# A Method for Photoinitating Protein Folding in a Nondenaturing Environment

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The early kinetic events of protein folding are an important part of the folding pathway, yet our understanding towards the process is limited. Information from the study of these early events can allow us to distinguish between the various models that have been proposed to describe the folding of a protein in real time. Unlike "typical" chemical kinetics with well-defined initial and final states, the initial state of a denatured protein is relatively ill-defined. This uncertainty introduces ambiguity in the interpretation of the experimental data on the early events in protein folding. Toward developing a unified theory of protein folding, it is necessary to begin the observation of the refolding process from a well-defined initial state, trigger folding as rapidly as possible, and to follow the protein in real time as it samples its conformational space over its highly complex free-energy landscape.

Traditional stopped-flow methods have been employed to follow the kinetic course of protein-folding reactions. However, such experiments are limited in time resolution and in that external denaturants are used to achieve a non-native state. It is not known exactly how denaturants interact with a protein and how they affect the kinetic pathways. In addition, substantial refolding occurs during the dead time of mixing in these stopped-flow experiments. To circumvent the time domain limitation, several groups have recently developed strategies based on temperature,[1] pH[2] or pressure jumps,[3] flash photolysis of heme ligands,[4] photoreduction of metalloproteins,[5] and the photolysis of engineered disulfides[6] (for a recent review see refs 7, 8). Although the above techniques have provided insight into protein folding, few studies have been done in the absence of denaturant. A general method to study the early events of folding in greater detail requires irreversibility, applicability to non-metalloproteins, fast triggering, and the elimination of external denaturants.

Our strategy for the initiation of protein folding is based on the picosecond flash photolysis of an organic cross-linker. Such a phototrigger may be placed within a cyclic form of a protein of interest. If chosen correctly, the resulting loop conformation will prohibit proper folding of the protein (Figure 1). Thus, instead of manipulating the external conditions, such as temperature, pH, or denaturant concentration, the covalent structure of the protein is altered to yield a conformationally constrained unfolded state. During the triggering event the linker is irreversibly cleaved to yield the "linear" protein that is free to fold.


**Figure 1.** The head-to-side chain cyclization scheme.

In 1971 Sheehan et al. showed that esters of 3′,5′-dimethoxybenzoin undergo efficient and clean photolysis under near-UV illumination[9] and can thus serve as photolabile protecting groups, otherwise known as caging compounds. The products of this photolysis are the phenylbenzofuran and the "free" form of the caged molecule, often a carboxylic acid. This protecting group has been converted into a linker by derivatization of the benzoin ring to give 3′-(carboxymethoxy)benzoin (CMB, Scheme 1, **1**).[10] Benzoinyl cages have the following properties which make them well-suited for these studies: high quantum yields (0.6−0.7), inert photoproducts, good water solubility, and fast photolysis rates (predicted $\sim 10^{10}$ $s^{-1}$, observed $> 10^{9}$ $s^{-1}$).[9,11]

The strategy described here involves forming a small loop from the N terminus of a protein to an internal amino acid side chain using CMB as a linker. To achieve selective linker attachment and subsequent cyclization of the peptide, the general synthetic strategy outlined in Scheme 1 was used. The first step in this method was to derivatize protected CMB (**1**) with bromoacetic acid to give compound **2** (BrAc-CMB).[12] Next, the fully side chain protected polypeptide was synthesized by standard solid-phase peptide synthesis, with the cysteine selectively protected by a weak acid-labile protecting group. Once the full-length peptide was synthesized and the terminal Fmoc group was removed, the BrAc-CMB linker was attached to the N terminus of the synthetic peptide through standard condensation coupling (**3**). The acid-labile Cys-protecting group was then removed selectively. Finally, cyclization through thioether formation was achieved using a non-nucleophilic amine base in an organic solvent. Upon completion, the resin was rinsed and neutralized. The peptide was cleaved from the solid support and purified by reverse-phase HPLC to yield the cyclized peptide **4**.

To demonstrate this general approach, we have used the small α-helical villin headpiece subdomain (Figure 2, inset).[13] This autonomous monomeric folding unit consists of 35 residues, is highly thermostable ($T_m \approx 70$ °C), and forms a compact primarily α-helical structure. This villin subdomain is expected to fold rapidly, and as such has been used as a model protein in a microsecond molecular dynamics simulation.[14] Residue Met 12 was chosen for mutation to Cys due to its close proximity in sequence to the N terminus and its apparent high solvent exposure as shown by the NMR structure.[15] Accordingly the peptide VHP-34 M12C was prepared with the *N*-terminal bromoacetylated CMB group attached. Cyclization and cleavage of the peptide and protecting groups as described above, yielded the cyclic form

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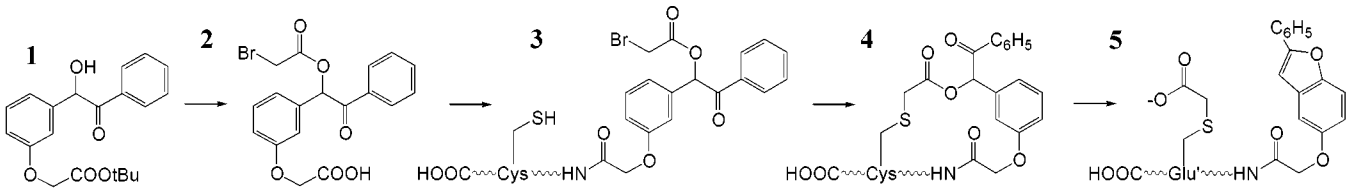
**Scheme 1.** General Strategy for Peptide Cyclization and Photolysis



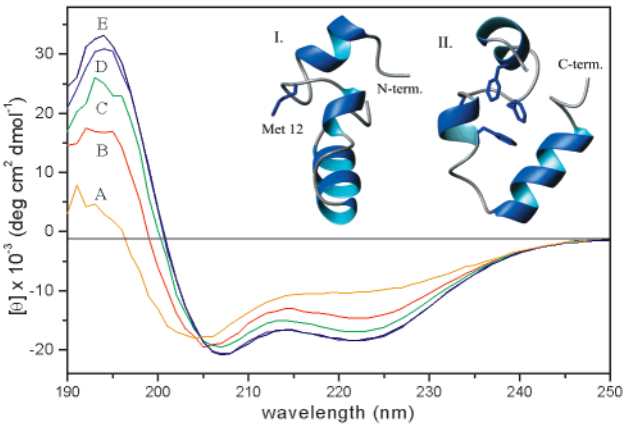




**Figure 2.** Steady-state photolysis of 28 $\mu$M cVHP M12C-CMB (4) in 10 mM $NaPO_4$ buffer, pH 7.4, irradiated from 300 to 400 nm using a filtered high-pressure mercury vapor arc lamp. "Linear" form at 95 °C (above the melting temperature of VHP-35) (A); cyclized form, irradiation time (s): (B) 0, (C) 10, (D) 30, (E) 90. Inset; Ribbon diagram of VHP-35 (PDB accession code 1vii).

of the peptide (cVHP-34 M12C-CMB, see Supporting Information for sequence). Upon photolysis, the cyclized peptide was cleaved to yield the "linear" form containing a carboxymethylcysteine at residue 12 (**5**), a solvent-exposed position where a charged residue should be tolerated.

To evaluate the secondary structure of the cVHP, steady-state photolysis was carried out and UV/vis and CD spectra were recorded. Irradiation of the cVHP-34 revealed a spectra indicative of a clean conversion of the benzoin to benzofuran, an event that corresponds to the conversion of the cyclized peptide to the "linear" form. This conclusion is corroborated by the molecular mass of the peptide before and after the photolysis, which were found to be 4191.9 and 4191.2 amu, respectively. The CD spectra (Figure 2) before photolysis (B) and after (E) show the expected formation of α-helical secondary structure concomitant with the photolysis event. The change in helix content was approximately 50% for this 12-residue loop form of the villin headpiece.[16] Examination of a cVHP-34 M12C-CMB model reveals that it is possible to maintain a hydrophobic core in the cyclized form of this very stable small protein. It is likely that the cyclization disrupts the helix contained in the first 12 residues and only partially disrupts the other two helices.

To ascertain the time course of the structural response of the peptide following linker cleavage, we have utilized time-resolved photoacoustic calorimetry (PAC) and photothermal beam deflection (PBD), two methods that are sensitive to photochemically induced volume changes.[17] Deconvolution of the experimental PAC data revealed two kinetic phases with time constants of approximately 100 and 400 ns (raw data presented in Supporting Information). PBD measurements revealed that the folding process was essentially complete within the dead time of our experimental setup (~10 $\mu$s). It is likely that fast helix formation was responsible for at least one of the phases in the observed PAC signal.

The observed rates of folding are in accord with existing theory and experiment. On the basis of a length-dependent diffusion calculation with an α-helical correction this peptide is predicted to fold between approximately 15−100 $\mu$s.[18] The two nanosecond phases observed by PAC are of the same magnitude as experimental and theoretical rates based on helix−coil transitions and other fast-folding experiments. Although the helix−coil relaxation rates yield important information, it is necessary to follow the coil−helix transition to obtain specific information about nucleation and chain propagation rates.

The head-to-side chain cyclization strategy outlined here has several advantages for the rapid triggering of protein-folding reactions. The synthetic scheme allows the somewhat sensitive BrAc-CMB group to be introduced at the end of the solid-phase synthesis process. With this technique loops of varying size may be formed, including small, synthetically accessible loops. Finally, the irreversible triggering event allows us to monitor the entire refolding process without kinetic competition from the reverse triggering reaction that was observed in earlier studies.[1−5] We are particularly interested in determining the effects of the initial unfolded state on the observed kinetic pathways. Our cyclization approach enables us to confine the protein to a subset of phase space, thus making it possible to map out sections of the folding landscape. This technique is not limited to aqueous media; therefore, the effect of viscosity and the effects of added denaturants on folding can be studied in alternative solvent systems. This method allows for the study of protein folding under more physiologically relevant conditions.

In summary, we have demonstrated an irreversible rapid triggering method to study protein folding that utilizes a conformational constraint to achieve an unfolded state so that the use of denaturant is unnecessary.

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**Supporting Information Available:** Procedures for the synthesis of **1**−**4**, UV/vis data, raw PAC data, and the sequence for cVHP-34 M12C-CMB (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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