In Vivo Imaging

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Fluorescence Imaging of Azobenzene Photoswitching In Vivo**

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The photoisomerization of azobenzene has been used to control a wide variety of molecular processes.[1] It has been applied to the photocontrol of biomolecular targets (peptides, proteins, and nucleic acids) in vitro[2–4] and in cell extracts.[5] Recently it has been applied to the photocontrol of coiled-coil proteins in living cells in culture[6] and to the photocontrol of ion channels in vivo.[7,8] These studies highlight the promise of azobenzene-modified biomolecules as general agents for the remote control of biomolecular function using light. In order to function as agents for controlling molecular events in complex living systems, however, the azobenzene-based photoswitches must be chemically stable in a variety of intracellular environments. At least for certain azobenzene photoswitches used for conformational control, glutathione in the intracellular environment can reduce and inactivate the photoswitch.[9] Enzyme-mediated reduction or other modification, as occurs with numerous azo dyes,[10,11] is also possible. Even if not inactivated, azobenzene photoswitches may exhibit different isomerization rates in a cellular environment. For instance glutathione has been found to catalyze thermal *cis*-to-*trans* isomerization of certain azobenzenes.[9,12] Fluorescence imaging of azobenzene photoswitching would enable a direct test of the feasibility of using azobenzene for intracellular photocontrol in a living organism. Since azobenzenes used for conformational control are not intrinsically fluorescent, we developed a fluorescence reporter for azobenzene photoswitching.

Peptides bearing pairs of Cys residues can be intramolecularly cross-linked with thiol reactive azobenzene-based photoswitches such as **1** (Figure 1). These *p*-amido substituted azobenzene derivatives are relatively electron-rich compounds among those that have been used for conformational control in vitro[2,8,9,13] and are resistant to reduction by glutathione in vitro (see Supporting Information). The metabolism of azo dyes has been found to be sensitive to their redox potentials, but also to the pattern and nature of ring substituents.[10] Photoisomerization of the azobenzene cross-linker **1** alters the conformation of the peptide depending on the location of the Cys residue attachment points (Figure 1).[3] Since a substantial conformational change occurs (Figure 2 a,b), we reasoned that attachment of a fluorescent dye near the photoswitch may result in a fluorescence change upon isomerization. We explored a variety of fluorescent dyes with both sulfonated and non-sulfonated photoswitches (see Supporting Information). Peptide **2**, shown in Figure 1, produced the largest change (a 40% decrease, Figure 2 c) in fluorescence emission intensity in vitro upon *trans*-to-*cis* isomerization. The dark-adapted reporter has the azobenzene switch in the *trans* state; incubation in the dark restores the *trans* isomer with a half life of 10.7 min at 25 °C. Irradiation in the UV range (350–390) causes *trans*-to-*cis* isomerization of the photoswitch as well as excitation of fluorescein. Irradiation with blue light (440–490) causes *cis*-to-*trans* isomerization as well as fluorescein excitation. Since the *cis* isomer of the peptide (**2a**-*cis*) has a lower quantum yield for fluorescence than the *trans* isomer (**2a**-*trans*), irradiation of a dark-adapted solution of reporter peptide with UV light produces a time-dependent fluorescence decrease (Figure 2 d). Conversely, irradiation of **2a**-*cis* with blue light causes a time-dependent fluorescence increase (Figure 2 d). The rates of these switching processes depend on the intensity and the wavelength of irradiation; action spectra are shown in the Supporting Information, Figure S1.

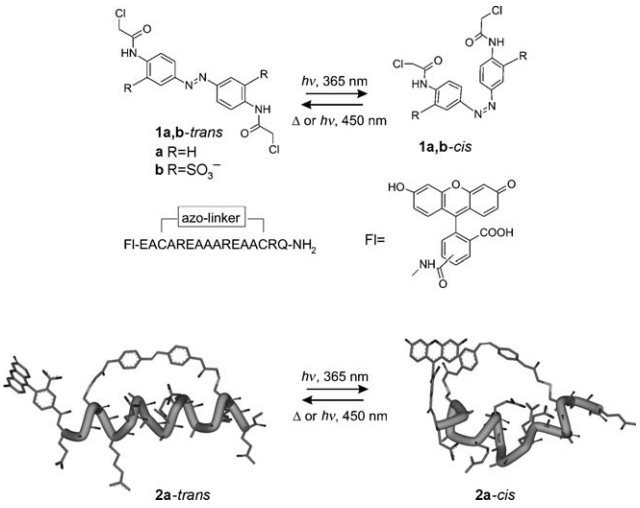

Figure 1. Structure of the photoswitches and the fluorescent peptide reporter.

A variety of mechanisms for the decrease in fluorescence intensity observed upon *trans*-to-*cis* isomerization are possible. Partial protonation of the fluorescein moiety due to an increase in its pK_a upon *trans*-to-*cis* isomerization is unlikely since the fluorescence response is unchanged between pH 7

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Supporting information for this article (details of UV/Vis, CD, and fluorescence measurements in vitro and fluorescence imaging in vivo) is available on the WWW under http://dx.doi.org/10.1002/anie.201006506.

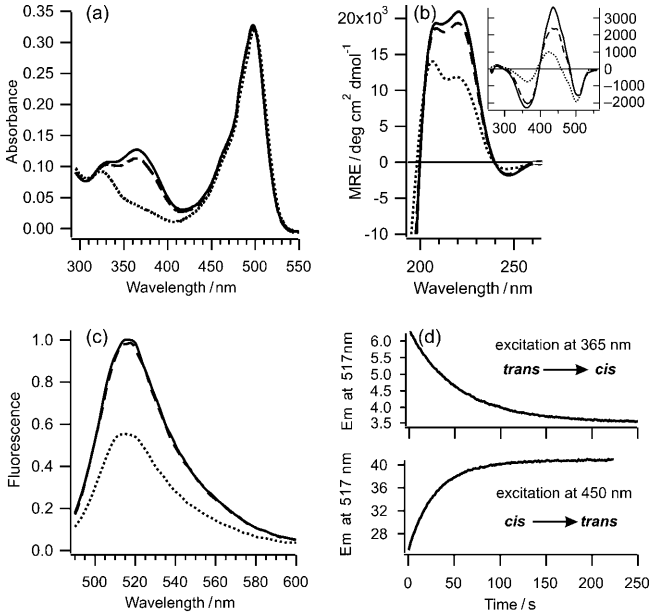

Figure 2. a) UV/Vis absorbance spectra of peptide **2a** in pH 7.0 phosphate buffer, dark-adapted (solid line), after UV irradiation (365 nm) (dotted line), and after irradiation at 450 nm (dashed line). b) CD spectra under the same conditions. c) Fluorescence emission spectra under the same conditions. d) Time-dependent changes in fluorescence emission with irradiation at 365 nm (upper panel) or 450 nm (lower panel).

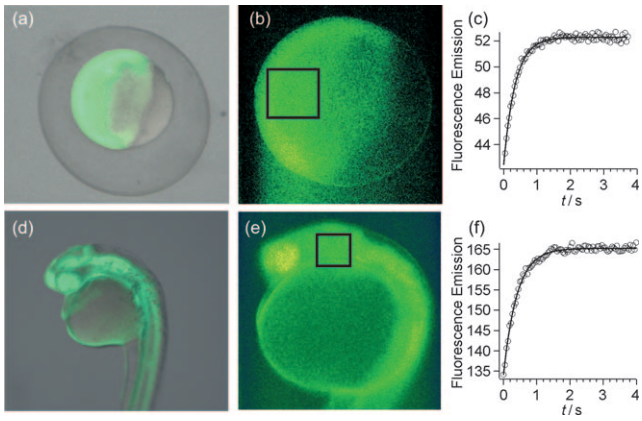

Figure 3. Fluorescence imaging of photoswitching in a–c) 8 h embryos and d–f) 30 h embryos. Images (a) and (d) show an overlay of green fluorescence with the bright-field image taken with a stereomicroscope. Images (b) and (e) show the embryos imaged using an epifluorescence microscope with a high-speed camera. Traces (c) and (f) show the time-dependent fluorescence response observed from the areas boxed in (b) and (e), respectively, upon shuttering on a blue excitation beam after embryos had been exposed to UV light for 2 s.

and pH 9 (Figure S2). Since the peptide cross-linked with the sulfonated photoswitch **1b** shows a substantially smaller change in fluorescence intensity (Table S1), while having a very similar absorbance spectrum to **1a**, a simple FRET mechanism is also unlikely. Interestingly, CD spectra of **2a** show, in addition to peptide structural changes, induced CD signals due to the azobenzene and fluorescein chromophores indicating these are electronically coupled to the peptide helix (Figure 2b). These induced CD signals are not seen with non-cross-linked peptides or peptides cross-linked with **1b**. Regardless of the mechanism, changes in fluorescein emission can be readily detected in vivo using fluorescence imaging methods making **2a** a good reporter peptide for azobenzene switching.

The D-peptide analog of **2a** (chosen to minimize the possibility of protease cleavage) was microinjected into zebrafish embryos at the 1–2 cell stage. Zebrafish are optically transparent while young.[14] Embryos were incubated at 28 °C and imaged using fluorescence microscopes (Figure 3). Wide-field illumination with a mercury lamp coupled to a UV bandpass filter was used to drive *trans*-to-*cis* isomerization. Irradiation was then shuttered off and the UV bandpass filter was replaced with a fluorescein filter set (blue excitation, green emission). Blue light was shuttered on and the fluorescence emission timecourse recorded with a high sensitivity camera. Figure 3 shows images of zebrafish at late blastula stage (8 h) and at the pharyngula period (30 h) with the corresponding timecourses for *cis*-to-*trans* photoswitching. Many cycles of photoswitching could be carried out (at least 30) with no loss of response (Figure S6). The peptide reporter appeared uniformly distributed throughout the embryo and switching was observed everywhere. The same fluorescently labeled peptide lacking the photoswitch did not show any time-dependent emission (Figure S7). Omitting the UV pre-irradiation step (failing to drive *trans*-to-*cis* isomerization) also resulted in no time-dependent emission (Figure S8). Robust photoswitching was observed for at least two days of embryo development (Figure S11). The embryos developed normally and no toxicity was evident (Figure S5). After two days, overall fluorescence signals became weaker so that reliable measurements of changes were more difficult. These results clearly indicate that the azobenzene photoswitch persists in vivo. The percent change in fluorescence observed in vivo (ca. 20%) is approximately half that observed in vitro, however, this does not appear to be due to irreversible reduction or enzymatic modification of the azobenzene since it is observed immediately after injection and remains constant for about 2 days. Neither does the decreased percent change in vivo appear to be due to a lower *cis* isomer content upon UV irradiation. The rate constants for *trans*-to-*cis* and *cis*-to-*trans* photoisomerization in vitro and in vivo were measured using the epifluorescence microscope by combining either a UV excitation bandpass filter or a blue excitation with a green emission filter (Figure S10). The rates were found to be the same indicating the same *cis*/*trans* isomer ratio in the photostationary state is achieved in both environments. Instead, we ascribe the decrease in percent emission change in vivo to an effect of the cellular environment on the size of the fluorescence response. Environmental changes in vitro (e.g. 6 M guanidinium or 50% methanol) also affect the size of the fluorescence change without affecting the *cis*/*trans* ratio (Figure S4).

The rate of thermal reversion in vivo was measured by inserting delay times before shuttering on the blue light. This gave a half life for thermal reversion of 7.5 min at 25 °C (Figure S9), a value slightly less than that measured in vitro

(10.7 min). The faster relaxation in vivo seems unlikely to be due to glutathione since this has no effect in vitro (Figure S3). Instead this slightly faster relaxation time is probably also an environmental effect since such effects are also observed in vitro.[15] We note that the corresponding L-amino acid peptide reporter did not show time-dependent emission behavior, a result we interpret to mean that the short peptide is rapidly degraded in a manner that decouples fluorescein from the photoswitch.

These results demonstrate that microinjection of appropriately designed bioactive photoswitchable molecules into zebrafish is a practical approach for achieving photocontrol of protein conformation in vivo. However, since more electron-deficient azobenzenes do suffer reduction,[9,11] careful choice of the photoswitch is required if in vivo application is intended. Also, clearly the biomolecule itself must remain intact for photocontrol of function to be achieved. The time window of 2 days over which photoswitching can be reliably observed is sufficient for the completion of numerous stages in early zebrafish development.[14] Azobenzene-modified biomolecules thus provide an opportunity to exert spatio-temporal control over a variety of molecular signaling processes involved in early development of zebrafish and probably also other organisms for which microinjection of embryos is possible.

Experimental Section

The peptide D-FK-11 (EACAREAAAREAACRQ-amide) was prepared using standard Fmoc-based solid-phase synthetic methods. Intramolecular cross-linking of cysteine residues on D-FK-11 was performed as follows: 1 mM peptide was incubated for 1 h at room temperature with 5 mM tris(carboxyethyl) phosphine in 50 mM sodium phosphate buffer (pH 8) to ensure cysteine residues were in their reduced state. An equivalent volume of dimethylformamide (DMF) was added to make a 50:50 (v/v) DMF: buffer mixture with a final concentration of 4 mM linker **1a**. The solution was stirred and heated to 40 °C for 24 h protected from light. The modified peptide was purified by HPLC (SB-C18 column) using a linear gradient of 5% to 65% acetonitrile/H_2O (+0.1% trifluoroacetic acid) over the course of 25 min; elution was observed at 43% acetonitrile. The modified peptide composition was confirmed by MALDI-MS [M+]: calcd $C_{81}H_{125}N_{31}O_{25}S_2 = 1996.00$ Da; obsd = 1997.30 Da. Labeling was achieved by adding a final concentration of 5 mM 5-(and 6-)carboxyfluorescein, succinimidyl ester (5(6)-FAM-SE) to a solution of 1 mM non-sulfoX-D-FK-11 in a 70:30 (v/v) dimethyl sulfoxide/50 mM sodium phosphate pH 8.0 mixture. The solution was stirred and heated to 40 °C for 24 h protected from light. The modified peptide was purified by HPLC as described above with elution at 52% acetonitrile. The modified peptide composition was confirmed by ESI-MS [M^+]: calcd for $C_{102}H_{135}N_{31}O_{31}S_2 = 2354.06$ Da; obsd = 2353.95 Da.

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