomer in Tris buffer was treated with GSH and GSSG at the GSH/GSSG/peptide ratio reported below for each experiment. The mixture was divided into two equal portions, one of which was irradiated at 360 nm for 30 min to induce the *trans* → *cis* isomerization. The two redox mixtures were allowed to equilibrate at 25 °C in the dark for at least 2 h. The enzyme reactivation was initiated by addition of reduced and denatured RNase A (24 μM) to the preequilibrated refolding mixtures, to obtain the following enzyme/GSH/GSSG/peptide ratios: 1:40:8:0.5, 1:40:8:1, 1:20:3:1, 1:20:2:2, and 1:20:0:4. The samples with the *cis* isomer were irradiated at 360 nm for 4 min every 2 h over 14 h and for further 4 min after 21 h and 24 h. At various time intervals, aliquots (60 μL) of the refolding mixtures were added to 800 μL of a cCMP solution (465 μM in MOPS buffer, pH = 7) and the absorbance was measured at 284 nm against the substrate solution.[28] Hydrolysis of cCMP was monitored at 25 °C over 3 min. The concentration of refolded RNase A was extrapolated from the initial rates of cCMP hydrolysis by use of a calibration curve calculated by assaying samples of native RNase A at known concentration (determined at 277.5 nm with an ε of 9800 M^{-1} cm^{-1}).[29] The time course of reactivation was plotted by using the average of triplicate measurements. The experimental curve was fitted by Equation (3), where A_{max} corresponds to the extrapolated maximum yield of the reactivation process and k_{app} is the "apparent rate constant" expressed in h^{-1}.

$$\%\ \text{RNase } A \text{ activity} = A_{max}(1 - e^{-k_{app}t}) \quad (3)$$

The initial rates were obtained from the first derivative of the graph calculated for $t = 0$ and converted into pmol min^{-1}.

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