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Tetrazine Phototriggers: Probes for Peptide Dynamics**

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Ultrafast photochemical triggers hold the promise of providing information on the dynamics of peptide and protein folding.[1] Prior to photolysis, bonding to the trigger constrains the peptide to have a narrow structure distribution. Photochemical triggering releases the constraints, permitting the molecule to evolve to a different equilibrium distribution. The structure evolution, even when ultrafast, can be followed by infrared probe or two-dimensional infrared spectroscopy. Fast phototriggering can thus reveal early kinetic events in protein dynamics by providing a means to explore the free energy landscape of folding and misfolding. Several phototriggers have been developed for this purpose,[1,2] but there remain significant challenges. For example, disulfide bonds in peptides can be used as a phototriggers. Deep UV light severs the disulfide bond and releases the structural constraints; such experiments have been carried out in short helical peptides,[1a,b] cyclic peptides,[1d] and β hairpins.[1c] Although disulfide photolysis offers the capability of initiating ultrafast structure equilibration, limitations preclude broad generality of this technique. Homolytic S–S bond scission reveals two reactive radicals that can undergo geminate recombination, as well as reactions with protein sidechains. Moreover, the UV excitation required to dissociate the disulfide bond also excites the peptide backbone. Another example is azobenzene which undergoes fast, reversible photoisomerization. When designed into a peptide a light pulse can be used to cause the system to reversibly shift between significantly different equilibrium configurations.[3]

In order to optimize knowledge of the early events in peptide/protein folding and unfolding, the triggering process should (1) be initiated on the ps timescale from a narrow structure distribution and be faster than the early events in conformational reorganization such as single bond rotations, hydrogen bond formation, or helix nucleation[4], (2) have a high photochemical yield, (3) produce inert products with negligible side reactions along the photolysis pathways, (4) have biocompatibility for ease of incorporation into peptides and proteins, and (5) be photochemically accessible to non-damaging light pulse frequencies. *s*-Tetrazine and its congeners represent a class of compounds that fulfill most of these requirements. For example, tetrazine photoproducts are relatively inert, unobtrusive nitriles and molecular nitrogen (Scheme 1).[5] In addition, the $n \rightarrow \pi^*$ transition at 532 nm permits excitation in the visible region of the spectrum with a photoproduct yield, in the case of the

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parent *s*-tetrazine, close to unity in the vapor phase.[6] The quantum yields and light induced pathways are environmentally sensitive,[7] and it is well established that the photoreaction of *s*-tetrazine occurs on the ground state following internal conversion.[8]

With these observations as background, we report here the potential use of incorporating the *s*-tetrazine structural motif in peptides as an ultrafast photochemical trigger to initiate early events in peptide/protein folding. Although the overall photochemical yield of substituted *s*-tetrazines has been suggested to decrease in the condensed phase compared with the gas phase,[5,9] the variation of yield with substituent has not been systematically examined. Our initial efforts therefore focused on investigating the condensed phase photophysical properties of the parent *s*-tetrazine along with dimethyl and diphenyl 3,6-substituted *s*-tetrazines as model compounds to guide the development of amino acid-based systems.

The photochemical quantum yield for this series of 3,6-disubstituted tetrazines was measured in the condensed phase by nanosecond flash photolysis, observing the bleach signal of the parent molecules around 542 nm after a 1 ms delay, when all photochemical processes are completed. The photolysis yield per shot was determined by the ratio of the ΔOD , the optical density of the bleach signal, and $OD(0)$, the optical density before irradiation at 532 nm. The mechanism of the photodissociation of simple tetrazines involves a barrier crossing on the electronic ground state.[8] The parent molecule, *s*-tetrazine ($R = H$), dissociates on the subnanosecond time scale with a high photolysis yield (~90%). When additional substituents are attached to the tetrazine, the number of modes accessible after internal conversion is greatly increased and the photolysis yield is reduced due to vibrational energy redistribution. For example, in the case of 3,6-dimethyl-*s*-tetrazine ($R = Me$), the yield is reduced to 18%. This trend continues for 3,6-diphenyl-*s*-tetrazine ($R = Ph$), where the yield is further reduced to 1–2% due to the involvement of the ring modes of the phenyl groups in the vibrational energy distribution after internal conversion. This overall reduction of the yield by substitution renders the utility of photodissociation reactions having this mechanism ineffective for triggering peptide systems.

A variety of heteroatom-substituted 3,6-tetrazines were evaluated in an effort to identify substituents that would lead to increased photoproduct yields. Pleasingly, 3,6-bis-thiobenzyl-*s*-tetrazine ($R = SBn$) displayed an acceptable photolysis yield of 24% at 355 nm and 12% at 410 nm upon flash photolysis. The thio-substitution causes an additional band centered at 410 nm to appear which has been attributed to either a separate $n \rightarrow \pi^*$ transition[10] or a charge-transfer band involving sulfur. This band is particularly useful because it presents the opportunity to initiate photolysis using a frequency doubled Ti:Sapphire laser pulse at ca. 400 nm that is not absorbed by a peptide or protein. While an explanation for the effect of the sulfur on the yield may involve the heavy atom effect,[11] whereby the sulfur slows down the energy flow out of the tetrazine ring, it is considered more likely that new photochemical pathways are opened up by sulfur substitution. An absorption band at ~510 nm, with a lifetime dependent on the oxygen concentration, is observed in the transient spectrum of 3,6-bis-thiobenzyl-*s*-tetrazine for up to 1 μs time delay which is attributed to the triplet-triplet absorption.[12] Fukasami and coworkers have observed intersystem crossing in 3,6-diphenyl-*s*-tetrazine compounds and have demonstrated that the triplet state is photostable.[13] The photoproducts in the present examples are therefore believed to arise through the singlet manifold with triplet formation being a non-productive pathway. The fluorescence lifetime was determined to be 585 ps for 3,6-bis-thiobenzyl-*s*-tetrazine suggesting photolysis on this timescale. Having identified a favorable tetrazine scaffold, the next step in designing a trigger suitable for incorporation within a peptide was to develop an amino acid-based tetrazine wherein the tetrazine trigger is incorporated between two cysteines.

Previous synthetic studies have demonstrated that when the electrophilic dichloro-*s*-tetrazine (**1**)[14] is employed, reaction with thiolates favors the symmetric bis-substituted derivatives.[15] The key to reproducibly high yields of tetrazine (–)-**3a** (Scheme 2) was exclusion of ambient light. This reaction can also be conducted under aqueous conditions using 1,4-dioxane as a co-solvent to provide (+)-**3b**. Introduction of the amino acid-based substituents did not significantly perturb the photochemical yield of the tetrazine fragmentation reaction: (–)-**3a** has a photoproduct yield of 11 % upon flash photolysis. The photolysis yield displayed a linear dependence on the irradiation intensity at lower energies (<10 mJ), while the yield saturates at higher energies due to two photon absorption processes that were not studied in detail.

We next sought to verify that the photofragmentation produced the anticipated thiocyanate product (–)-**4b** (Scheme 3). The steady-state photolysis was conducted with $\lambda = 256$ nm due to the higher absorbance of (+)-**3b** at this wavelength. The photolysis of (+)-**3b**, monitored by ^1H and ^{13}C NMR spectroscopy (Figure 1), indicated quantitative conversion to (–)-**4b**, as exemplified by the disappearance of the ^{13}C signal corresponding to the tetrazine ring, with concomitant formation of the SCN resonance at $\delta_{\text{C}} = 113$ ppm.[16]

The fluorescence of tetrazine (–)-**3a** decays mainly within a lifetime of 851 ps (with a few percent component of 1.67 ns). To further investigate the timescale of photolysis, femtosecond transient IR measurements were performed; the results demonstrated that the ester carbonyl modes of (–)-**3a** are sensitive to photolysis. The transient difference spectrum obtained after a 1 ns time delay was similar to the difference spectrum obtained after complete photolysis from the stationary spectrum of (–)-**4b** (Figure 2). The IR transient absorption peaks at 1765 cm^{-1} and 1745 cm^{-1} arise from the ester absorption after the molecule was photolyzed, while the bleach signals at 1730 cm^{-1} and 1755 cm^{-1} are due to the removal of parent molecules. These results demonstrate that the photolysis occurs before 1 ns and does not involve a triplet intermediate.

The thiocyanate vibrational mode of the photoproduct was monitored at different delays between the optical pump and infrared probe. The spectrum of the transient absorption monitoring the photolysis of (–)-**3a** in the SCN region reveals the presence of one peak at 2163 cm^{-1} at 1 ns (inset of Figure 3, black curve). The peak corresponds to the 2163 cm^{-1} band observed in the FTIR spectrum of the compound after 400 nm irradiation (inset of Figure 3, red curve). The thiocyanate signal was estimated from the number of photons and the photochemical yield of 166 μOD , assuming two identical thiocyanates having similar stationary line shapes formed upon photolysis. The observed thiocyanate signal was $\sim 120\text{ }\mu\text{OD}$ at the 2163 cm^{-1} peak at 1 ns time delay for the photolysis of (–)-**3a**. However, the integrated absorption prediction and measurement are in close agreement, suggesting that indeed two thiocyanates are formed. The thiocyanate transient at the peak maximum was fit to an exponential growth (Figure 3) and the time constant was determined to be 177 ps for (–)-**3a**. The rate constant is faster than the S_1 lifetime, suggesting that the dissociation producing the SCN groups occurs from a highly excited state.

Having validated that the tetrazine fragmentation reaction occurs within a few hundred picoseconds upon photolysis, the development of a method to insert the trigger within a cyclic peptide system was undertaken. The peptide oxytocin was chosen as a readily available model system to develop a method for insertion of the bis-(*S*, *S*)-tetrazine trigger motif. Initial efforts to reduce the disulfide of the native peptide with immobilized tris(2-carboxyethyl)phosphine (TCEP), followed by application of the aqueous tetrazine insertion conditions proved irreproducible. A solid-phase peptide synthesis strategy employing the orthogonal Mmt *S*-protecting group to mask the pivotal thiol groups was developed to overcome these limitations (Scheme 4). Standard techniques were used to construct the

peptide, with the *N*-terminus capped as the acetamide. Removal of Mmt groups liberated the thiols of the Cys residues within the resin-bound peptide. Subsequent treatment with **1** introduced the tetrazine bridge. The pseudodilution effect induced by immobilizing the peptide precludes formation of tetrazine-bridged peptide dimers. Standard deprotection/resin cleavage conditions and purification[17] then provides the target peptide: **NHAc-1,6-*S,S*-Tet-oxytocin**.

Detailed NMR studies support the location of the tetrazine ring between the two Cys side-chains. Assignment of the amide NH resonances and calculation of the $^3J(\text{NH}, \text{H}\alpha)$ values permitted simulation of the peptide backbone angles.[18] The position of the tetrazine bridge was further refined by analysis of the 3J coupling constants between the well-resolved Cys(*H* α) and Cys(*H* β) resonances. This analysis permits assignment of the tetrazine-bridged peptide structure as illustrated in Scheme 3. Details of this analysis and representative peptide structures for the lowest energy conformational families are provided in the Supporting Information.

Having achieved an effective method to insert a tetrazine trigger within a peptide construct, we next evaluated the photochemical properties of this system within the constrained cyclic peptide. The fluorescence decay of **NHAc-1,6-*S,S*-Tet-oxytocin** displays three components: a fast component of 200 ps (66% of the amplitude) and two slower components at 1.09 ns (26%) and 4.09 ns (8%). The large contribution of the 1 ns component is likely to be from another conformer as suggested by the NMR analysis. The rate of formation of the SCN stretching peak upon photolysis was again measured by femtosecond transient IR, and the thiocyanate transient was fit to a single exponential having a time constant of 207 ps for **NHAc-1,6-*S,S*-Tet-oxytocin** (Figure 3). The quantum yield of the model compound, (–)-**3a**, is dependent on excitation wavelength. There is a two-fold increase in SCN transient absorption upon excitation at 355 nm compared with 400 nm. Clearly, the photochemistry occurs from unrelaxed states following excitation.

In summary, this study demonstrates that an *S,S*-tetrazine system can be readily incorporated within a bis-thiolate peptide motif by treatment with **1**. The photolysis rate of this new phototrigger was directly measured by femtosecond transient absorption to occur on the picosecond time scale, providing rapid access to non-equilibrium states. As far as we can ascertain the two SCN groups are formed at the earliest measured times (ca. 25 ps). The photochemical yield upon flash-photolysis is wavelength dependent being 22% at 355 nm. The photolysis can be run to complete conversion with standard photochemical irradiation ($\lambda = 256$ nm). The principal advantages of the *S,S*-tetrazine triggering system are that the photolysis can be initiated with 330 to 400 nm or at lower yield with visible irradiation, making the tetrazine triggering system safely bio-compatible. Upon incorporation into a cyclic peptide system, the photolysis rate and photolysis yield of the tetrazine trigger are similar to the acyclic versions [e.g., (–)-**3a**], demonstrating that the mechanism of dissociation is not influenced by rigidity of the peptide system or the presence of other side chain functionalities. Furthermore, site selective $^{13}\text{C}=^{18}\text{O}$ replacements in the peptide backbone will permit FTIR or 2D-IR tracking at the residue level[19] of the resulting peptide structure.

Experimental Section

Detailed synthetic procedures and full characterization data for all new compounds along with a description of the methods employed to obtain the photophysical data are provided as Supporting Information, available on the WWW from <http://www.angewandte.org>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

¹³C NMR ($\delta = 200\text{--}100$ ppm) spectral monitoring of the steady state photolysis of (+)-**3b** (20 mg/mL, CD₃OD, quartz NMR tube). Top: (+)-**3b** starting material; Bottom: After 4 h of irradiation complete conversion to (–)-**4b** is observed.

Figure 2.
Femtosecond IR transient absorption of carbonyl ester region of (–)-**3a** in CD₃OD (blue) and stationary FTIR of the photolysis product AcHN-Cys(SCN)-COOMe (red).

Figure 3. Time dependence spectra of the SCN transient absorption following the photolysis of (-)-**3a** (triangles – raw data, red line – fit) and AcNH-1,6-S,S-Tet-oxytocin (squares – raw data, blue line – fit) in CHCl₃/MeOH (95:5 v/v) mixture. Inset: Femtosecond IR transient absorption spectra of (-)-**3a** (black) in SCN region at 1 ns delay and FTIR absorption (red) band of SCN stretching frequency after steady-state irradiation of (-)-**3a** at 400 nm. The asymmetry of the transient absorption peak is not physically meaningful, but is caused by the detector.

Scheme 1.
Photofragmentation of *s*-tetrazines.

Scheme 2.
Synthesis of Cys-derived tetrazines and X-ray crystal structure of (-)-**3a**.

Scheme 3.

Scheme 4.
 Solid-Phase synthesis of **AcNH-1,6-S,S-Tet-oxytocin**.