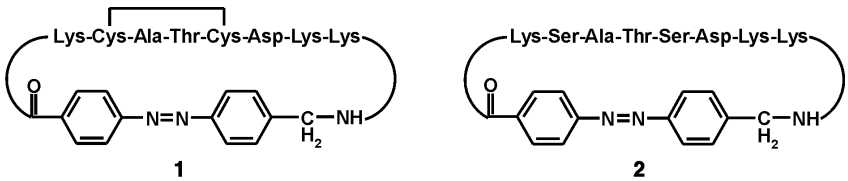
Photoresponsive Cyclic Bis(cysteinyl)peptides as Catalysts of Oxidative Protein Folding**

Anna Cattani-Scholz, Christian Renner, Chiara Cabrele, Raymond Behrendt, Dieter Oesterhelt, and Luis Moroder*

Folding and disulfide bridging of cysteine-containing proteins are thermodynamically coupled, generally slow processes in which rapidly formed nonnative intermediates have to be reshuffled by redox systems into the native cysteine network.[1] Disulfide bond formation and rearrangement are catalyzed in vivo by specialized enzymes such as protein disulfide isomerase (PDI) in eukaryotes[2] and disulfide bond (Dsb) proteins in prokaryotes.[3] These enzymes belong to the thioredoxin superfamily, exhibit the thioredoxin fold,[4] and contain the active-site consensus motif Cys-Xaa-Yaa-Cys that is located at the N-terminus of an α-helix. The cysteine residue nearer to the N-terminal of the two cysteine residues in the active site is solvent-exposed and because of its low pK_a value it acts as the nucleophile in the thiol/disulfide exchange reactions required for the correct oxidative folding pathway.[5] In vitro, the glutathione redox system (GSH/GSSG) is routinely used as auxiliary oxidative refolding agent[6] to mimic the natural environment in the endoplasmic reticulum,[7] and thus to allow through thiol/disulfide exchange reactions the thermodynamically most stable oxidation product to be formed, although often with significantly lower efficacy than in the presence of the thiol/disulfide oxidoreductases.

Despite the common thioredoxin fold, the thiol/disulfide oxidoreductases differ significantly in their redox potentials (E'_0) ranging from -270 mV for thioredoxin[8] to -122 mV for DsbA,[9] as well as in the pK_a value of the N-terminal cysteine of the active site (3.5 for DsbA, 4.5 for PDI, and about 7 for thioredoxin).[9] These large differences in the redox and nucleophilic properties of the thiol functions have been attributed to the different sequences of the α-helix-folded active-site motif,[8–10] whereas a decisive role of vicinal charged residues is more controversial.[11] In a previous study we analyzed whether the well differentiated redox properties of the enzymes of the thioredoxin family are retained in linear synthetic replicates of the active-site fragments.[12] Although the bis(cysteinyl)peptides showed moderately different redox potentials, their values were all close to -200 mV, thus enabling only minor catalytic effects on oxidative folding of RNase A as model protein.[13] These results clearly confirmed that the sequence-intrinsic free energy of formation of the disulfide-bridged Cys-Xaa-Yaa-Cys motif as well as the pK_a values of the two thiol functions are significantly affected and amplified by the structural constraints imparted by the overall protein fold. Consequently, conformationally restricted cyclic bis(cysteinyl)peptides might mimic in a more appropriate manner the active sites of the enzymes in terms of redox properties and disulfide reshuffling activity. In fact, cyclization of the active-site fragments mentioned above led to significantly more oxidizing redox potentials (unpublished results).

Backbone cyclization of the active-site fragment *H*-Ala-Cys-Ala-Thr-Cys-Asp-Gly-Phe-*OH* [134–141] of *E. coli* thioredoxin reductase by the photoresponsive 4-(4-aminophenylazo)benzoic acid or 4-(4-(aminomethyl)phenylazo)benzoic acid led to peptide systems that are characterized by a fully reversible *cis* $\leftrightarrow$ *trans* photoisomerization of the azobenzene moiety.[14] This isomerization is accompanied both in the reduced monocyclic and oxidized bicyclic form by conformational transitions. The low-energy structures of *trans* azo isomers are well-defined whereas *cis* azo isomers are less restricted conformational ensembles.[15] Owing to the water insolubility of these photoresponsive cyclic peptides, in the present study a water-soluble cyclic peptide was synthesized by appropriately replacing three residues outside the Cys-Ala-Thr-Cys motif with lysines (**1**, Scheme 1). Photomodulation of the redox properties of this analogue in aqueous media was attained and exploited to differently affect rates and efficacy of oxidative folding of RNase A in redox buffer.



Scheme 1. Structures of synthetic cyclic peptides related to the active site of *E. coli* thioredoxin reductase and containing 4-(4-aminomethyl)phenylazo)benzoic acid as backbone constituent.

To unambiguously characterize the NMR solution conformation of the cyclic bis(cysteinyl)peptide in its oxidized and reduced state as *trans* and *cis* isomer,[16] in addition to the bicyclic compound **1**, the related monocyclic bis(seryl)peptide **2** (Scheme 1) was synthesized[17] as the isomorphous analogue of the reduced bis(cysteinyl)peptide. The Cys $\rightarrow$Ser replacement assures full stability under the non-strictly anaerobic conditions of the NMR experiments. As already observed in our previous study for the water-insoluble cyclic azobenzene peptides in DMSO,[15] the NMR-derived conformations of both compounds **1** and **2** in the *trans*-azo configuration are mainly dictated by the extended structure of the chromophore which stretches the C-terminal portion of the peptide into a parallel alignment to the azobenzene moiety and allows a bending only in the N-terminal part as imposed by the cyclic structure of the construct rather than by the disulfide bridge (Figure 1). In fact, in the absence of a disulfide bond and, thus, upon relief from the conformational restrictions imparted by the 14-membered ring, that is in the bis(seryl) analogue **2**, the overall structure is largely retained. The side chains of the two cysteine residues, mimicked by the serine residues in com-

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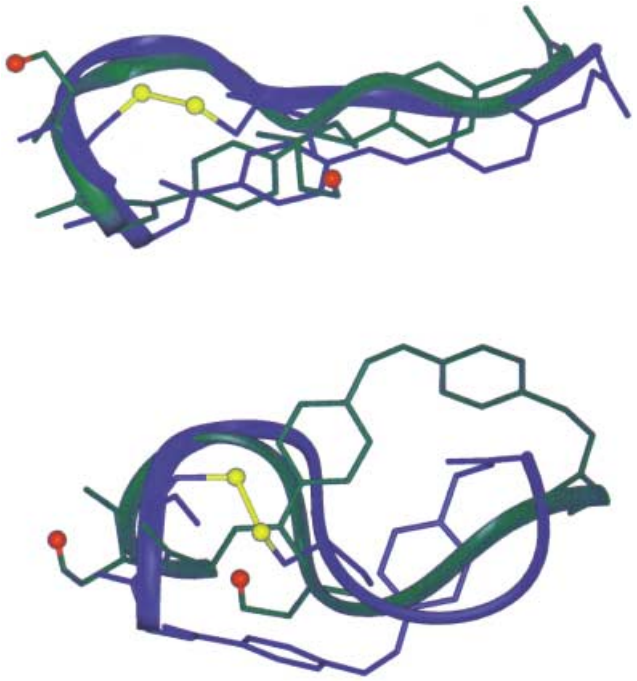

Figure 1. Representative structures from the lowest energy conformational families in aqueous solution derived from NMR conformational analysis of the *trans* isomers (top) and *cis* isomers (bottom) of the bicyclic compound **1** and the bis(seryl)peptide **2** as mimic of the reduced compound **1**. Backbone conformations are shown as ribbons, and the cysteine/serine residues as well as the azobenzene moiety as ball-and-stick models: blue for **1** and green for **2.**

pound **2**, are released from the ring-structure into the bulk water with minimal translational and rotational motions of the peptide backbone. Conversely, in the *cis*-azo configuration the bent structure of the chromophore significantly enhances the conformational freedom of the peptide moiety in both the monocyclic (**2**) and bicyclic (**1**) compounds. This favors in compound **1** the onset of a more helix-like turn involving the Cys-Ala-Thr-Cys motif, but the restraints of the disulfide bridge lead to an overall frustrated system that flip-flops between multiple conformational states. In the monocyclic peptide **2** a significantly more convergent family of energetically relaxed conformers is observed which are characterized by an S-shaped conformation. This is further supported by the observation that the activation energy of the thermal *cis* → *trans* isomerization of the monocyclic compound **2** is 12 kJ higher than that of the oxidized bicyclic form **1**.

As observed for the analogous systems in DMSO,[15] the *trans* ↔ *cis* photoisomerization of compound **1** upon irradiation at 360 nm and 450 nm, respectively, is fully reversible and occurs with high quantum yields following first-order kinetics with two isosbestic points located at 396 nm and 291 nm, respectively. Based on these photoresponsive properties as well as on the slow thermal relaxation of the isomerization in the darkness at room temperature, the redox properties of compound **1** in the *trans*-azo and *cis*-azo configuration were analyzed.[18] For this purpose, thiol/disulfide exchange experiments were performed with GSH/GSSG as reference redox pair, as previously described for the linear active-site fragments of the enzymes of the thioredoxin family.[12a] A typical chromatograph (HPLC) of the redox mixture of the *cis* and *trans* isomers with excess glutathione is shown in Figure 2. Reproducible K_{ox} values were obtained at

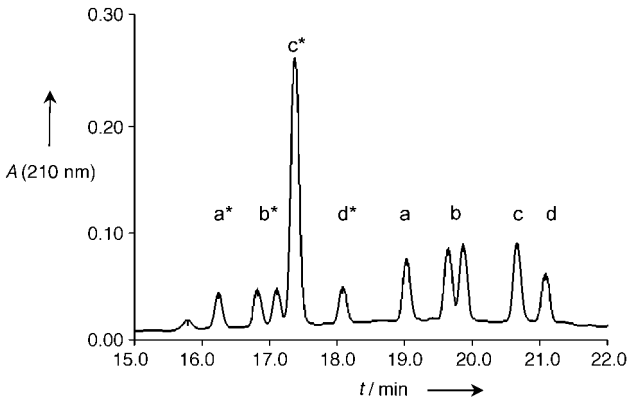

Figure 2. Chromatogram (HPLC) of an equilibrated redox mixture of **1** (*cis*/*trans* isomers) with excess glutathione (see ref. [18]). The species relative to the *cis*-azo configuration are marked by ∗: a) mixed bis(glutathione)/peptide disulfide; b) a pair of mixed mono(glutathione)/peptide disulfides; c) reduced peptide, and d) oxidized peptide.

different GSH/GSSG ratios from which the average redox potentials were derived by using $E_0' = -240$ mV for glutathione[19] (Table 1). Despite cyclization and incorporation of three lysine residues, the *trans* isomer exhibits a redox potential of -200 mV which differs only marginally from that of the parent linear active-site fragment *H*-Ala-Cys-Ala-Thr-Cys-Asp-Gly-Phe-*OH* [134–141] (-210 mV),[12a] con-

Table 1. Apparent redox potentials of the linear and cyclic active-site peptides of *E. coli* thioredoxin reductase.

Compound	K_{ox} [M]	E_0' [mV]
thioredoxin reductase		-250[a]
linear active-site fragment [134–141]	0.123[b]	-210[b]
cyclic peptide **1** as *trans*-azo isomer	0.050[c]	-200[c]
cyclic peptide **1** as *cis*-azo isomer	0.0008[c]	-146[c]

[a] Determined at pH 7.[21] [b] Determined in 0.1 M phosphate buffer containing 0.1 M NaCl and 1 mM EDTA (pH 7.0) at 20 °C and referred to a redox potential of -240 mV for GSH/GSSG.[12a] [c] Determined in the present study in 0.1 M phosphate buffer containing 0.1 M NaCl and 1 mM EDTA (pH 7.0) at 25 °C using $E_0' = -240$ mV for GSH/GSSG.[18, 19] The K_{ox} and E_0' values are the average of three experiments.

firming the minor effect of charged residues on the redox potentials.[11] Conversely, in the *cis*-azo configuration the redox potential is significantly shifted to a more oxidizing value ($E_0' = -146$ mV), which makes the peptide system more similar to the native enzymes PDI ($E_0' = -147/-159$ mV) and DsbA ($E_0' = -122$ mV).[9] These differences in the redox potentials between the *trans* and *cis* isomers correlate well with the NMR conformational analysis. In the *trans*-azo configuration both the oxidized and reduced species show a very similar overall conformation and the free energy of formation of the 14-membered ring in the oxidized form should be mainly dictated by the entropic contributions associated with the proximal display of the two cysteine side

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chains, while the conformational enthalpy and entropy terms should affect only marginally the K_{ox} value. In the *cis*-azo configuration, however, formation of the disulfide bridge is highly unfavorable, with a K_{ox} value that is nearly three orders of magnitude lower than that of the *trans* isomer. This lower propensity for the bicyclic state may be attributed to the penalty by the large conformational entropy change deriving from freezing the cysteine side chains into a defined topology, which is not compensated by a favorable conformational enthalpy term. In fact, a transition takes place from a lower energy conformation to a frustrated system in the oxidized form, as the lower activation energy for the *cis* →*trans* thermal relaxation of **1** shows. An additional contribution to the difference in the redox potentials and a possible effect on the pK_a values of the two thiols might also arise from electronic contributions of the azobenzene moiety that is spatially displayed in a significantly different mode in the *trans*-azo and *cis*-azo configuration relative to the disulfide group. Because of the sensitivity of the azobenzene group toward strong reducing agents such as 1,4-dithiothreitol (DTT) or tricarboxyethylphosphane, quantitative reduction of the cystine system in **1** for the measurement of the thiol pK_a values was prevented. In this context it is worthy to note that the pK_a values of the two cysteine residues in the cyclic bis(cysteinyl)peptides mentioned above are approximately 8 and 10, respectively, independent of the redox potentials that range from -130 mV to -200 mV (unpublished results).

Generally high redox potentials, low thiol pK_a values, and the presence of a peptide binding site, that is a chaperone activity that enables preferred interactions with the substrate, are the major features of natural protein disulfide isomerases. Although small bis(cysteinyl)peptide systems cannot be expected to exert chaperone activity, their redox properties and pK_a values of the thiols should govern the catalytic efficiency in the oxidative folding processes of proteins. This catalytic effect on the oxidative refolding of reduced and denaturated RNase A was analyzed for **1** in the *trans*-azo and *cis*-azo configuration.[20] For this purpose nonoptimal GSH/GSSG concentrations, that is an RNase A/GSH/GSSG ratio of 1:20:4, were used to avoid a buffering effect of glutathione on the auxiliary catalyst **1**. As shown in Figure 3, by replacing one equivalent of GSSG with **1** as *cis* isomer an acceleration of the initial rate of activation by 30% is obtained and, more significantly, the yield of active RNase A is increased from 56% to 80% after 30 h, with an extrapolated maximal reactivation of 94% ($\pm$3%) versus 64% ($\pm$2%) in the absence of **1**. Conversely, by replacing the *cis* isomer with the *trans* isomer the initial folding rate remains unaffected, but the refolding efficiency of the redox system is enhanced (extrapolated maximal reactivation of 83% $\pm$3%). The latter effect may derive from the higher nucleophilicity of at least one of the two peptide thiols compared to glutathione, as it can be expected by analogy to the differentiated pK_a values of the cyclic bis(cysteinyl)peptides mentioned above. An increase in the amount of compound **1** as *cis* isomer in the refolding mixture to two equivalents leads to a further acceleration of the initial folding rate (52% higher compared to the GSH/GSSG system). In contrast, increase in the concentration of the *trans* isomer is accompanied only by an increase in the yield of the process, with an extrapolated maximal reactivation of 100%.

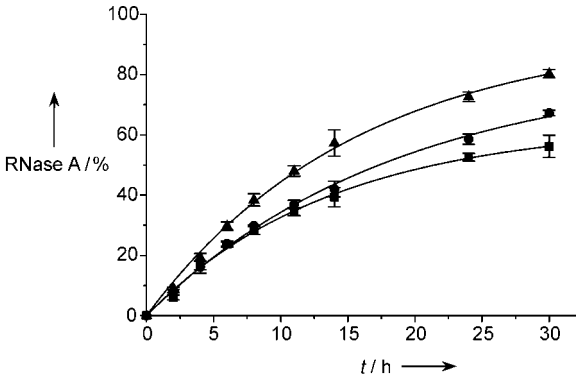

Figure 3. Time-course for the reactivation of reduced RNase A at 30 °C and pH 7.4 with glutathione at an RNase A/GSH/GSSG molar ratio of 1:20:4 (■), glutathione plus **1** as *trans* isomer (●) or *cis* isomer (▲) at an RNase A/GSH/GSSG/**1** molar ratio of 1:20:3:1. Each point is the average value from three experiments. The data points are fitted to the equation $[\text{active RNase A}]_t/[\text{reduced RNase A}]_{t=0} = a(1-e^{-kt})$, where a is the extrapolated maximal reactivation and k the first-order kinetic constant.

The experimental results confirm that conformationally restricted bis(cysteinyl)peptide systems can mimic the active sites of thiol/disulfide oxidoreductases and exert disulfide isomerization activity that are reminiscent of the foldase properties of the natural protein disulfide isomerases. Moreover, by incorporation of the azobenzene moiety into the cyclic bis(cysteinyl)peptide, a photomodulation of the efficiency of such folding adjuvants was achieved, a fact that may offer the possibility of a time-resolved modulation of functional properties.

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[16] ^{1}H NMR experiments were acquired on 3 mM samples in 9:1 (v:v) H_2O/D_2O at 500 MHz (Bruker DRX500 spectrometer) as described in reference [15b,c] with water suppression achieved by presaturation or WATERGATE technique (V. Sklenár, M. Piotto, R. Leppik, V. Saudek, *J. Magn. Reson. A* **1993**, *102*, 241 – 245). Conversion of NMR data to geometrical constraints and structure calculations (100 structures) were performed as described in reference [15b,c].
[17] The linear peptide precursors were synthesized on chlorotrityl resin by using the 9-fluorenylmethoxycarbonyl (Fmoc)/*t*Bu chemistry as described in reference [14]. Cyclization of the resulting side chain protected peptides was performed in DMF at a concentration of 0.5 mM by PyBOP/HOBt (benzotriazolyl-1-oxy-tris(pyrrolidino)phophonium hexafluorophosphate/1-hydroxybenzotriazole), and deprotection was carried out with trifluoroacetic acid/CH_2Cl_2 (95:5) containing triethylsilane as scavenger. Oxidation of the cyclic bis(cysteinyl)peptide was performed by air-oxygen in ammonium acetate buffer (pH 8) at a concentration of 10^{-4} M. **1**: ESI-MS: *m*/*z*: 1113.6 [M+H$^+$]; M_r = 1112.4 calcd for $C_{49}H_{72}N_{14}O_{12}S_2$; amino acid analysis (6 M HCl, 110 °C, 24 h): Asp 1.00 (1), Thr 0.90 (1), Ala 0.97 (1), Cys 1.46 (2), Lys 2.95 (3); peptide content: 71 %. **2**: ESI-MS: *m*/*z*: 1085.8 [M+H$^+$]; M_r = 1082.6 calcd for $C_{49}H_{74}N_{14}O_{14}$; amino acid analysis (6 M HCl, 110 °C, 24 h): Asp 1.00 (1), Thr 0.99 (1), Ser 1.98 (2), Ala 0.98 (1), Lys 2.90 (3); peptide content: 75 %.
[18] For the determination of the K_{ox} values, the *trans* isomer and mixtures of the *cis*/*trans* isomers of **1** (0.1 mM) in argon-saturated 0.1 M phosphate buffer (pH 7) containing 0.1 M NaCl and 1 mM EDTA were equilibrated at 25 °C for 3 – 4 h under argon with 100-fold excess glutathione at varying GSH/GSSG ratios. Aliquots of the equilibrated redox mixtures were quenched with 1 M phosphoric acid and analyzed by HPLC. Baseline separation of all components was achieved and these were identified as the two sets of the *cis* and *trans* isomer species by ESI-MS and by comparison with the redox mixture obtained for the pure *trans* isomer (see Figure 2). The concentrations of the oxidized and reduced forms of **1** at equilibrium were obtained by integration of the corresponding HPLC peak areas.
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[20] Fully reduced and denatured RNase A was prepared from bovine pancreatic RNase A (Aldrich, approximately 100 Kunitz units mg^{-1} protein) and refolding assays were performed following essentially the protocols previously described (Y. Konishi, T. Ooi, H. A. Sheraga, *Biochemistry* **1982**, *21*, 4734 – 4740). Reoxidation of the reduced RNase A (24 μM) in 0.1 M Tris-HCl buffer, 1 mM EDTA, pH 7.4, was carried out at 30 °C under argon in the glutathione redox buffer at an RNase A/GSH/GSSG molar ratio of 1:20:4 and in the presence of **1** at an RNase A/GSH/GSSG/**1** molar ratio of 1:20:3:1 and 1:20:2:2. Compound **1** was used as *trans* isomer (100 % after thermal relaxation at 50 °C overnight) and, upon irradiation at 360 nm, in the *cis*-azo configuration, that is in the photostationary state (*cis*/*trans* ratio: 80:20 ± 3). The reactivation reactions were initiated by addition of RNase A to the preequilibrated (at least 2 h at room temperature) refolding mixtures. The samples with the *cis* isomer were irradiated at 360 nm for 4 min every 2 h over 14 h and for a further 4 min after 21 h and 28 h. The time course of correctly folded RNase A was determined by measuring on aliquots of the refolding mixtures the initial rates of enzymatic cCMP hydrolysis (432 μM in 0.1 M MOPS buffer, pH 7.0) at 284 nm and at a final RNase A concentration of 1.7 μM.
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Oligo(phenylenevinylene)s with Terminal Donor – Acceptor Substitution**

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Conjugated oligomers[1] such as the oligo(phenylenevinylene)s (OPV)s have been extensively investigated for a number of years because they exhibit properties of interest to materials science in regard to application in nonlinear optics (NLO) and as photoconductors and electroluminophores. Typical of such a class of compounds is the convergence of absorption and fluorescence with the increasing number n of repeating units.[2] The determination of the effective conjugation length (ECL)[1q] is important for the characterization of the oligomers, as well as for their function as model compounds for the corresponding polymers. A simple algorithm, based upon exponential functions as natural growth functions, has been demonstrated to be effective for the determination of convergence and ECL in more than 20 series of conjugated compounds.[3]

We recently demonstrated that with a terminal push – pull substitution of conjugated oligomers the expected monotonic bathochromic shifts of absorption and fluorescence with increasing number n of repeating units need not necessarily be present.[4] Since such series are very important, especially for NLO materials, we have investigated the effect more thoroughly. OPV systems were prepared which have solubilizing bis(2-hexyloctyl)amino residues as the donor (D). Different acceptors (A) were introduced at the other end of the OPV chain (Scheme 1). The involvement of zwitterionic resonance structures should have a decisive influence upon

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