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A Nearly Isosteric Photosensitive Amide-Backbone Substitution Allows Enzyme Activity Switching in Ribonuclease S

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Abstract: ψ[CS−NH]^4-RNase S, a site specific modified version of RNase S obtained by thioxylation (O/S exchange) at the Ala^4-Ala^5- peptide bond, was used to evaluate the impact of protein backbone photoswitching on bioactivity. ψ[CS−NH]^4-RNase S was yielded by recombination of the S-protein and the respective chemically synthesized thioxylated S-peptide derivative. Comparison with RNase S revealed similar thermodynamic stability of the complex and an unperturbed enzymatic activity toward cytidine 2′,3′-cyclic monophosphate (cCMP). Reversible photoisomerization with a highly increased *cis*/*trans* isomer ratio of the thioxopeptide bond of ψ[CS−NH]^4-RNase S in the photostationary state occurred under UV irradiation conditions (254 nm). The slow thermal reisomerization ($t^{1/2} = 180$ s) permitted us to determine the enzymatic activity of *cis* ψ[CS−NH]^4-RNase S by measurement of inital rates of cCMP hydrolysis. Despite thermodynamic stability of *cis* ψ[CS−NH]^4-RNase S, its enzymatic activity is completely abolished but recovers after reisomerization. We conclude that the thioxopeptide bond modified polypeptide backbone represents a versatile probe for site-directed photoswitching of proteins.

Introduction

Numerous attempts have been made to reversibly control the bioactivity of a protein by introducing a photoresponsive element into the protein chain.[1−3] The success of the approach was demonstrated by generation of photoswitchable variants of enzymes,[4−7] neuronal ion channels,[8−10] and an *E. coli* transcriptional activator.[11] Typically, azobenzene and spiropyrane groups can be easily introduced in polypeptides and convey them to respond to irradiation with a conformational change. The *cis*/*trans* isomerization of azobenzene moieties involves a fairly large conformational change that is rapid on the time scale of conformational interconversions of a polypeptide chain.[12,13] A practical approach involves using substituted azobenzene moieties as site-specific side chain or backbone modifications that allow polypeptide chains to sample two regions of the conformational space determined by the azobenzene isomerization.[3,14−16] However, conformational flexibility of the linkages coupling the photoresponsive element to the polypeptide chain is likely to dampen effects of the azobenzene group isomerization and makes effects difficult to interpret.[17] Consequently, the phototriggered *cis*/*trans* isomerization of the phenylazophenylalanine residue at position 11 of RNase S, which is near its active site, did not lead to substantial effects on enzyme activity.[14] The hypothesis was made that the task of faithfully converting a light signal into a predetermined structural change of a protein is best accomplished by photoswitching the conformation of the backbone directly.

It is worth noting that the natural protein backbone itself provides a conformational change that has some features in common with the *cis*/*trans* isomerization of the azobenzene moiety. The *trans* to *cis* interconversion of the peptide bond (C_α−C(=O)−NR−C′$_\alpha$; R= H, alkyl) shortens the distance

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between the C_α and the C_α' atom by approximately 0.8 Å, and the *cis*/*trans* ratio increases in the photostationary state.[18,19] This isomer-specific chain displacement, in some proteins, propagates into the distal part of the molecule.[20] Unfortunately, the wavelength range (<220 nm) of peptide absorbance, which must be used for photoswitching, is not compatible with the photochemical stability of proteins. In addition, fast re-equilibration rates prevent the photoinduced *cis* population from applying isomer-specific effects on a coupled reaction. We have been developing a new approach to the peptide bond as a photoresponsive element of a protein that utilizes features of the near-isosteric thioxopeptide bond (—C(═S)—NR—; R = H, alkyl) substitution.[21−23] The process of photoisomerization of short thioxopeptides is completed within the picosecond time scale at a high quantum yield and does not exhibit a more prolonged reaction time for a longer peptide chain.[24,25] Biologically, the O/S exchange causes minor effects as long as a bioreaction does not directly involve the respective amide carbonyl group. In particular, it does not greatly affect secondary structure formation, and the bioactivity of the thioxopeptide derivative of ligands was found to be similar to that of the oxopeptide congener.[26−29] However, the lack of methods is evident for the site-specific thioxylation of a mature polypeptide chain and the chemical synthesis of an enzyme thioxylated at a predefined position. In addition, classical strategies of chemical ligation, which are normally used for synthesis of site-specific modified polypeptides, are not suitable because the presence of thioxopeptide bonds is not compatible with the subsequent synthesis of the thioester moiety which is necessary for the ligation procedure.

One way to reconcile the latter drawback would be for a protein which constitutes a bioactive molecule by noncovalently recruiting an oligopeptide. In this case, the oligopeptide can be prepared in the form of a mono- or polythioxylated molecule.

We were tempted to utilize RNase S to prepare an enzymatically active protein containing a single thioxopeptide bond. RNase S is composed of two RNase A fragments obtained by subtilisin cleveage, the S-peptide (consisting of residues 1 to 20), and the S-protein (consisting of residues 21 to 124) that constitute the active enzyme after mixing both components in solution.[30] It is well-known that RNase S dissociates and associates in a pH-dependent manner allowing the substitution of the natural S-peptide of RNase S by chemically modified versions (in the case of the recombination of modified S-peptides with S-protein, the term RNase S' is also in use).[14,31−34] We present results herein that suggest that backbone peptide bond thioxylation located in the *N*-terminal region of the S-peptide provides a reversible on/off photoswitch for the enzymatic activity of RNase S.

We provide evidence that the incorporation of the *trans* Ala^4-ψ[CS−NH]-Ala^5- segment into the S-peptide chain does not impair the formation of the noncovalent (Ala^4-ψ[CS−NH]-Ala^5) S-peptide/S-protein complex (ψ[CS−NH]^4-RNase S) and its hydrolytic activity against cytidine 2′,3′-cyclic monophosphate (cCMP). Irradiation of a ψ[CS−NH]^4-RNase S sample resulted in a markedly increased nonequilibrium population of *cis* ψ-[CS−NH]^4-RNase S that retained less than 1% cCMP cleaving activity. The data of this study are indicative of a major chemical change in the active site due to a predefined one-bond phototrigger at a remote site.

Experimental Section

General Considerations. All chemicals and reagents were purchased with the highest purity commercially available (Fluka, Merck, Aldrich, Sigma, Bachem, Novabiochem, Advanced ChemTech). The S-protein (Grade XII) was purchased from Sigma. Peptide synthesis was performed using a Syro II multiple peptide synthesizer (MultiSynTech, Germany). Analytical HPLC was performed using solvent gradients of B (ACN, 0.1% TFA) in A (H_2O, 0.1%TFA) on a Pharmacia LKB HPLC system in combination with the peak integration software "PC Integration Pack" (Kontron Instruments) and a LiChroCART 125-4 RP 8 (5 μm) column (Merck). Detection was performed at 220 nm, and the solvent flow rate was 1 mL/min. Preparative HPLC was performed on a Hibar RT 250-25 LiChrosorb RP-select B (7 μM) column using an HPLC system from SYKAM (Germany). Concentrations of the S-protein, S-peptide, and ψ[CS−NH]^4-S-peptide were determined using the extinction coefficients ϵ_{280} = 9560 M^{-1} cm^{-1}, $\epsilon_{257.5}$ = 195 M^{-1} cm^{-1}, and ϵ_{266} = 12 000 M^{-1} cm^{-1}, respectively.

Peptide Synthesis. Whereas the S-peptide was synthesized using a standard protocol of solid-phase peptide synthesis, a special protocol was used for the ψ[CS−NH]^4-S-peptide and Ac-Ala-ψ[CS−NH]-Phe-NH_2 as described elsewhere.[35] Peptides were purified by preparative HPLC.

Ac-Ala-ψ[CS−NH]-Phe-NH_2: [M + H^+] calcd *m*/*z* = 294.1; [M + H^+] found *m*/*z* = 294.0; t_R, 19.8 min (10−25% B in 30 min). S-peptide: [M + H^+] calcd *m*/*z* = 2148.1; [M + H^+] found *m*/*z* = 2148.4; t_R, 18.85 min (5−35% B in 30 min). ψ[CS−NH]^4-S-peptide: [M + H^+] calcd *m*/*z* = 2164.1; [M+H^+] found *m*/*z* = 2164.1; t_R, 18.86 min (5−35% B in 30 min).

Isothermal Titration Calorimetry. Calorimetric experiments were performed using a VP-ITC titration calorimeter (MicroCal, Inc, Northampton, MA) at 25 °C. All solutions were degassed under vacuum prior use. Solutions of the respective S-peptide derivative and S-protein were dialyzed against 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl). The volume of the protein solution (50 μM) in the sample cell was 1.4 mL. The injection syringe was filled with 300 μL solution of the respective S-peptide derivative (200 μM). Each titration experiment consisted of a single 1 μL injection followed by 16 identical 5 μL injections of the peptide solution.

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CD Spectroscopic Measurements. Measurements were performed at 25 °C in 50 mM sodium acetate buffer (pH 6.0, 0.1 M NaCl) using a Jasco-710 spectropolarimeter. The CD spectrum of ψ[CS−NH]^4-RNase S was yielded by mixing of the S-protein (45 μM) and ψ[CS−NH]^4-S-peptide (29.25 μM) and succeeding incubation at 25 °C for 15 min. According to the association constant, which was determined under the same conditions by ITC measurements, 96% of the thioxylated S-peptide is complexed by the S-protein. The CD spectrum of this mixture was corrected regarding the part of the S-protein and thioxylated S-peptide that are still free in solution, and the resulting spectrum was normalized to a ψ[CS−NH]^4-RNase S concentration of 50 μM. The CD spectrum of RNase S was obtained according to the same procedure with concentrations of 45 μM and 225 μM for the S-protein and the S-peptide, respectively (99.5% saturation of the S-protein), and was also normalized to an RNase S concentration of 50 μM.

Stopped-Flow Measurements. Determination of k_{off}: Measurements were performed using a Pistar-180 stopped-flow spectrophotometer (Applied Photophysics) at 25 °C in 50 mM sodium acetate buffer (pH 6.0, 0.1 M NaCl). The 2.5 mL injection syringe of the stopped-flow spectrometer was filled with a 6.9 mM solution of the S-peptide, and the 1 mL injection syringe, with a 126 μM solution of ψ[CS−NH]^4-RNase S (mixture containing 135 μM S-protein and 135 μM ψ[CS−NH]^4-S-peptide preincubated for 15 min at 25 °C). Concentrations in the sample cell (path length: 10 mm) directly after mixing of both solutions, meaning at the time point t_0 of the kinetic measurement, were 33.7 μM ψ[CS−NH]^4-RNase S and 4.93 mM S-peptide. Detection was performed using the CD signal of the thioxo peptide bond n−π* transition at 336 nm.

Determination of k_{on}: Measurements were performed at 25 °C in 50 mM sodium acetate buffer (pH 6.0, 0.1 M NaCl) using an SX-18 MV stopped-flow spectrophotometer (Applied Photophysics). The final overall concentration of the S-protein within the sample cell was 5 μM. All data were obtained under pseudo-first-order conditions ([S-peptide derivative] ≥ 5 × [S-protein]). Emission was detected at 315 nm (excitation: 280 nm). The rate constant k_{obsd} was calculated from the respective time course using a single-exponential equation.

NMR Measurements. All NMR experiments were performed using a DRX500 spectrometer (Bruker, Rheinstetten, Germany). All spectra were processed with the XWINNMR 3.5 software (Bruker) and referenced to external 2,2-dimethyl-2-silapentane-5-sulfonate.

Fluorescence Measurements. Spectra were recorded using a Fluorolog 3 fluorescence spectrometer (Jobin Yvon) and a 10 × 10 mm quartz cuvette. All spectra are corrected for buffer fluorescence and device specific effects.

Determination of the Enzyme Kinetic Constants. Measurements were performed in 0.1 M MES buffer (pH 6.0, 0.1 M NaCl) at 25 °C, according to Wang et al.[36] The respective S-peptide derivative was incubated for 15 min with the S-protein, and the measurement was then started by substrate addition (cCMP). Final concentrations in the samples were 2 μM for the S-protein, 200 μM for the S-peptide, and 100 μM for the ψ[CS−NH]^4-S-peptide. Accordingly, it was guaranteed that at least 99% of the S-protein is in the complex with the respective S-peptide derivative.

Irradiation Experiments. Irradiation was performed using a Xenon/Mercury lamp in combination with a monochromator (Photon Technology International). The light was conducted to the sample by a fiber optic cable. The ψ[CS−NH]^4-RNase S was yielded by preincubation (15 min) of a mixture containing the S-protein and ψ[CS−NH]^4-S-peptide at the indicated concentrations.

In all UV and CD spectroscopic measurements it was guaranteed that the UV light of the spectrometer has no significant influence on the thioxo peptide bond *cis*/*trans* ratio.

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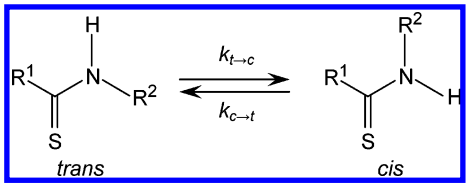

Figure 1. *Trans* and *cis* isomeric forms of an amidic thioxo peptide bond (R^1, R^2 = alkyl).

Results and Discussions

Preparation and Biophysical Characterization of ψ[CS-NH]^4-RNase S. Figure 1 shows the isomerization reaction underlying the phototriggered backbone conformational change. The sequence of the designed thioxylated S-peptide is H_2N-Lys-Glu-Thr-Ala-ψ[CS−NH]-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Nle-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH (ψ[CS−NH]^4-S-peptide) and was synthesized as described elsewhere.[35] Because of common problems with oxidation, the Met^{13} of the natural S-peptide was replaced by norleucine (Nle), a substitution that is tolerated by RNase S.[37,38] To generate ψ[CS−NH]^4-RNase S the S-protein and ψ[CS−NH]^4-S-peptide were allowed to recombine by mixing at pH 6.0 (50 mM acetate buffer) at 25 °C for 15 min. As a reference RNase S was prepared by recombination of S-protein with the corresponding non-thioxylated S-peptide that also contained an Nle at position 13. Comparative analysis of the S-peptide/S-protein complex stabilities by isothermal titration calorimetry (ITC) revealed that ψ-[CS−NH]^4-RNase S (K_a = (1.4 ± 0.04) × 10^6 M^{-1}) was thermodynamically slightly more stable than RNase S (K_a = (1.0 ± 0.04) × 10^6 M^{-1}). These values are in the range reported for the S-peptide/S-protein interaction under our conditions.[39,40] When free in solution, the S-peptide adopts a random coil conformation at 25 °C. In contrast, this part of the molecule forms an α-helix that spans the amino acids at position 3 to 13 of RNase S.[41−43] The peptide carbonyl group of Ala^4 is engaged in a hydrogen bond donated by the NH group of Phe,[8] whereas the Ala^5−NH is solvent exposed (PDB file 2RLN). Based on near-UV CD spectra the chromogenic properties of the thioxo peptide bond allowed the determination of microscopic rate constants of ψ[CS−NH]^4-RNase S formation according to eq 1.

$$\psi\text{[CS−NH]}^4\text{-S-peptide} + \text{S-protein} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \psi\text{[CS−NH]}^4\text{-RNase S} \quad (1)$$

In its unbound state, ψ[CS−NH]^4-S-peptide showed two thioxo group derived bands in the near-UV CD spectrum, representing the π−π* and n−π* transition at 265 and 336 nm, respectively (Figure 2). Examination at 336 nm revealed ψ-[CS−NH]^4-RNase S formation by a change $\Delta[\Theta]_{MRW}$ = 7900 deg cm^{-2} dmol^{-1} from positive to negative ellipticity in the

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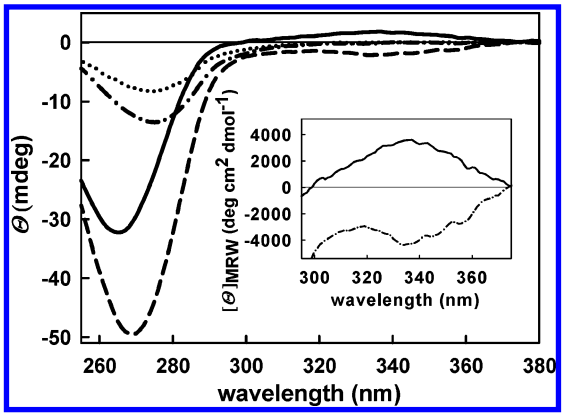

Figure 2. CD measurements. The figure shows the near UV−CD spectra of the ψ[CS−NH]^4-S-peptide (solid line), S-protein (dotted line), RNase S (dashed dotted line), and ψ[CS−NH]^4-RNase S (dashed line) at 50 μM concentration (path length 10 mm). Insert: zoom of the range between 300 and 380 nm in the case of the ψ[CS−NH]^4-S-peptide (solid line) and ψ-[CS−NH]^4-RNase S (dashed line). Measurements were performed at 25 °C in 50 mM sodium acetate buffer (pH 6.0, 0.1 M NaCl).

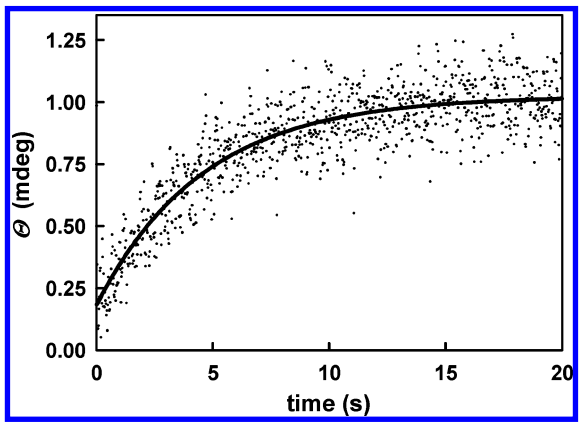

Figure 3. Determination of k_{off}. The figure shows the time course of displacement of the ψ[CS−NH]^4-S-peptide from the S-protein by addition a large excess of the S-peptide to the ψ[CS−NH]^4-RNase S. Measurements were performed using the CD signal at 336 nm. Concentrations in the sample cell (path length: 10 mm) were 33.7 μM ψ[CS−NH]^4-RNase S and 4.93 mM S-peptide. The excess was shown to be sufficient by increasing the concentration of the S-peptide until the observed rate constant was concentration independent. Using a single-exponential equation, a k_{off} value of (0.22 ± 0.01) s^{-1} was determined. The solid line represents the first-order fit. All measurements were performed at 25 °C in 50 mM sodium acetate buffer (pH 6.0, 0.1 M NaCl).

bound state. Notably, the spectral region above 320 nm lacked CD signals for S-peptide, S-protein, and RNase S. Thus, the supply of an excess of S-peptide to preformed ψ[CS−NH]^4-RNase S displaced ψ[CS−NH]^4-S-peptide from the complex and gave rise to a time-dependent increase in the CD signal at 336 nm that followed pseudo-first-order kinetics (Figure 3). In the RNase S dissociation reaction the rate of displacement of fluorescently labeled S-peptides was already shown to be similar to that of k_{off}.[44] The half time of dissociation of ψ[CS−NH]^4-RNase S was found to be 3.1 s (k_{off} = (0.22 ± 0.1) s^{-1}). We next determined k_{on} for ψ[CS−NH]^4-RNase S formation by using stopped-flow fluorescence at Tyr emission of S-protein. Using this method, it was also possible to determine k_{on} for the S-peptide/S-protein recombination under the same conditions (Figure 4). A k_{on} value of about 4.4 × 10^5 M^{-1} s^{-1} was obtained for both, the modified and unmodified RNase S, thus showing

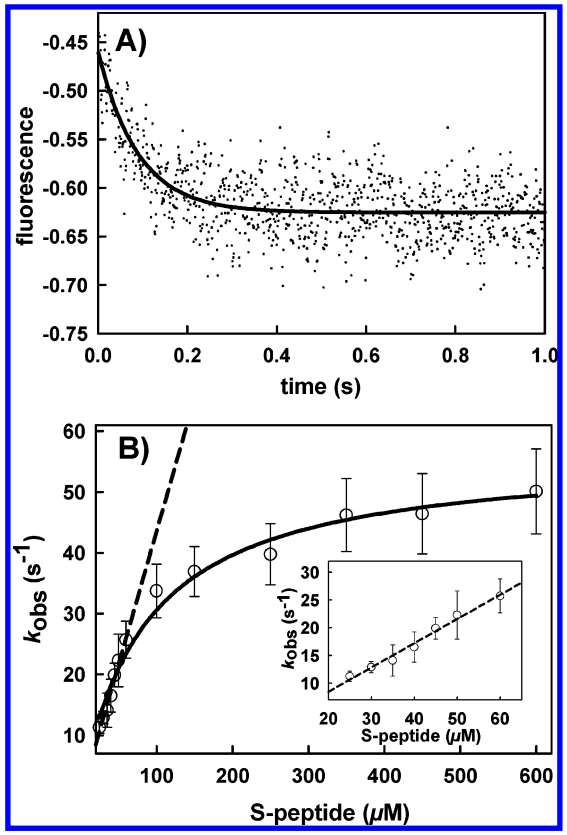

Figure 4. Determination of the k_{on} value of the S-peptide/S-protein-complex (RNase S) formation. (A) Time course of the fluorescence decrease during formation of RNase S. (B) Concentration dependence of the kinetics reaction. Measurements were performed at 25 °C in 50 mM sodium acetate buffer (pH 6.0; 0.1 M NaCl). Overall concentration of the S-protein within the sample cell was 5 μM. All data were obtained under pseudo-first-order conditions ([S-peptide] ≥ 5 × [S-protein]). Emission was detected at 315 nm (excitation: 280 nm). The rate constant k_{obsd} was calculated from the respective time course using a single-exponential equation (solid line). As shown in (A), for an S-peptide concentration of 25 μM a value of k_{obsd} = (11.2 ± 0.7) s^{-1} was obtained. The k_{on} value of the S-peptide/S-protein complex formation is equivalent to the initial slope of the curve displayed by the dashed line in (B) and was determined to be (4.37 ± 0.24) × 10^5 M^{-1} s^{-1}.

Table 1. Parameters Describing the Interaction between the S-Protein and the Respective S-Peptide Derivative

S-peptide derivative	k_{on}, 10^5 M^{-1} s^{-1}	k_{off}, s^{-1}	K_a,[a] 10^6 M^{-1}	K_a,[b] 10^6 M^{-1}
Ψ[CS−NH]^4-S-peptide	4.40 ± 0.55	0.22 ± 0.01	1.4 ± 0.04	2.0 ± 0.34
S-peptide	4.37 ± 0.24	0.44 ± 0.04[c]	1.0 ± 0.04	

[a] Determined by isothermal titration calorimetry. [b] Calculated with the equation $K_a = k_{on}/k_{off}$. [c] Calculated with the equation $k_{off} = k_{on}/K_a$; K_a from ITC measurements. Measurements were performed in 50 mM sodium acetate buffer (pH 6.0; 100 mM NaCl) at 25 °C.

that thioxylation at position 4 of the S-peptide has no effect on k_{on}. Furthermore, K_a for ψ[CS−NH]^4-RNase S formation, as calculated from the equation $K_a = k_{on}/k_{off}$ = (2.0 ± 0.34) M^{-1}, is rather similar to the value measured by isothermal titration calorimetry (Table 1). The rate constant k_{off} for RNase S, which is not directly available under our conditions, can then be calculated from $k_{off} = k_{on}/K_a$ = (0.44 ± 0.04) s^{-1}. This indicates that the O/S exchange contributed little to the noncovalent interactions determining RNase S stability, and this small contribution is exclusively found in the k_{off} rate. Taken together these results demonstrate that, as a precondition for the suitability of this thioxo peptide bond in photoswitching

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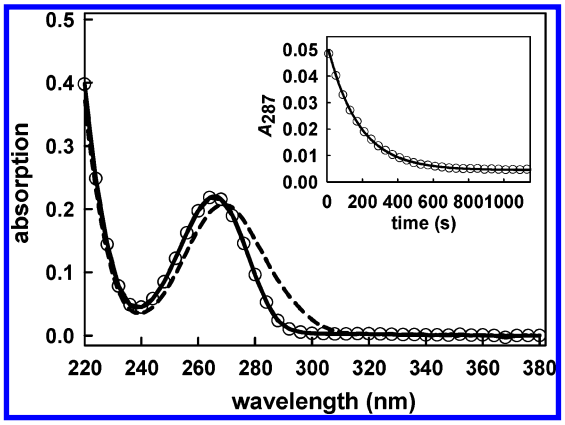

Figure 5. Photoisomerization of ψ[CS−NH]^4-S-peptide: UV spectroscopic characterization. The figure shows the UV spectrum of the ψ[CS−NH]^4-S-peptide measured before irradiation (solid line), in the photostationary state of irradiation at 254 nm (dashed line) and after 5 cycles of irradiation/re-equilibration (circles). Insert: The time dependence of re-equilibration (circles) has revealed a first-order rate constant $k_{c/t}$ of (5.7 ± 0.005) × 10^{-3} s^{-1} and was measured following the absorption at 287 nm. The solid line represents the first-order fit. All measurements were performed at 10 °C with 18.3 μM of the ψ[CS−NH]^4-S-peptide in 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl, path length: 10 mm). Irradiation was performed for 5 min at 254 nm.

experiments, the O/S substitution at position 4 has only a minor influence on the thermodynamic constants which characterize the S-peptide/S-protein complex.

Photoisomerization of Thioxo Compounds. The Ala^4-ψ-[CS−NH]-Ala^5 moiety of the ψ[CS−NH]^4-S-peptide provides an example of a isosteric replacement of a secondary amide peptide bond that probably all have >99% *trans* conformation in the ground state of unstructured polypeptides.[23,45,46] Irradiation of the ψ[CS−NH]^4-S-peptide with UV light at 254 nm has resulted in a bathochromic shift of the 265 nm UV absorption band and a slightly reduced maximum intensity (Figure 5) that is typical of an increased *cis*/*trans* ratio.[21,23] The thermal re-equilibration after irradiation is characterized by a first-order rate constant $k_{c/t} = 5.7 \times 10^{-3}$ s^{-1} at 10 °C about 1000-fold slower as compared to an oxopeptide bond (Figure 5).[45,47] This slow rate of isomerization allowed us to explore the individual catalytic parameters for each of the two isomeric forms of the enzyme. These forms exist as transient populations in the photostationary state contingent to a *cis*/*trans* isomerization at a single predefined peptide bond.

The lack of an NMR-sensitive isomeric reporter group has limited direct determination of the *cis*/*trans* ratio in ψ[CS−NH]^4-S-peptides.[45] Fortunately, an indirect method, which is based on the nearly sequence-independent $\pi-\pi^*$ transition in the UV/vis spectra of thioxopeptides could serve as a basis for determining the *cis*/*trans* ratio of the ψ[CS−NH]^4-S-peptide and ψ[CS−NH]^4-RNase S in their photostationary states. If the isomerizing thioxopeptide bond is the linker group between phenylalanine and alanine such as those occurring in Ac-Ala-ψ[CS−NH]-Phe-NH_2, the alanine β-methyl group experienced a large isomer specific anisotropy shift of 0.7 ppm that is upfield for the ^{1}H NMR signal of the *cis* isomer. The intensity pattern of the two sets of methyl signals, the *cis* isomer and *trans* isomer

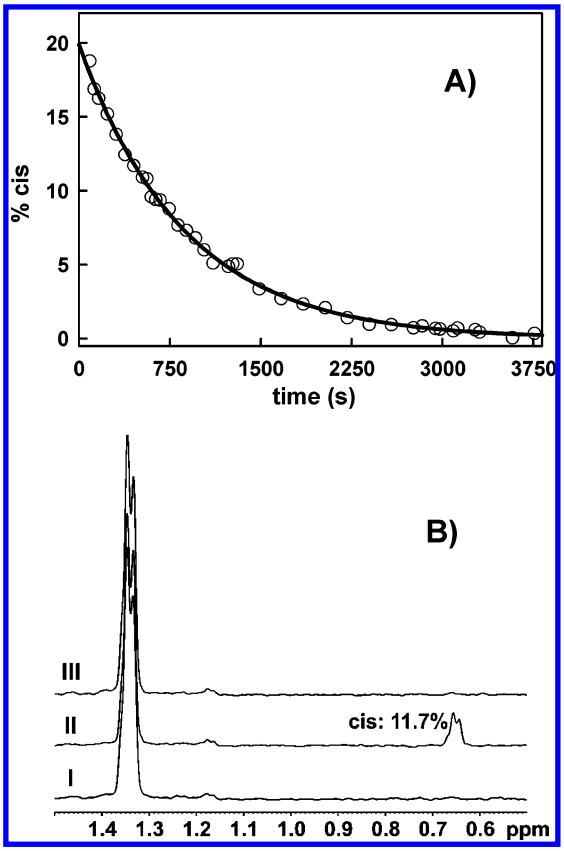

Figure 6. Ac-Ala-ψ[CS−NH]-Phe-NH_2: Determination of the *cis* content in the photostationary state of 254 nm irradiation by ^{1}H NMR measurements. A sample containing 3.07 mM Ac-Ala-ψ[CS−NH]-Phe-NH_2 in 33 mM sodium phosphate buffer (pH 7.5, 5% D_2O) was irradiated with UV light at 254 nm until the photostationary state was reached and then the ^{1}H NMR measurement started. (A) From the time course of the *cis*/*trans* ratio (circles) a value of (1.15 ± 0.03) × 10^{-3} s^{-1} was calculated for the first-order rate constant $k_{c/t}$ characterizing the re-equilibration process at 5 °C. The solid line represents the first-order fit. By extrapolation of the regression curve to the time point $t = 0$, a maximum *cis* content of 19.9% in the photostationary state was determined. The *cis* isomer percentage was calculated by integration of the respective thioxo alanine β-methyl proton signal (*trans*, 1.33 ppm; *cis*, 0.63 ppm) as shown in (B) (I, before irradiation; II, after 7.5 min of re-equilibration; III, after 1 h of re-equilibration). The following parameters were used for the NMR data collection: relaxation time, 1.2 s; number of scans per experiment, 16; spectral width, 14 ppm (7002.8 Hz); 90° puls length, 7.4 μs (2 dB); time domain size, 4 K; spectrometer frequency, 500.13 MHz.

doublets, permitted us to calculate the *cis*/*trans* ratio in the photostationary state after irradiation at 254 nm (Figure 6). The *cis* content increased from ≪0.5% to 19.9% in the photostationary state at 3.07 mM peptide under the conditions of the NMR experiment. If examined under similar conditions the NMR-based knowledge about the *cis*/*trans* ratio gave rise to the UV/vis spectrum of the pure *cis* and *trans* isomers (Figure 7). The Ac-Ala-ψ[CS−NH]-Phe-NH_2 exhibited absorption maxima in its *cis* and *trans* state at 281 and 265 nm with molar absorption coefficients of $\epsilon_{281} = 15\,215$ M^{-1} s^{-1} and $\epsilon_{265} = 12\,000$ M^{-1} s^{-1}, respectively. Notably, the *cis*/*trans* ratio of the photostationary state rises to 40% if the peptide concentration was lowered from 3 mM to 20 μM (see the Supporting Information). The reason is that at 3 mM the transparency of the sample is too low for an efficient photoisomerization. Corresponding isomer-specific band positions for the ψ[CS−NH]^4-S-peptide were calculated to be 281 (*cis*) and 265 nm (*trans*).

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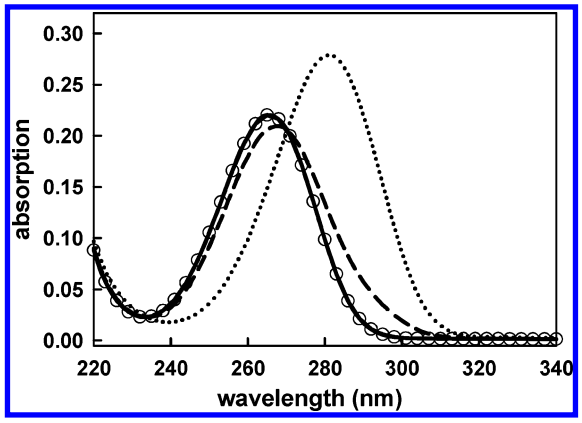

Figure 7. UV spectroscopic characterization of the thioxo peptide bond *cis*/*trans* photoisomerization of Ac-Ala-ψ[CS−NH]-Phe-NH_2. The following UV spectra are shown: Ac-Ala-ψ[CS−NH]-Phe-NH_2 before irradiation (solid line), in the photostationary state of irradiation at 254 nm (dashed line), after five cycles of irradiation/re-equilibration (circles) and the calculated spectrum of the pure *cis* thioxo peptide bond isomer form (dotted line). The measurements were performed at 5 °C in 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl, path length: 10 mm). For irradiation (duration: 5 min, wavelength: 254 nm), the same solution as in the case of the NMR measurements was used. After irradiation this concentrated solution was transferred into a cuvette containing the appropriate amount of buffer, resulting in a final thioxo peptide concentration of 18.35 μM. The measurement was then started immediately. Using a value of 19.9% for the *cis* content of the irradiated sample, as determined by the NMR measurements, the UV spectrum of the pure *cis* isomeric form was calculated. Thereby, it was assumed that the UV spectrum of the nonirradiated sample represents the spectrum of the pure *trans* isomeric form, because the NMR measurements have revealed that without irradiation the *cis* content is $\ll$ 0.5%.

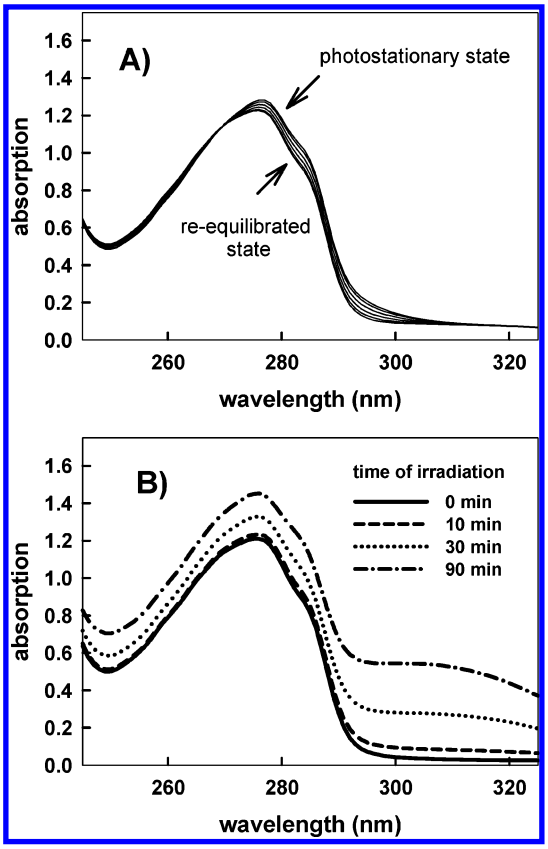

Figure 8. Irradiation of ψ[CS−NH]^4-RNase S with UV-light at 254 nm. (A) UV spectroscopic characterization of the re-equilibration process after thioxo peptide bond *cis*/*trans* photoisomerization of ψ[CS−NH]^4-RNase S. The measurements were performed at 10 °C in 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl, path length: 10 mm). The sample contained 79.6 μM S-protein and 36 μM ψ[CS−NH]^4-S-peptide ensuring that >98% of the S-peptide derivative is bound to the S-protein. Before irradiation (duration, 5 min; wavelength, 254 nm), the sample was stirred for 15 min. After irradiation the first measurement was started immediately followed by further measurements after increasing times of re-equilibration. (B) Long-time irradiation at 254 nm leads to irreversible changes in the UV spectrum of ψ[CS−NH]^4-RNase S. Measurements were performed using the same conditions as described in (A). Before the respective measurement was started, the sample was irradiated the indicated time and then stirred for a further 60 min without irradiation (re-equilibration of the thioxo peptide bond isomeric state).

Short-time irradiation of ψ[CS−NH]^4-RNase S leads also to reversible changes in the UV spectrum. But during long-time irradiations an irreversible change in the UV spectrum of ψ-[CS−NH]^4-RNase S was observed (Figure 8). On the contrary to the reversible effect, the irreversible effect does not depend on the thioxo peptide bond isomerization because it was also observed in the case of the S-protein and RNase S, respectively.

Given the similarity of isomer-specific changes of the C═S-derived $\pi-\pi^*$ transition[23] for Ac-Ala-ψ[CS−NH]-Phe-NH_2 and ψ[CS−NH]^4-S-peptide, spectra monitored in the photostationary states of ψ[CS−NH]^4-S-peptide and ψ[CS−NH]^4-RNase S can be used to calculate the *cis*/*trans* ratio of the irradiated samples (see the Supporting Information). At a concentration level of 18.3 μM we obtained 30.3% and 27.5% *cis* for ψ[CS−NH]^4-S-peptide and ψ[CS−NH]^4-RNase S, respectively. Ac-Ala-ψ[CS−NH]-Phe-NH_2 and ψ[CS−NH]^4-S-peptide subjected to five cycles of irradiation/re-equlibration did not show any sign of an irreversible event (Figures 5, 7).

Re-equilibration after irradiation of thioxopeptides gave rise to rate constants of the reversible thioxo peptide bond *cis*/*trans* isomerization by monitoring the time-dependent decrease in absorbance at 287 nm (Figure 9). At 10 °C the first-order rate constants for thioxopeptide bond isomerization in the case of the ψ[CS−NH]^4-S-peptide and ψ[CS−NH]^4-RNase were $k_{c/t} = (5.7 \pm 0.005) \times 10^{-3}\ s^{-1}$ and $k_{c/t} = (3.9 \pm 0.02) \times 10^{-3}\ s^{-1}$, respectively. Importantly, the order of irradiation and recombination steps involving S-protein and ψ[CS−NH]^4-S-peptide does not affect the observation of a lower *cis*/*trans* isomerization rate for ψ[CS−NH]^4-RNase. In the presence of an excess of unmodified S-peptide, which displaced ψ[CS−NH]^4-S-peptide from the irradiated ψ[CS−NH]^4-RNase, the isomerization rate constant $k_{c/t}$ is similar to that found for the unbound ψ[CS−NH]^4-S-peptide. This indicates that only the specific binding of the ψ[CS−NH]^4-S-peptide by the S-protein is responsible for the effect of the S-protein on $k_{c/t}$. These results are consistent with a stable *cis* ψ[CS−NH]^4-RNase molecule that does not dissociate into the components after switching the Ala^4-ψ[CS−NH]-Ala^5 moiety in *cis* conformation.

Definitive proof of the stability of *cis* ψ[CS−NH]^4-RNase S required investigation of (ψ[CS−NH],^{4}Trp^8)-RNase S that was obtained from chemically synthesized (ψ[CS−NH],^{4}Trp^8)-S-peptide and S-protein by recombination. The association constant characterizing the stability of the (ψ[CS−NH],^{4}Trp^8)-RNase S at 10 °C, the same temperature used in the irradiation experiments, has a value of $(5.6 \pm 0.4) \times 10^5\ M^{-1}$ and is significantly lower than the value measured at 25 °C for ψ-[CS−NH]^4-RNase. In this context it is also important to note that at 10 °C compared to 25 °C the S-peptide/S-protein complex stability is about 10-fold higher.[37]

The fluorescence of the singular Trp residue found in the whole complex is considerably quenched due to (ψ[CS−

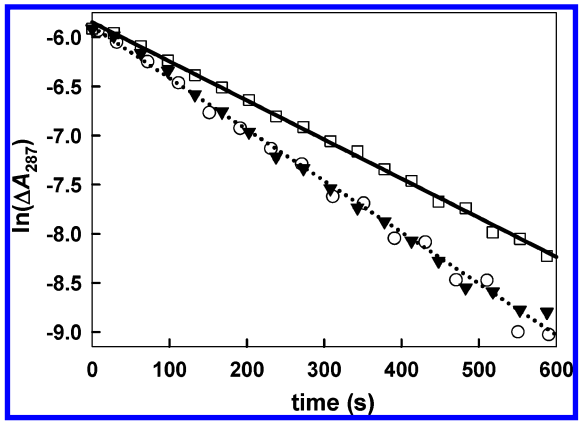

Figure 9. Re-equilibration process after the thioxo peptide bond *trans* to *cis* photoisomerization in the ψ[CS−NH]^4-S-peptide: influence of the S-protein on $k_{c/t}$. All measurements were performed at 10 °C in 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl). Before starting the measurements, each sample was irradiated for 5 min with UV light at 254 nm. The re-equilibration process was followed at 287 nm. In every case, the concentration of the ψ[CS−NH]^4-S-peptide was 2 μM. (Circles) free ψ[CS−NH]^4-S-peptide: $k_{c/t} = (5.7 \pm 0.03) \times 10^{-3}$ s^{-1}; (squares) in presence of 60 μM S-protein: $k_{c/t} = (3.9 \pm 0.02) \times 10^{-3}$ s^{-1}; (triangles) in presence of 60 μM S-protein and 200 μM S-peptide (the S-peptide was added directly after termination of irradiation): $k_{c/t} = (5.7 \pm 0.03) \times 10^{-3}$ s^{-1}. For the first and second case the respective linear regression is shown by the dashed and solid line, respectively.

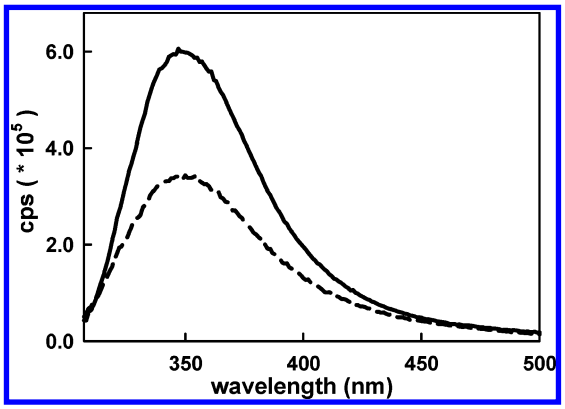

Figure 10. Fluorescence emission spectra of the (ψ[CS−NH]^4,Trp^8)-S-peptide and (ψ[CS−NH]^4,Trp^8)-RNase S. Measurements were performed at 10 °C in 50 mM sodium acetate buffer (pH 6.0) containing 100 mM NaCl. The concentration of the (ψ[CS−NH]^4,Trp^8)-S-peptide (solid line) and (ψ[CS−NH]^4,Trp^8)-RNase S (dashed line) was 10 μM. The sample of the (ψ[CS−NH^4,Trp^8)-RNase S contained 60 μM of the S-protein ensuring that >96% of the (ψ[CS−NH^4,Trp^8)-S-peptide (final concentration in the sample: 10 μM) is bound to the S-protein. Fluorescence resulting from the nonbounded, excess S-protein was subtracted from the respective spectrum. Samples were excitated at 297 nm. The quenching of the tryptophan fluorescence results from specific binding of (ψ[CS−NH]^4,Trp^8)-S-peptide by the S-protein. This was demonstrated by addition of a large excess of the S-peptide to the preformed (ψ[CS−NH]^4,Trp^8)-RNase S that replaces the (ψ[CS−NH]^4,Trp^8)-S-peptide from the S-protein (see the Supporting Information).

NH],^{4}Trp^8)-RNase S formation (Figure 10) allowing sensitive detection of complex dissociation during irradiation. After irradiation at 254 nm we found fluorescence quenching completely retained with the consequent preservation of the original population of *cis* (ψ[CS−NH],^{4}Trp^8)-RNase S (Figure 11).

Photocontrol of ψ[CS−NH]^4-RNase S Activity. The cleavage of a PO bond in cytidine 2′,3′-cyclic monophosphate (cCMP) has been investigated to compare the enzymatic properties of RNase S and ψ[CS−NH]^4-RNase S. Conditions of quantitatively formed RNase S complexes from the constituents are readily attained by using appropriate concentrations of starting materials. In essence, only minor alterations were found for k_2 (rate constant of the slowest step of the enzyme catalyzed substrate hydrolysis) and K_m values of both ψ[CS−NH]^4-RNase S and RNase S (Table 2), and the constants were similar to those reported for RNase S.[36] Because the thioxopeptide site does not make contact with the S-protein and the thioxocarbonyl is located in a space a long distance from the catalytic residues of RNase S, insensitivity of the catalytic parameter to this substitution is comprehensible. To assess how ψ[CS−NH]^4-RNase S responds to the light-induced *trans* to *cis* interconversion of the Ala^4-ψ[CS−NH]-Ala^5 moiety, initial rates of cCMP cleavage have been investigated before and after irradiation at 254 nm. Briefly, preformed ψ[CS−NH]^4-RNase S was irradiated and the hydrolytic reactions were started by addition of cCMP into the sample cell. Time courses of cCMP hydrolysis were measured by monitoring the absorbance increase at 290 nm at 10 °C and were found to be linear over the initial phase of the reaction (10 s). This indicates that cCMP depletion was negligible and alterations of the activity of irradiated ψ[CS−NH]^4-RNase S were absent in this time range (Figure 12A). Furthermore, the results of the irradiation experiments with (ψ-[CS−NH],^{4}Trp^8)-RNase S (Figures 10 and 11) discussed above

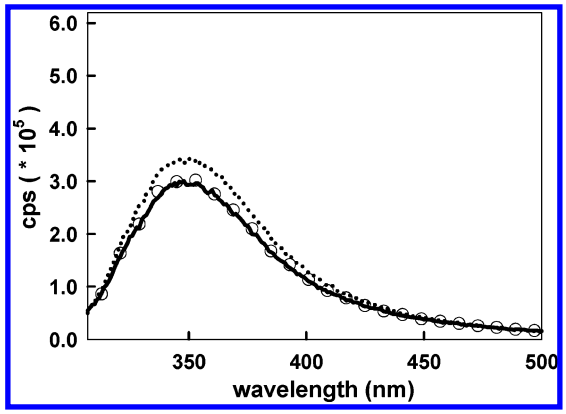

Figure 11. The thioxopeptide bond photoisomerization does not influence the fluorescence emission spectrum of the (ψ[CS−NH]^4,Trp^8)-RNase S. The spectra represent the (ψ[CS−NH]^4,Trp^8)-RNase S before irradiation (dotted line), in the photostationary state of the thioxopeptide bond photoisomerization (solid line) and after 2 h of re-equilibration (circles). The irreversible decrease in fluorescence results from the small amount of the tryptophan photobleaching during irradiation at 254 nm (see the Supporting Information). Measurements were performed at 10 °C in 50 mM sodium acetate buffer (pH 6.0) containing 100 mM NaCl. The concentration of the (ψ[CS−NH]^4,Trp^8)-RNase S was 10 μM. The sample contained 60 μM of the S-protein ensuring that >96% of the (ψ[CS−NH]^4,-Trp^8)-S-peptide (final concentration in the sample: 10 μM) is bound to the S-protein. Fluorescence resulting from the nonbounded, excess S-protein was subtracted from the respective spectrum. The fluorescence excitation was performed at 297 nm. Irradiation was performed for 5 min at 254 nm.

Table 2. Enzyme Kinetic Constants for Hydrolysis of the Substrate Cytidine 2′,3′-Cyclic Monophosphate (cCMP) by RNase S and ψ[CS−NH]^4-RNase S

enzyme	K_m (mM)	K_p[a] (μM)	k_2[b] (s^{-1})	k_2/K_m (M^{-1} s^{-1})
Ψ[CS−NH]^4-RNase S	0.6 ± 0.02	75 ± 1.1	3.6 ± 0.2	6000 ± 533
RNase S	0.6 ± 0.02	75 ± 0.6	3.9 ± 0.13	6500 ± 433

[a] The K_p value represents the dissociation constant of the enzyme/product complex, since the reaction product, cytidine 3′-monophosphate, is an inhibitor of RNase S. [b] k_2 represents the reaction rate constant of the slowest step of the catalyzed substrate hydrolysis. Measurements were performed in 0.1 M MES buffer (pH 6.0, 0.1 M NaCl) at 25 °C according to Wang et al.[36]

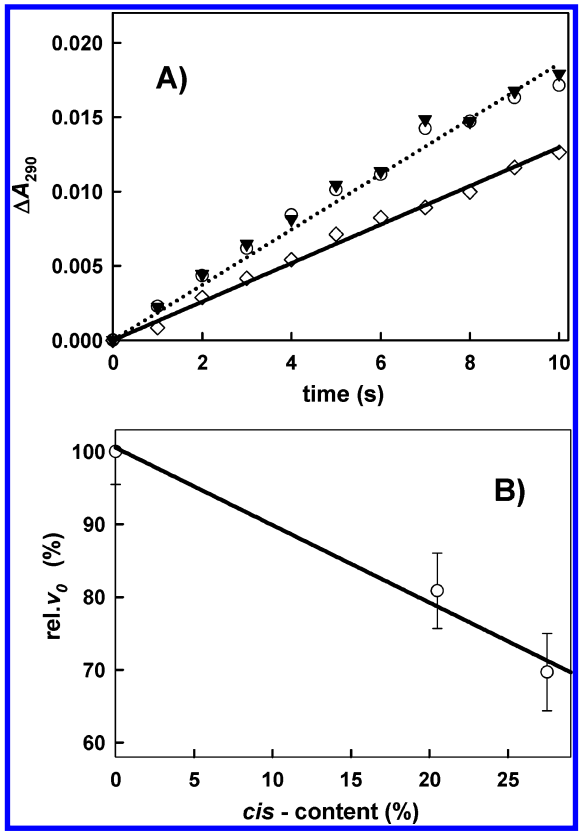

Figure 12. Initial rates of the ψ[CS−NH]^4-RNase S catalyzed cCMP hydrolysis: Influence of the thioxopeptide bond photoisomerization. (A) Enzyme kinetic measurements were performed before irradiation [circles; $v_0 = (1.88 \pm 0.04)$ μM/s], at the photostartionary state of irradiation [diamonds; $v_0 = (1.31 \pm 0.02)$ μM/s] and after re-equilibration (triangles; $v_0 = (1.93 \pm 0.04)$ μM/s]. In the first and second case the corresponding regression line is shown by the dotted and solid line, respectively. Kinetic measurements were performed at 10 °C in 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl). Final concentrations in the sample were 60 μM S-protein, 2 μM ψ[CS−NH]^4-S-peptide where >98% of the ψ[CS−NH]^4-S-peptide is complexed by the S-protein. Irradiation at 254 nm was performed for 5 min. Data collection was started immediately after substrate addition (100 μM cCMP). Thereby every 1 s one data point was collected. Because of the re-equilibration process of the thioxo peptide bond *cis*/*trans* equilibrium, a small part of ΔA_{290} in the case of the irradiated sample results from the small amount (~1%) of the thioxo peptide bond *cis* to *trans* isomerization that happens in parallel during the time of the enzymkinetic measurement. This small amount of signal change was subtracted guaranteeing that the graph of the irradiated sample in the figure represents only the substrate hydrolysis. (B) Correlation between the effect of irradiation on the ψ[CS−NH]^4-RNase S activity and the content of the *cis* thioxo peptide bond after irradiation. *Cis* contents of 27.5% and 20.5% were yielded by irradiation with UV light at 254 and 260 nm, respectively. The kinetic measurements were performed as those described above. The slope of the regression line is -1.07 ± 0.13, indicating that the ψ[CS−NH]^4-RNase S species with the *cis* thioxo peptide bond seems to be inactive.

along with the linear signal response in the activity measurements (Figure 12A) indicate that *cis* ψ[CS−NH]^4-RNase S does not dissociate in the constituents after its formation. Surprisingly initial rates of cCMP hydrolysis were found to be (1.88 ± 0.04) μM/s and (1.31 ± 0.02) μM/s for the nonirradiated state and the photostationary state of ψ[CS−NH]^4-RNase S, respectively. Furthermore, using the same conditions, irradiation of the nonthioxylated enzyme has no effect on the acitivity. Thus, the activity loss of 30% of ψ[CS−NH]^4-RNase S after irradiation appears to result from a partial interconversion of *trans* ψ[CS−NH]^4-RNase S into *cis* ψ[CS−NH]^4-RNase S. Two different approaches provided proof of this. First, the rate constant of

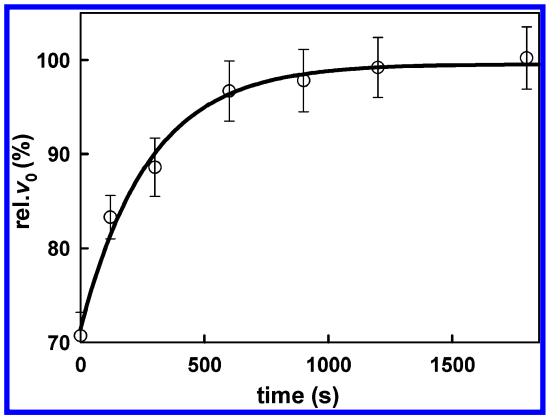

Figure 13. Regain of the ψ[CS−NH]^4-RNase S activity after the thioxopeptide bond photoisomerization. Kinetic measurements were performed at 10 °C in 50 mM sodium acetate buffer (pH 6.0, 100 mM NaCl). A mixture of 60 μM S-protein and 2 μM ψ[CS−NH]^4-S-peptide was preincubated for 15 min. At these concentrations, >98% of ψ[CS−NH]^4-S-peptide is complexed by the S-protein. The mixture was then irradiated for 5 min at 254 nm. After the respective time of re-equilibration the measurement was started by addition of 100 μM cCMP and the corresponding initial velocity v_0 was determined. The relative initial rate rel. v_0 was calculated by setting the initial rate of the nonirradiated sample to 100%. For the regain of the enzymatic activity, data analysis using a single-exponential equation resulted in a rate constant of $(3.6 \pm 0.5) \times 10^{-3}$ s^{-1}.

activity regain was found to follow first-order kinetics with $k = (3.6 \pm 0.5) \times 10^{-3}$ s^{-1} that agrees well with the value $k_{c/t} = (3.9 \pm 0.02) \times 10^{-3}$ s^{-1} directly measured for the thioxo peptide bond *cis*/*trans* isomerization of ψ[CS−NH]^4-RNase S (Figures 9, 13). Second, different *cis* levels of ψ[CS−NH]^4-RNase S in the photostationary state, which could be achieved by variations of the irradiation wavelength, were linearly correlated with initial rates of cCMP hydrolysis (Figure 12B). To ensure reversibility of the photoswitching, photoisomerized ψ[CS−NH]^4-RNase S was allowed to re-equilibrate for 60 min followed by enzyme activity monitoring (Figure 12A). The original level of enzyme activity (1.93 ± 0.04) μM/s was achieved after this procedure. What is most striking is that the magnitude of the *cis* ψ[CS−NH]^4-RNase S fraction of the irradiated ψ[CS−NH]^4-RNase S and the relative loss of ψ[CS−NH]^4-RNase S activity can be quantitatively matched (Figure 12B). Given a stable complex structure the *cis* ψ[CS−NH]^4-RNase S complex must be considered as an RNase inactive enzyme variant. This last result sets ψ[CS−NH]^4-RNase S apart from [phenylazophenylalanine]^4-RNase S since the latter side-chain photomodulatable enzyme variant hardly affected catalytic properties after adopting the *cis* conformation.[14,33] It is somewhat a mystery why a remote peptide bond *trans* to *cis* isomerization caused reduction of the level of cCMP hydolysis to <1 % of those for the *trans* ψ-[CS−NH]^4-RNase S variant. From our data it is unlikely that photoisomerization displaces *cis* ψ[CS−NH]^4-S-peptide from *trans* ψ[CS−NH]^4-RNase S with a concomitant total loss of enzyme activity.

Notably, it is the first turn of the short α-helix encompassing residues 3−13 that includes the Ala^4-ψ[CS−NH]-Ala^5 moiety. The *cis* conformation can be postulated to produce a local interruption of the helical conformation leaving other helical turns intact. However, from the invariance of K_a to the *O*/*S* replacement it is suggested that the C═S · · · · H−N Phe^8 hydrogen bond plays only a minor role for the helix stability, and thus removal has a low impact on helix positioning. In contrast, the insertion of a *cis* peptide linkage at the *N*-terminus

might apply compensatory components to the helix dipole that has been implicated in acid/base properties of pendent side chains.[48] At the helix termini the pK_a of a histidine side chain can be shifted up to 2 units when compared with the normal value of histidinium residues.[49,50] Major residues involved in RNase S catalysis include His^{12} and His^{119} that display pK_a values (RNaseA) of 5.8 and 6.2, respectively.[51] The unperturbed intrinsic pK_a of a proteinaceous His residue was given to be 6.5 for both surface and buried histidine.[52] The C-terminus of the S-peptide derived α-helix presents His^{12} prone to such a helix dipole induced pK_a shift. Thus, an upward shift of the pK_a value by reducing the helical dipole might severely interfere with the role of His^{12} as an acid/base catalyst. Alternatively, an isomerization-mediated chain displacement of the N-terminus of ψ[CS−NH]^4-RNase S might indirectly affect its active site structure. Although the N-terminal corrupted, the core part of the ψ[CS−NH]^4-S-peptide helix would remain connected to the S-protein. New ψ[CS−NH]^4-S-peptide/S-protein contacts could be formed in the *cis* isomer that could not be inferred from available RNase S structures with an all-*trans* S-peptide segment. However, because of results which were obtained with des-Lys, ^{1}Glu, ^{2}Thr^3-RNase S, and [Pro^6]-RNase S, respectively, the total loss of enzymatic activity by isomerization-mediated chain displacement of the *N*-terminus ore interruption of the first turns of the S-peptide α-helix of ψ[CS−NH]^4-RNase S seems to be less likely.[53−55]

There is evidence by using theoretical investigations of concerted conformational fluctuations to suggest a correlation between the network of protein vibrations and rate enhancement by enzymes.[56] In light of these data, uncoupling of backbone motions from substrate turnover steps might also account for the loss of enzymatic activity in the *cis* ψ[CS−NH]^4-RNase S variant. Experimental hints have emerged that such a mechanism could be effective in dihydrofolate reductase catalysis.[57,58] However, it is currently not known whether internal protein motions differ between *trans* ψ[CS−NH]^4-RNase S and the *cis* ψ[CS−NH]^4-RNase S variant variants.

Conclusion

UV/vis irradiation of the site-specifically thioxylated 20-mer S-peptide of RNase S or its ψ[CS−NH]^4-RNase S complex, in which the ground state all-*trans* conformation of the S-peptide portion is nearly complete, resulted in a photostationary state with about 30% *cis* isomer for the secondary amide thioxopeptide bond. A slow first-order reaction of thermal re-equilibration allowed sufficient time for assaying the influence of the increased *cis* population on enzymatic reactions. Photoswitching *trans* ψ[CS−NH]^4-RNase S thus provides the unique feature of giving an enzyme the opportunity to respond to a well-defined one-bond conformational change situated at a predefined peptide bond of the enzyme.

A surprising result of the first application of this method is that the conformation of the remote Ala^4-ψ[CS−NH]-Ala^5 moiety, which was never considered to be important for catalysis, has been implicated in the control of the catalytic machinery of RNase S. Whatever the reason, the one-bond photoswitching of the backbone is likely to find utility in the evaluation of folding-function relationships. A combination of a thioxopeptide scan through polypeptide segments with photoswitching may be especially informative.

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Supporting Information Available: Determination of content of the *cis* ψ[CS−NH]^4-S-peptide and *cis* ψ[CS−NH]^4-RNase S and additional control experiments about photoswitching of (ψ[CS−NH],^{4} Trp^8)-RNase S are described in detail. This material is available free of charge via the Internet at http://pubs.acs.org.

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