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Photochemical Regulation of an Artificial Hydrolase by a Backbone Incorporated Tertiary Structure Switch

N. Johan V. Lindgren, Miranda Varedian, and Adolf Gogoll*[a]

Abstract: A stilbene chromophore has been incorporated into the turn region of a 42 amino acid peptide, linking two helical peptide sections. Spatial proximity between these sections, as well as aggregation into dimers, is required to facilitate the catalytic function of this artificial hydrolase. Photomodulation of the hydrolase activity results in an increase of the activity of 42% upon switching from the *trans* to the *cis* isomer of the chromophore. This is rationalized by a change in the aggregation state of the peptidomimetic, which is supported by diffusion coefficients obtained from PFG-NMR experiments. The results show that incorporation of a small, relatively flexible chromophore into a large peptide is capable of inducing a considerable change in tertiary structure and thus, functionality.

Keywords: aggregation · chromophores · enzyme catalysis · peptidomimetics

Introduction

Nature relies on photoswitchable molecules to trigger biological functions essential for life. One of the most familiar examples is the *cis–trans* isomerization of retinal, a small molecule covalently attached to opsin. The protein changes its conformation when retinal is isomerized, and this conformational change activates an associated G-protein and triggers a second messenger cascade, ultimately resulting in the perception of vision.[1] Another example is provided by the kindling fluorescent proteins (GFP, green fluorescent proteins) of certain marine invertebrates, in which chromophore isomerization triggers bioluminescence.[2] Recently, the enzyme-catalyzed *cis–trans* isomerization of backbone proline residues has attracted attention as a natural mechanism to modulate protein function.[3]

Incorporation of photoswitchable chromophores into peptides and proteins opens new possibilities to modulate the structure and hence the function of these compounds.[4] Azobenzenes constitute the dominating chromophores in these studies,[4,5] probably due to their well understood photochemistry and ease of synthetic accessibility. The photomodulated properties of the resulting peptidomimetics include change of secondary structures such as β-hairpins and α-helical motifs,[6,7] and modulation of gramicidin channel permeability for ionic currents.[8] A few examples address the control of catalytic activity. Thus, a photoinduced change in the rate of hydrolysis of *para*-nitrophenol acetate by a cyclodextrin-peptide hybrid tagged with azobenzene has been reported.[9] Woolley et al. showed that juxtaposing a phenylazophenylalanine adjacent to the catalytic residue in RNase S led to small changes of enzyme activity between the isomers of the azobenzene derivative for the hydrolysis of RNA.[10] Both of these examples use the strategy of attaching azobenzene derivatives to amino acid side chains, a concept that previously has been shown to affect the conformation of the peptide to a much smaller extent than the backbone approach.[11]

In this work we report a profiling on the incorporation of the stilbene photoswitch into the backbone of a peptide, thus triggering a change of tertiary rather than secondary peptide structure. The target peptide of choice is a previously reported artificial hydrolase (HN1), comprising two parallel helical segments linked by a Gly-Pro-Val-Asp turn (Figure 1). Spatial proximity of the two helical segments has been established to be crucial to the enzymatic function of this artificial hydrolase.[12,13] The prime objective was whether it would be possible to modulate this enzymatic activity with a rather flexible photoswitch[14] that was to be incorporated into the peptide backbone. Examples of this so called optical nanotweezer approach are very rare,[4a] as opposed to side chain incorporation. Since photomodulation of large

[a] N. J. V. Lindgren, M. Varedian, Prof. A. Gogoll
Department of Biochemistry and Organic Chemistry
Uppsala University, Box 576, 751 23 Uppsala (Sweden)
Fax: (+46) 18-471-3818
E-mail: adolf.gogoll@biorg.uu.se

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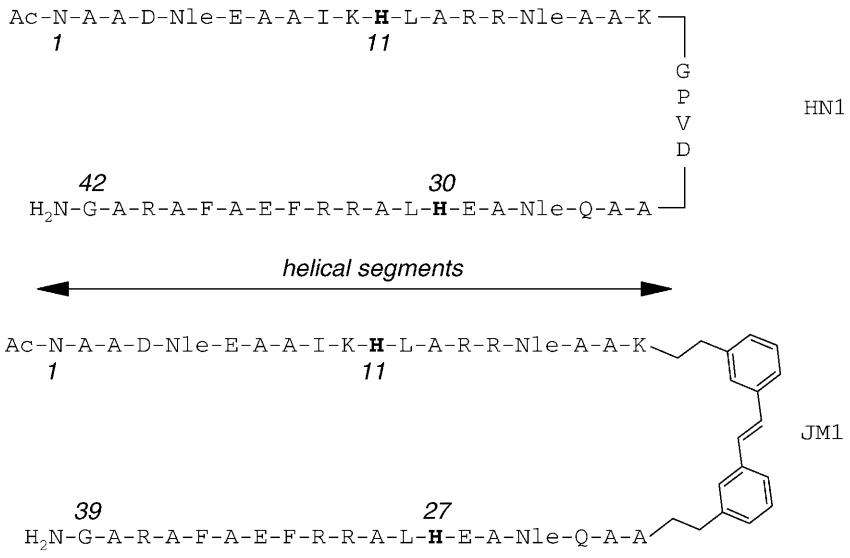


Figure 1. Sequences of the HN1 and JM1 peptides (Nle = norleucin). The C-terminal was amidated and the N-terminal was acetylated.

molecular entities, for example, dendrimers ($M \approx 6500$), by incorporation of stilbene chromophores has been reported,[15] this task appeared to be viable.

The hydrolytic activity of the target peptide depends on the proximity of histidines, which then promote a general acid–general base catalysis.[16,17] The sequence of the HN1 peptide (Figure 1) is based on the template SA42 and has earlier been reported to catalyze the hydrolysis of an RNA mimic.[18] The role of each residue has not been unequivocally determined but the proximity of several histidines to each other is of utmost importance. The two helical segments are amphiphilic and designed to interact through the hydrophobic effect. They are connected head to tail by the tetrapeptide sequence Gly-Pro-Val-Asp, which acts as helix breaker and forms a loop region. The design can be described in terms of the heptad repeat pattern[19] $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, in which the residues in the a and d position form the hydrophobic core, the residues in the c and g position form the exposed surface of the dimer, and the residues in the b and e position are at the dimer interface and control the dimerization. The design of the polypeptide in this manner drives it to fold into antiparallel dimers, constituting four-helix-bundles. Here, it has to be noted that the accurate secondary and tertiary structures of these peptides have not been established yet.

The stilbene switch substituted for the Gly-Pro-Val-Asp sequence (Figure 2), has previously been used to photochemically regulate the secondary structure of a β-hairpin.[14] It provides access to two photoisomers of different geometry (i.e., *cis* and *trans* isomers) but has at the same time an inherent conformational flexibility. Hence, it is likely to have the potential to adopt to the secondary structure of a peptide, if other interactions, for example, such as those between parallel peptide strands, overpower the conformational preferences of the chromophore itself.[14,20] Therefore, we hypothesized that the exchange of the entire GPVD sequence with a *meta*-substituted stilbene derivative, might mimic the loop upon isomerization to its *cis* isomer. Conversely, the *trans* isomer might generate a turn sequence less favorable for the hydrophobic interaction between the helical segments (Figure 2).

Thioaurones are another type of photoswitchable compounds and have recently been used to regulate the helical conformation of a peptide by linkage of two amino acid side chains in the peptide strand.[8,21] Conformational studies have indicated that thioaurone derivatives are more rigid than stilbenes, and thus potentially impose larger conformational restraint differences between their *E*- and *Z*-isomers onto peptides upon isomerization.[15] However, the use of thioaurones in photo-

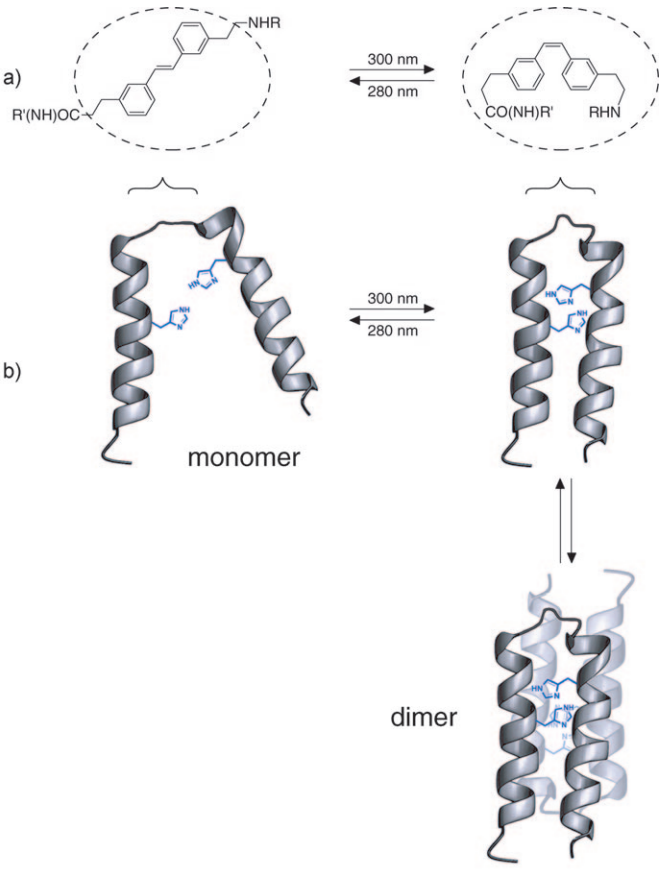
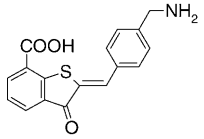


Figure 2. Photoisomerization of the stilbene chromophore and of the photoswitchable peptidomimetic JM1.

switchable peptidomimetics appears to be limited, due to their chemical instability during solid-phase peptide synthesis conditions.[22,23]

To test the performance of this chromophore, we synthesized another peptide sequence, JM2, in which the GPVD sequence was replaced by the thioaurone switch[24] in the loop region of the polypeptide (Scheme 1).



Scheme 1. The thioaurone chromophore incorporated into the peptide sequence JM2.

Results and Discussion

Previous studies of the stilbene photoswitch (Figure 2a) have shown that both *cis* and *trans* isomers, due to their intrinsic flexibility, promote a loop motif in peptidomimetics, albeit of different geometry.[14,25] Photoisomerization of the peptide JM1 was followed by analysis with analytical HPLC (see Figure S1 in the Supporting Information). This indicated a 1:9 *cis*:*trans* ratio of the stilbene moiety after synthesis, which is supported by the ^{1}H NMR data. Photoisomerization for 90 min at 300 nm changed this to a 4:1 *cis*:*trans* ratio, comparable to other peptidomimetics with the stilbene chromophore.[14,20,25]

CD spectroscopy of the peptidomimetics indicates a high helical content for a broad range of peptide concentrations (see Supporting Information for data). The JM1 peptide has a higher helical content compared to HN1. No significant differences were detected between the *cis*-JM1 and the *trans*-JM1 structures. Estimates of helical contents have previously been used to obtain indirect information about the aggregation state of this type of peptides. However, in the absence of detailed structural information, it is difficult to draw clear conclusions from the current CD data.

Measurements of translational diffusion coefficients by PFG-NMR experiments provide comparably straightforward information on the molecular weight of peptides and their aggregates in solution, as has been elaborated by Gräslund et al.[26] These parameters are presented in Table 1. The considerable divergence between the diffusion coefficients for *trans*-JM1 and *cis*-JM1, is unlikely to originate only in an alteration in peptide tertiary structure such as a different orientation of the two helical segments (Figure 2b). More likely, it indicates a change of peptide aggregation state, that is, from monomers (*trans*-JM1) into dimers (*cis*-JM1). Interestingly, although the above-mentioned relationship between peptide molecular weight (M_r) and diffusion coefficient D_t was devised for spherical peptides, calculated diffusion coefficients based on the molecular mass of JM1 are surprisingly similar to the observed values for *trans*-JM1 and *cis*-JM1. This suggests that *trans*-JM1 exists predominantly as monomers, whereas *cis*-JM1 forms dimers. The validity of this interpretation receives further support from investigations on the HN1 peptide. Analogues of HN1 have previously been shown, by ultracentrifugation, to exist in dimeric form in aqueous solution, and HN1 has been reported to behave similarly.[18] Again, the diffusion coefficient, as determined by PFG-NMR experiments, fits well with this assumption (Table 1). Furthermore, when HN1 is dissolved in D_2O/CD_3CN (65:35 vol%), which is known to suppress peptide aggregation,[27] the diffusion coefficient increases to the value that would be expected for the monomeric peptide. Here, the different viscosity of the solvent mixture has been accounted for.

The hydrolysis of *para*-nitrophenylacetate was used to monitor the enzymatic performance of the peptidomimetics. Second-order rate constants k_2 for the peptides HN1, and the photostationary states of *cis*-JM1 and *trans*-JM1 are presented in Table 2. A reference peptide HN1-ref in which the histidines are replaced with alanines is also included. As expected, the HN1-ref peptide shows almost no effect of general acid–general base catalysis ($k_2^{rel}=0.07$). The small residual effect (i.e., $k_2^{rel}\neq 0$) can be attributed to secondary effects, most likely transition-state stabilization by the positively charged arginines.[17] The lower value of $k_2^{rel}=0.88$ for *trans*-JM1 as compared to $k_2^{rel}=1.25$ for *cis*-JM1 shows that the histidines are situated in a less favorable position in the *trans* isomer. The higher k_2^{rel}-value for *cis*-JM1 compared to that for the reference peptide HN1 ($k_2^{rel}=1.00$), is likely due to different geometries of the loop regions.

Table 2. Second-order rate constants of the peptides HN1, JM1 and HN1-ref. The third column shows the rate constants normalized to the rate constant of the HN1 peptide.

Peptide	k_2 [M^{-1} s^{-1}]	$k_2^{rel}=k_2$ (pept)/k_2 (HN1)
HN1	0.049	1.00
trans-JM1	0.043	0.88
cis-JM1	0.061	1.25
HN1-ref	0.004	0.07

Table 1. Observed (obs) and calculated (calcd) diffusion coefficients for HN1 and JM1 peptides.

Peptide	Solvent	M_r [g mol^{-1}]	D_{obsd} 10^{10} [m^2 s^{-1}]	D_{calcd} 10^{10} [m^2 s^{-1}][26]
HN1 (dimer)	D_2O	9018	1.14	1.10
HN1 (monomer)	D_2O:CD_3CN (65:35)	4509	1.48	1.50
trans-JM1 (monomer)	D_2O	4416	1.58	1.51
cis-JM1 (dimer)	D_2O	8832	1.03	1.11

Thus, it appears that the loop provided by the *cis*-stilbene promotes arrangement of the helical peptide segments into a structure even more suitable for catalytic activity than what the Gly-Pro-Val-Asp sequence does in the original HN1 peptide.[28] In addition,

and most likely even more important, the dimerization of the peptidomimetic, which promotes its catalytic activity, is augmented for the *cis* isomer. It should also be noted that *trans*-JM1 appears to be monomeric while retaining its high helix content, in contrast to previously reported congeners without the stilbene motif.[12]

To test the hypothesis that a more rigid switch might generate a larger structural and thus catalytic difference between the photoisomers, the thioaurone switch was incorporated in the novel sequence JM2. The synthesis of JM2 was successful according to MALDI-TOF MS and HPLC data (see Supporting Information for data). Following cleavage from the resin, subsequent purification steps, which were monitored by MALDI-TOF MS, indicated successive decomposition, which also was detected by changes in retention times and the UV spectrum. A likely explanation is an irreversible chemical conversion of the thioaurone moiety, probably due to intramolecular attack by nucleophiles present in the peptide sequence.

Conclusion

Herein we have shown that when replacing the loop region in a catalytically active helix-loop-helix peptide with a photoswitchable stilbene derivative, the rate of hydrolysis of an activated substrate was not only retained, but slightly improved. Photoisomerization resulted in a modulation of catalytic activity, with a 42% higher rate constant for the *cis* isomer. Most likely, in the photoswitchable peptidomimetics the inter-helix distances are more (*cis* isomer) or less (*trans* isomer) optimal for the catalytic activity than in the reference peptide HN1. Thus, incorporation of a comparatively small and even somewhat flexible photoswitchable unit into a large peptidomimetic appears to be a viable concept for photomodulation of functionality in a biocatalyst. Optimization of the switch structure to render larger conformational differences between the photoisomers, for example, by choosing a more rigid switch, holds the promise to generate more drastic effects. However, such a switch must also show sufficient chemical stability.

Experimental Section

Synthesis: The synthesis of the photoswitchable *meta*-substituted stilbene derivative was conducted according to the literature.[14] Characterization and detailed synthetic procedures can be found in the Supporting Information. The peptide JM1 was synthesized on an automated peptide synthesizer (Pioneer, Applied Biosystems) by standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry on a 0.2 mmol scale. The solid phase used was Fmoc-PAL-PEG-PS (Applied Biosystems) with a loading of 0.20 mmol g^{-1}. The Fmoc-protected amino acids were coupled in a fourfold excess by benzotriazol-1-yl-*N*-tetramethyluronium hexafluorophosphate (HBTU; 0.8 M in DMF) and diisopropylethylamine (DIPEA; 1 M in DMF) as activators. The Fmoc protecting group was deprotected by using 20% piperidine in DMF (v/v). Standard coupling times for the amino acids were 60 min except for His and Gln (90 min), Arg and Asn (120 min). The N-terminal was capped with acetic anhydride and the C-terminal was amidated upon cleavage from the resin. The Fmoc-protected stilbene derivative was, together with the two following amino acids, introduced manually in the sequence (to minimize the excess) using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBop) as activating agent (0.6 M), with a threefold amino acid excess allowing a coupling time of 120 min. The global cleavage of the peptide was done in a mixture containing trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (95:2.5:2.5 v/v) for four hours. The solution was concentrated and the peptide was precipitated with cooled diethyl ether. The crude material was lyophilized prior to purification by reversed-phase HPLC (semi-preparative hypersil C-18 column 250×20 mm, 5 μm particle size) using a gradient of 40–50% acetonitrile containing 0.1% TFA (v/v) for 60 min. The purified peptide was obtained upon lyophilization of the collected HPLC-fractions. Identification of the peptide was done by MALDI-TOF MS (Applied Biosystems, Voyager-DE PRO). The sample was mixed with a saturated solution of α-cyano-4-hydroxy cinnamic acid in acetonitrile/H_2O (1:1) containing 0.1% TFA. Purity was confirmed by amino acid analysis (Uppsala University Amino Acid Analysis Center) and analytical HPLC (Hypersil C-18 250×4.6 mm, 5 μm particle size) using a gradient of 40–60% acetonitrile containing 0.1% TFA for 40 minutes. No impurities were detected (diode-array detector 229–400 nm). The peptide JM2 was synthesized in a similar way, but due to stability problems of the thioaurone switch during solid-phase peptide synthesis, cleavage conditions were altered by replacing triisopropylsilane by anisole.

CD spectroscopy: CD spectroscopy (JASCO J-810) was done at 298 K using peptide concentrations of 600, 60, and 6 μM in 10 mM phosphate buffer. The pH was set to 7.0 prior to measuring.

Photoisomerization: The photochemical reactions were carried out for acetonitrile/water under N_2 gas flow using an Oriel 1000 W Xe ARC light source and a 300 or 280 nm Oriel UV filter. The emitted light intensity was determined by using a UV enhanced Si photodiode (5.8 mm^2) attached to a current meter.

Kinetics: The second-order rate constant k_2 of JM1 for the hydrolysis of *para*-nitrophenylacetate was determined by linear regression of the plot of the observed velocity (v_{obs}) at three different concentrations of the catalyst against the catalyst concentration. The v_{obs} was determined by diluting a stock solution of peptide of known concentration determined by amino acid analysis to a new stock solution, making it 2 mM of peptide in 50 mM sodium acetate as buffer. The pH was fixed to 5.0. From the 2 mM peptide solution, three concentrations of peptide (0.4, 0.6, and 0.8 mM) were made by dilution with buffer in separate Eppendorf tubes. The substrate was dissolved in acetonitrile to give a stock solution of 20 mM. Measurement of the product formation started as soon as possible after addition of 4 μL of substrate stock to 796 μL of peptide solution, thorough mixing, and division of each peptide into two 0.1 cm cuvettes for dual samples. The evolution of the hydrolysis was studied at 320 nm for detection of the *para*-nitrophenol on a CARY 100 BIO equipped with a temperature controller. The reaction proceeded at 298 K. The concentration of product formed was determined from the absorption of the *para*-nitrophenol species using an absorption coefficient of 10000 $cm^{-1}M^{-1}$. The reactions were monitored to complete hydrolysis of the substrate. The v_{obs} was determined from the initial rate for the first 60 min of the measurement.

Diffusion measurements: For the LED-PGSE diffusion experiments,[29] z gradients were employed and 64 scans were acquired. A relaxation delay of 0 s, 9 ms gradient pulse duration, 40 ms diffusion delay, 5 ms storage delay was used and the gradient pulse strength was arrayed between 0 and 20 Gauss cm^{-1} (20 steps). The diffusion coefficients were calculated based on the diffusion coefficient of HDO (1.902×10^{-9} m^2s^{-1}),[30] with an accuracy of approximately 3%.

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