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Structure-Based Approach to the Photocontrol of Protein Folding

Fuzhong Zhang,[†] Arash Zarrine-Afsar,[‡] M. Sameer Al-Abdul-Wahid,[§] R. Scott Prosser,[§] Alan R. Davidson,[‡] and G. Andrew Woolley*[,†]

Department of Chemistry, University of Toronto, 80 Saint George Street, Toronto M5S 3H6 Canada, Department of Biochemistry, University of Toronto, 1 King's College Circle, Toronto, M5S 1A8, Canada, and Department of Chemistry, University of Toronto at Mississauga, 3359 Mississauga Road North, Mississauga, Ontario, L5L 1C6, Canada

Received October 7, 2008; E-mail: awoolley@chem.utoronto.ca

Abstract: Photoswitchable proteins offer exciting prospects for remote control of biochemical processes. We propose a general approach to the design of photoswitchable proteins based on the introduction of a photoswitchable intramolecular cross-linker. We chose, as a model, a FynSH3 domain for which the free energy of folding is less than the energy available from photoisomerization of the cross-linker. Taking the experimentally determined structure of the folded protein as a starting point, mutations were made to introduce pairs of Cys residues so that the distance between Cys sulfur atoms matches the ideal length of the cis form, but not the trans form, of the cross-linker. When the trans cross-linker was introduced into this L3C-L29C-T47AFynSH3 mutant, the protein was destabilized so that folded and unfolded forms coexisted. Irradiation of the cross-linker to produce the cis isomer recovered the folded, active state of the protein. This work shows that structure-based introduction of switchable cross-linkers is a feasible approach for photocontrol of folding/unfolding of globular proteins.

Introduction

Light-sensitive proteins control processes as diverse as vision in vertebrates to photomorphogenesis in plants.[1] In many of these cases, light appears to trigger long-range conformational transitions in the protein structure including folding/unfolding transitions.[2] Light-sensitive proteins designed-to-order offer exciting prospects for external manipulation and probing of complex biochemical systems.[3−5] As with other types of protein engineering, the development of a light-sensitive protein may be approached via a structure-based approach, a selection-based approach, or a combination of the two.[6,7] Although photoswitchable amino acids can be introduced into proteins using non-natural amino acid mutagenesis technology,[8,9] and photoswitchable domains may be co-opted from natural photoswitches,[3,5] without a framework for focusing mutagenesis efforts, the combinatorial possibilities are vast. Trauner, Isacoff, Kramer, and colleagues have taken a structure-based approach to achieve photocontrol of glutamate receptor proteins by coupling an allosteric effector to a photoswitchable tether, thereby capitalizing on the fact that allosteric effector binding can trigger productive long-range conformational changes.[10,11]

Here we explore an approach based on designed introduction of photoswitchable intramolecular cross-linkers. Effects of intramolecular cross-links on protein structure and stability are considered one of the more tractable aspects of protein engineering.[12,13] The impact of engineered disulfide bonds on protein stability can often be explained in terms of the decrease in loop entropy of the unfolded state.[14] In favorable cases, covalent cross-links can also be used to probe the presence or absence of particular structures in protein folding transition states.[15,16] Photocleavable cross-linkers have been used to trigger protein folding processes.[17,18]

Cross-linkers containing the azobenzene chromophore can undergo a change in length upon isomerization. Azobenzene

[†] Department of Chemistry, University of Toronto.
[‡] Department of Biochemistry, University of Toronto.
[§] Department of Chemistry, University of Toronto at Mississauga.

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exhibits robust, reversible cis−trans isomerization about the N=N double bond.[19] It has been used extensively in the design of switchable small molecules, nucleic acids, and protein secondary structure elements—helices and turns (e.g., refs 20−22). The trans conformation of azobenzene is ∼10 kcal/mol more stable than the cis conformation in the dark[23] and the thermal barrier to the interconversion of cis and trans isomers is ∼18 kcal/mol.[24] These values should be more than sufficient to drive substantial conformational change in proteins where typical folding free energies are between 5 and 15 kcal/mol.[25] Thus, photocontrol of global folding/unfolding of globular proteins using azobenzene should be possible.

To explore whether an azobenzene-based cross-linker could be applied productively to the photocontrol of protein folding, we introduced the BSBCA (3,3′-bis(sulfonato)-4,4′-bis(chloroacetamide) azobenzene)[26] linker into a β-sheet protein, the SH3 domain from Fyn-tyrosine kinase (Figure 1). The FynSH3 protein is well-characterized and extensively used as a model for protein folding studies.[27−29] The free energy of folding of wild-type FynSH3 is ∼4.2 kcal/mol,[30] but the wealth of experimental data available for FynSH3 mutants[29,31,32] enables one to choose proteins of virtually any desired stability.

Materials and Methods

Molecular Modeling. To identify possible sites for cross-linking, the software program sGAL[33] was applied to the Fyn tyrosine SH3 domain from crystal structure (PDB: 1SHF). The distance range scanned for the cis form of the cross-linker was 11−13 Å; the distance range for the trans form cross-linker was 16−18 Å. Candidate residues were then mutated to Cys using the program HyperChem (Hypercube Inc.) and sulfur-to-sulfur distances were calculated for all combinations of side chain rotamers. DSViewer Pro (Accelrys, Inc.) and DeepView (http://www.expasy.org/spdbv/) were used to measure distances between side chain γ atoms in the FynSH3 domain NMR structure ensemble (PDB: 1A0N) and to test for steric clashes.

Preparation of Cross-Linked Proteins. The double-cysteine mutant FynSH3 protein plasmids were created by site-directed mutagenesis from the wild-type FynSH3 plasmid.[34] The mutant proteins were then covalently cross-linked using the azobenzene cross-linker, 3,3′-bis(sulfonato)-4,4′-bis(chloroacetamide)azobenzene (BSBCA).[26] ESI-Mass spectra were used to characterize the cross-linked and un-cross-linked proteins. The absence of free Cys residues after cross-linking was confirmed using Ellman's reagent.

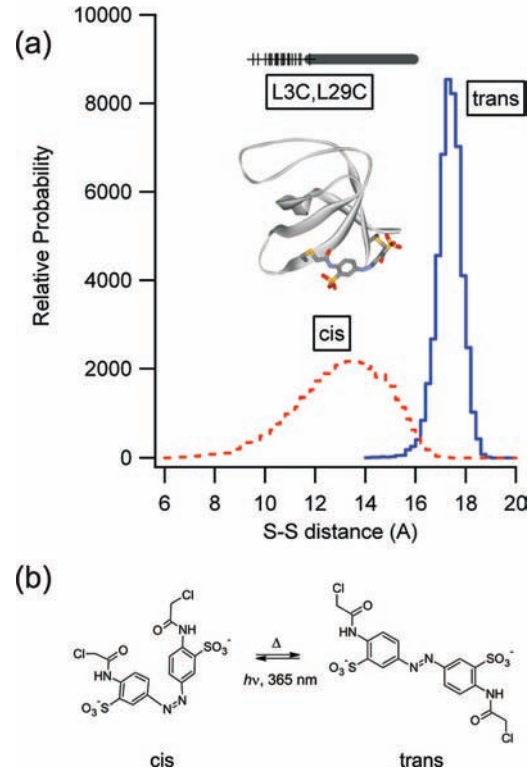

Figure 1. (a) S−S interatomic distance distributions for trans (—) and cis (− − −) BSBCA cross-linkers obtained from MD simulations compared to L3C−L29C distances in X-ray (solid bars) and NMR (crosses) structures of FynSH3 proteins. A model of *cis*-BSBCA linked L3C-L29C-FynSH3 is inset. (b) Chemical structure of the thiol-reactive BSBCA linker in cis and trans forms.

CD and UV Spectra. CD spectra were obtained at 20 °C for both dark-adapted and irradiated proteins. Each spectrum was acquired from 260 to 205 nm (1 nm step) with an integration time of 5 s at each wavelength and three scans were averaged, smoothed, and baseline subtracted. All CD measurements were carried out in a 1 mm cuvette with a protein concentration of 50 μM in 0.5 mM TCEP, 10 mM sodium phosphate buffer at pH 7.0. For CD spectra of irradiated protein, samples were irradiated using a high intensity 365 nm UV LED (∼200 mW, Opto Technology, Inc., Wheeling, IL, Model OTLH-0480-UV) directly in the CD instrument. This procedure produced samples that were ∼85% cis as judged by UV.[35] CD spectra are uncorrected for the percentage cis isomer. UV spectra were measured on a Shimadzu UV-2401PC UV−vis recording spectrophotometer. After irradiation, scans were taken at 5 min intervals from 600 to 240 nm (3 nm/s). The spectrophotometer was set at 600 nm until the next scan started and the slit width was set to 0.2 nm to ensure that effects of the measurement beam on the relaxation rate were minimal. Thermal relaxation

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curves were fitted to a single exponential equation to calculate the relaxation lifetime.

NMR Spectroscopy. Protein samples for NMR spectroscopy were prepared using Bioexpress cell growth ^{15}N-media (Cambridge Isotope Laboratories, Inc.). NMR experiments were performed on a 600 MHz or an 800 MHz Varian Inova spectrometer equipped with a Z-gradient HCN 5 mm cold probe, at a temperature of 293 K. For irradiation, the sample was transferred from the NMR tube to a cylindrical cuvette placed flat in an ice-bath and irradiated with stirring using the high intensity 365 nm UV LED described above. After irradiation, the sample was transferred back to the NMR magnet in darkness and shimmed. Due to the high optical density of the concentrated NMR solution and the delay required for sample transfer and shimming, a maximum of 60% cis isomer could be obtained using this protocol. HSQC spectra were acquired with an interscan delay of 1 s, 64 scans per increment, and 128 increments in the indirect dimension, spanning 2600 Hz. The SOFAST-HMQC[36] experiment was employed with an interscan delay of 175 or 250 ms, yielding spectra with high signal-to-noise in 20 or 25 min. HMQC spectra were acquired with 32 scans and 64 increments in the indirect dimension, spanning 1944 Hz. Spectra were processed with the NMRPIPE processing suite,[37] using the included addNMR program to obtain difference FIDs where appropriate. FID signals consisting of 1024 complex points in the direct dimension were linear predicted to twice the original data size, apodized using a shifted-squared-sinebell function, and then zero filled twice prior to Fourier transformation. In the indirect dimension, FID signals consisting of 128 (HSQC) or 64 (HMQC) complex points were processed in the same manner as for the direct dimension.

Isothermal Titration Calorimetry. The isothermal titration calorimetry (ITC) experiments were performed on a VP-ITC MicroCalorimeter from MicroCal, Inc. The substrate peptide VSLARRPLPPLP was prepared using standard Fmoc-based solid-phase peptide synthesis followed by HPLC purification. All ITC titration experiments were performed at 5 °C. The titration pipet contained 300 μL of 1.7 mM substrate peptide in 10 mM sodium phosphate buffer pH 7.0 and the sample cell contained 1.4 mL of 100 μM un-cross-linked L3C-L29C-T47AFynSH3 or 200 μM cross-linked protein in 10 mM sodium phosphate buffer pH 7.0. Substrate (10 μL) injections were 10 s, and a 240 s spacing time was used. To measure the binding to irradiated protein, X-L3C-L29C-T47AFynSH3 was irradiated as in CD experiment while incubating the sample on ice. After irradiation, the sample was immediately transferred into the calorimeter sample cell at 5 °C to minimize the extent of thermal relaxation to the trans form. All the data were processed using Microcal Origin from Microcal Software Inc. Single-site and two-site binding models were used to analyze all the data. The un-cross-linked L3C-L29C-T47AFynSH3 data could be fitted well to a single-site model. When fitting the dark-adapted X-L3C-L29C-T47AFynSH3 data to a single-site model a very poor fit was obtained unless the number of peptide binding sites per protein was ~0.5, i.e., approximately half the protein was inactive with respect to peptide binding. A two-site model provided a significantly better fit as described in the main text. Upon irradiation of the cross-linked sample, a two-site model also gives a better fit than a single site model. In this case, a single site-model requires $N = 0.82$ and $K_d = 0.67$ μM.

Results and Discussion

The first step of the design was to identify potential sites for intramolecular cross-linking. We used the computer program sGAL[33] to systematize this process. Taking the wild-type FynSH3 domain crystal structure (1SHF) as input, sGAL mutates each residue to cysteine sequentially and calculates the exposed surface area of the γ atom (the sulfur atom). The program then searches for pairs of γ atoms that meet specified criteria regarding degree of surface exposure and interatomic distance. Figure 1 shows S−S distance ranges expected for one pair identified by sGAL: L3C-L29C. S−S distance ranges are calculated on the basis of the X-ray structure of the wild type protein with all possible combinations of Cys side-chain rotamers, as well as distances observed between corresponding gamma atoms in the 25-structure NMR-derived ensemble (PDB: 1A0N[38]).

Although there is a significant range of S−S distances associated with these structures, it is clear that the L3C-L29C mutant is more compatible with a cis cross-linker than a trans cross-linker. Figure 1a (inset) shows a modeled structure of cis-linked L3C-L29C-FynSH3 (cis-X-L3C-L29C-FynSH3); no steric clashes or substantial distortions were evident upon analysis using molecular visualization software (see Materials and Methods section). In the dark, at equilibrium, the azobenzene-based linker is expected to be in the trans state. We therefore predict that cross-linking will lead to distortion of L3C-L29C-FynSH3, in the dark. Irradiation to produce the cis state of the cross-linker should permit the native folded structure of L3C-L29C-FynSH3 to be regained.

The L3C-L29C-FynSH3 mutant and two other mutants, L29C-T47C and T14C-E33C, identified using the same structure-based approach, were expressed, cross-linked and screened for photocontrol of conformation using circular dichroism (CD) spectroscopy (see Supporting Information for models of these mutants and CD spectra). Protein solutions were exposed to a ~25 mW source of 365 nm light for 5 min to produce ~85% cis isomer as judged by UV−vis spectroscopy.[35] Of the three mutants, X-L3C-L29C-FynSH3 showed the largest change in CD signal upon irradiation. HSQC NMR spectroscopy of the L3C-L29C-FynSH3 mutant indicated that it was >95% folded at 20 °C (see Supporting Information). We therefore introduced an additional T47A mutation that has been shown to destabilize wild-type FynSH3 by ~0.7 kcal/mol[34] to enhance the susceptibility of L3C-L29C-FynSH3 to effects of the cross-linker.

Figure 2 shows UV−vis and CD spectra obtained for the resulting X-L3C-L29C-T47AFynSH3 mutant. UV spectral changes are as expected for BSBCA isomerization; thermal cis-to-trans relaxation occurs with a half-live of 114 ± 5 min at 20 °C (Table 1).

Although the FynSH3 domain is composed primarily of β sheet secondary structure, its CD spectrum shows positive ellipticity at 222 nm that has been attributed to contributions from aromatic side chains in the structure.[27] Unfolding of FynSH3 by heat or denaturant is accompanied by a substantial decrease of ellipticity at wavelengths near 220 nm.[27] The CD spectrum for dark-adapted X-L3C-L29C-T47AFynSH3 (Figure

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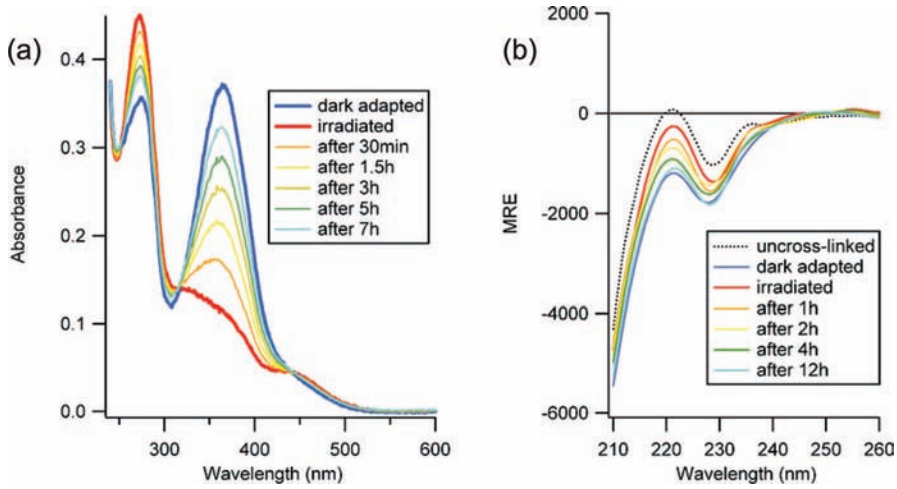

Figure 2. (a) UV−vis spectra of cross-linked L3C-L29C-FynSH3. The blue curve is the spectrum of 20 μM dark-adapted cross-linked in 10 mM sodium phosphate buffer at pH 7.0, 20 °C. The red curve is the spectrum obtained immediately after exposure of the solution to 365 nm light (~25 mW) for 5 min (~85% cis). Intermediate curves (from yellow to green) were obtained at the indicated times after photo irradiation (b) CD spectra of X-L3C-L29C-T47AFynSH3. The dashed line represents un-cross-linked L3C-L29C-T47AFynSH3 and the blue solid line represents dark-adapted X-L3C-L29C-T47AFynSH3. The red solid line was obtained immediately after irradiation of the solution (~85% cis). Intermediate curves were obtained after 1, 2, 4, and 12 h of relaxation after irradiation.

Table 1

measurement	$\tau_{1/2\ (min)}$
UV−vis	114 ± 5
CD	118 ± 10
NMR	119 ± 27

2b) indicates significant loss of folded structure. Irradiation shifts the spectrum toward that observed for the folded state (dashed line). After irradiation the CD signal at 222 nm relaxes with a time constant of 118 ± 10 min (Table 1) consistent with the expectation that protein conformational changes are driven by isomerization of the cross-linker.

We used NMR spectroscopy to further characterize the conformational changes induced by the cross-linker in X-L3C-L29C-T47AFynSH3. (^{1}H,^{15}N) HSQC spectra of un-cross-linked and cross-linked L3C-L29C-T47AFynSH3 are shown overlaid in Figure 3. Whereas un-cross-linked L3C-L29C-T47AFynSH3 exhibits a well-resolved spectrum with the expected number of peaks, the spectrum of the cross-linked species exhibits approximately double the number of peaks with a large fraction of these appearing in the center of the spectrum (^{1}H, 8−8.3 ppm), a region associated with unfolded protein structure.[39] For example, eight Gly resonances are observed in the cross-linked sample, whereas four are observed in the un-cross-linked sample (Figure 3 inset). The coexistence of folded and unfolded species in slow exchange on the NMR chemical shift time scale has been observed previously with drkN and Grb2 SH3 domains.[40,41] Thus, it appears that introduction of the trans cross-linker destabilizes L3C-L29C-T47AFynSH3 to the extent that folded and unfolded forms are approximately equally populated at 20 °C. Many of the peaks associated with folded state of the cross-linked sample overlay closely with peaks in the un-cross-linked sample indicating that the folded, trans-cross-linked species adopts a similar structure to un-cross-linked L3C-L29C-T47A-FynSH3 (Figure 3).

Next, we collected (^{1}H,^{15}N) HMQC spectra after irradiating the sample and at 20 min intervals thereafter until the protein had completely relaxed back to trans state. Under the conditions of irradiation, a yield of ~60% of the cis form was produced (see Materials and Methods section). In order to extract the cis-form spectrum we calculated a difference HMQC spectrum by subtracting FIDs of the last spectrum acquired (fully relaxed, trans form) from the spectrum acquired immediately after irradiation (Figure 4). Positive peaks in the difference spectrum (colored red, Figure 4a) are thus associated with the cis-form of X-L3C-L29C-T47AFynSH3 while negative peaks (colored green, Figure 4a) are associated with peaks that are diminished by trans-to-cis isomerization.

A first inspection of the difference spectrum reveals that numerous residues are affected by irradiation; there is a global rather than a local structural rearrangement triggered by isomerization. There are approximately equal numbers of red and green peaks in Figure 4a, each corresponding to the number of peaks associated with a single state of the protein. Overall, the red set of cross-peaks (cis) shows greater chemical shift dispersion whereas the green set is gathered in the center of the spectrum, typical of an unfolded protein.[39] Overlaying the green set of peaks with the spectrum of un-cross-linked L3C-L29C-T47AFynSH3 shows little overlap (Figure 4b), whereas overlaying the red set of peaks with un-cross-linked L3C-L29C-T47AFynSH3 (Figure 4c), shows that many of the peaks produced by the cis isomer are only slightly shifted from those in the un-cross-linked form. This result indicates that trans-to-cis isomerization of the BSBCA cross-linker induces a shift from unfolded to folded protein with the folded state being similar to the un-cross-linked species. The intensity of individual well-resolved cross-peaks in the difference spectrum showed single exponential relaxation curves with a lifetime consistent with CD and UV data (Table 1).

The function of SH3 domains is to recognize and bind to proline-rich motifs in partner proteins that function as components in signal transduction cascades.[42] The recognition function of FynSH3 is expected to be impaired by misfolding/unfolding

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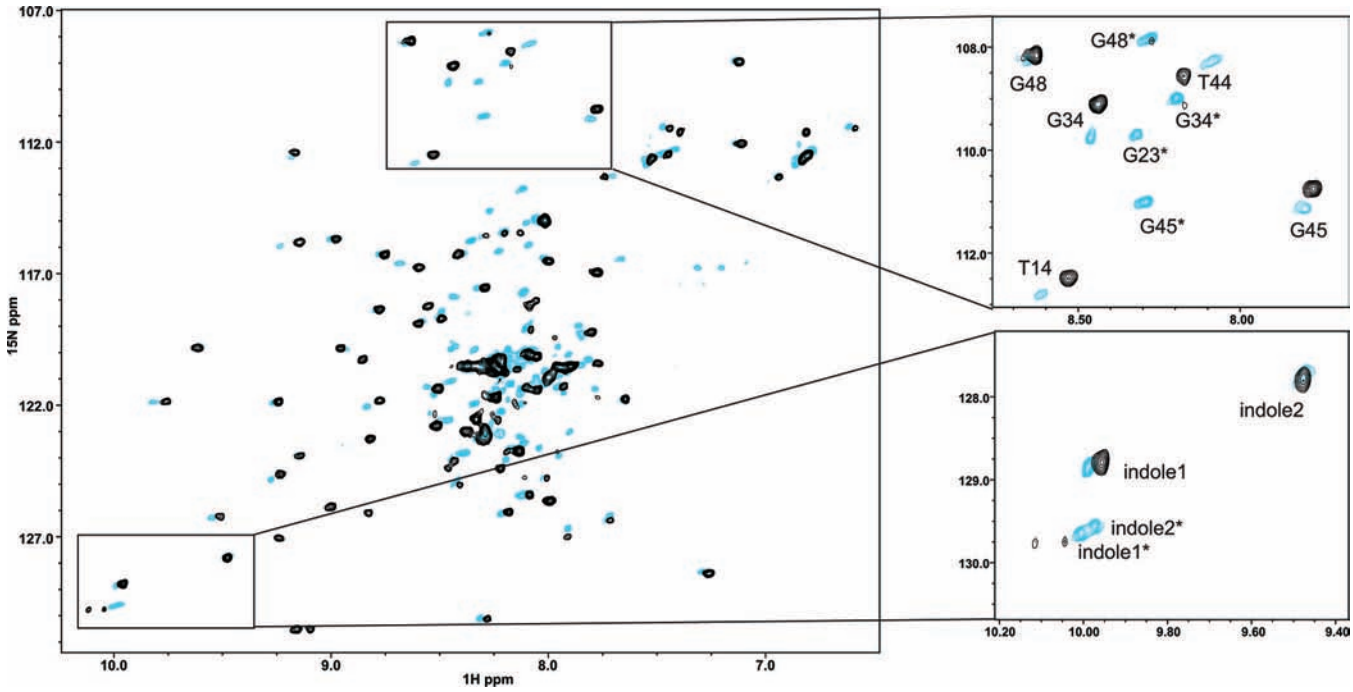

Figure 3. (^{1}H,^{15}N) HSQC spectra of un-cross-linked L3C-L29C-T47AFynSH3 (black) and dark-adapted, cross-linked X-L3C-L29C-T47AFynSH3 (blue). Inset (top): expanded view showing the doubling of Gly resonances in the dark-adapted, cross-linked X-L3C-L29C-T47AFynSH3 spectrum (The ^{15}N and ^{1}H coordinates of Gly23(folded) are at 115.5 and 9.05 ppm, respectively (out of this region). Inset (bottom): expanded view showing the doubling of indole NH resonances in the dark-adapted, cross-linked X-L3C-L29C-T47AFynSH3 spectrum (blue). Approximately 5% unfolded indole NH signal is also present in the un-cross-linked L3C-L29C-T47AFynSH3 (black).

of the protein. To test the effect of the cross-linker on the ability of X-L3C-L29C-T47AFynSH3 to bind target protein, we measured binding to a test peptide using isothermal titration calorimetry. The position of the cross-linker is not expected to directly interfere with peptide binding since it is on the opposite side of the protein from the peptide binding cleft.[43] A temperature of 5 °C was employed to minimize thermal relaxation of the cis isomer after irradiation.

These data indicate that un-cross-linked L3C-L29C-T47A FynSH3 binds target peptide with a K_d of 1.0 ± 0.1 μM, a similar affinity to wild type.[43] Dark-adapted X-L3C-L29C-T47AFynSH3, which contains ~60% unfolded protein, as judged by NMR, shows a binding curve that cannot be fit to a single site model with $N \approx 1$, but instead fits a model in which there are two types of binding sites: ~55% of the protein shows very low affinity (K_d > 10 mM) for peptide, and the remainder (~45%) shows slightly reduced affinity ($K_d = 3.4 \pm 0.8$ μM). Finally, irradiation to produce ~85% cis-X-L3C-L29C-T47AFynSH3 substantially restores the binding ability of the protein. The irradiated X-L3C-L29C-T47AFynSH3 binding curve can be fit to a model in which there are two types of binding site with the predominant binding site (81%) showing wild-type affinity ($K_d = 0.56 \pm 0.09$ μM) and the second (19%) showing low affinity (K_d > 10 mM).

Summary

Models of the un-cross-linked and cross-linked L3C-L29C-T47AFynSH3 proteins are shown in Figure 6 together with energy diagrams illustrating the effects of the cross-linker on the stability of folded and unfolded states. The un-cross-linked L3C-L29C- T47AFynSH3 protein is ~97% folded at 20 °C as judged by NMR, so that the folded state is ~2 kcal/mol lower in energy than the unfolded state. Introduction of a cross-linker is expected to decrease the conformational entropy of the unfolded state and thereby increase its free energy by an amount approximated by $\Delta G_l = 2.4RT \ln (l_o/l)$,[14] where l is the effective length of the cross-linker (trans ≈ 17.5 Å, cis ≈ 13.5 Å) and l_o is the average sulfur to sulfur distance between L3C and L29C residues in the unfolded state (calculated to be 33.5 Å using the program Foldtraj[44]). The introduction of the BSBCA cross-linker thus destabilizes the unfolded state of the protein by 0.9 kcal/mol (dark adapted, trans state) or 1.3 kcal/mol (cis state). The length of the trans form of the cross-linker is not compatible with the normally folded conformation of the protein and so also destabilizes that form significantly. As a consequence, folded and unfolded populations of the protein coexist in a ratio of 40:60, as determined by NMR (Figure 6). Irradiation produces the cis form of the cross-linker, which has a ~4 Å shorter mean end-to-end distance that is more compatible with the folded structure (Figure 6). Trans-to-cis isomerization thus increases the population of the folded state, with a restored ability to bind to target peptide sequences. On the basis of the CD data and the percentage cis produced by irradiation (~85%), the cis form of the cross-linked protein is ≥95% folded (folded is ≥1.7 kcal/mol more stable than unfolded), similar to that of the un-cross-linked protein.

These results provide a first indication that, using a structure-based approach, photoisomerization of an intramolecular cross-linker can be used to photocontrol the global folding of a globular protein. Only a small number of cross-linker sites had to be tested to discover a site that led to productive coupling between cross-linker isomerization and a global change in protein structure. We speculate that the L3C-L29C site is

(43) Feng, S.; Kasahara, C.; Rickles, R. J.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12408–12415.

(44) Feldman, H. J.; Hogue, C. W. *Proteins* **2000**, *39*, 112–131.

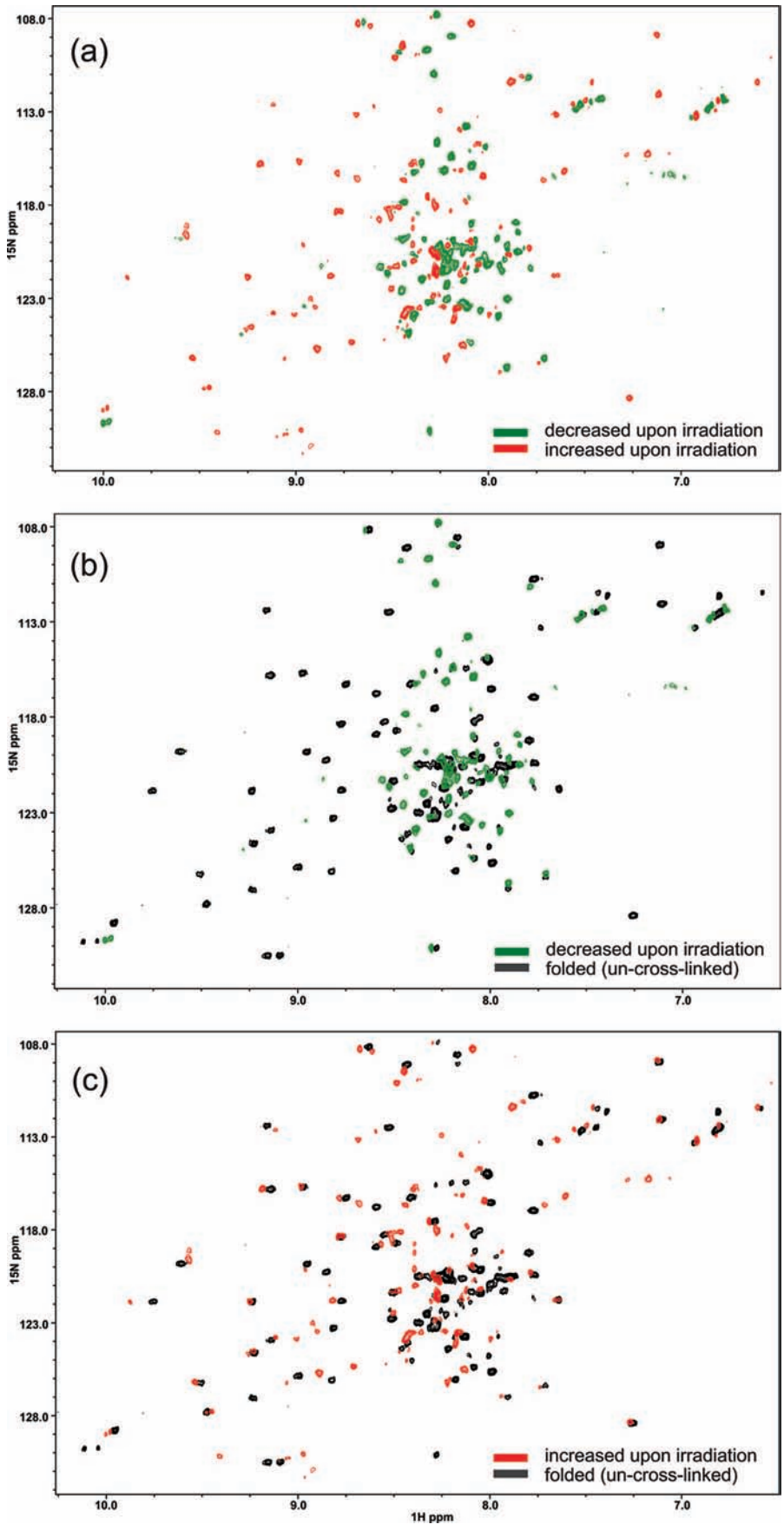

Figure 4. (a) Difference (^{1}H,^{15}N) HMQC spectrum of X-L3C-L29C-T47AFynSH3. Signals that increased upon irradiation are shown in red; signals that decreased are shown in green. (b) Overlay of negative peaks from difference spectrum (green) with un-cross-linked L3C-L29C-T47AFynSH3, (c) Overlay of positive peaks (cis X-L3C-L29C-T47AFynSH3) (red) with un-cross-linked L3C-L29C-T47AFynSH3.

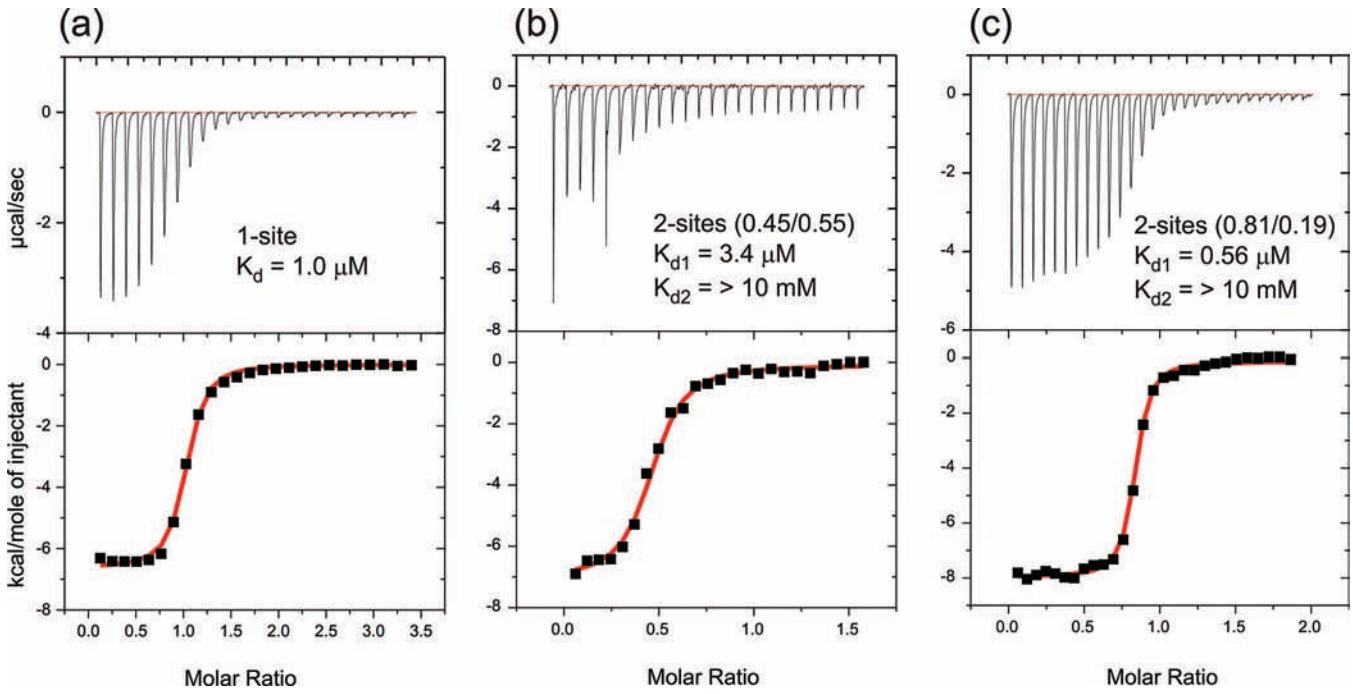

Figure 5. Isothermal titration calorimetry of (a) un-cross-linked L3C-L29C-T47AFynSH3, (b) dark-adapted X-L3C-L29C-T47AFynSH3, and (c) irradiated X-L3C-L29C-T47AFynSH3. The titration pipet contained 1.7 mM VSLARRPLPPLP peptide in 10 mM phosphate buffer pH 7.0 and the sample cell contained 100 μM un-cross-linked L3C-L29C-T47AFynSH3 or 200 μM cross-linked protein in 10 mM phosphate buffer pH 7.0. Temperature was 5 °C.

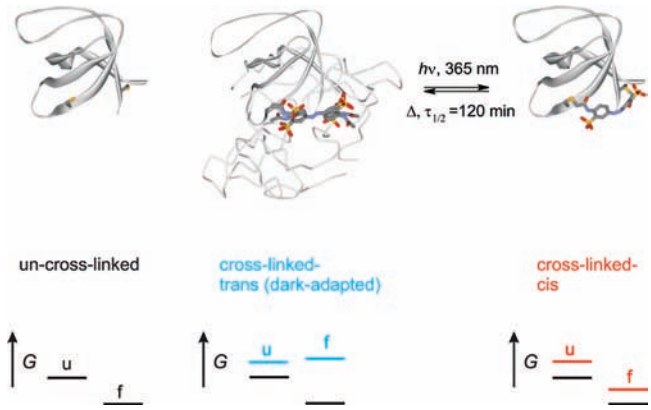

Figure 6. Models of un-cross-linked and cross-linked L3C-L29C-T47AFynSH3 and corresponding free energy diagrams. The folded (f) state of the un-cross-linked protein is ~2 kcal/mol more stable than the unfolded (u) state. The trans cross-linker (dark-adapted) destabilizes the unfolded state by ~0.9 kcal/mol (blue (u)) due to loss of conformational entropy. The trans cross-linker also destabilizes the folded state (blue (f)) due to mismatch with the ideal L3C-L29C S−S distance (Figure 1) so that the folded state is 0.24 kcal/mol less stable than the unfolded state. The cis cross-linker destabilizes the unfolded state (red (u)), to a similar extent as the trans cross-linker, due to loss of conformational entropy. However, the cis cross-linker matches the ideal S−S distance so that a normal folded state (red (f)) can form. The energies of the folded and unfolded states of the un-cross-linked protein are shown for reference (black lines).

effective because it produces strain close to the hydrophobic core (i.e., residues Phe4 and Ile28[29]) of the protein. The less pronounced effect observed with X-T14C-E33C-FynSH3 may be because the cross-linker was inserted between two flexible loops of the protein. Only ~2 kcal/mol of an available ~10 kcal/mol energy difference between cis and trans forms of the cross-linker was used in this case. Linkers that undergo more dramatic length changes upon photoisomerization and/or optimization of protein attachment sites may produce even larger destabilization/stabilization effects and thereby offer a general approach to photocontrol of protein activity.

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Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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