DNA Binding

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Photochemical Regulation of DNA-Binding Specificity of MyoD**

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The first step in the expression of genetic information, namely the synthesis of RNA from a DNA template, is tightly regulated by DNA-binding proteins called transcription factors. The recognition of DNA by many transcription

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factors relies on short α helices that bind in the major groove. Isolated α-helical peptides could therefore be used to interfere selectively with protein–DNA interactions. However, isolated peptides do not normally adopt well-defined secondary structures, and the recognition helices in DNA binding proteins often rely on interactions with other proteins for stability with the potential for selectivity modulation.[1–7] For instance, the activity of the muscle-specific transcription factor MyoD is regulated through interaction with coactivators from the myocyte enhancer factor-2 (MEF-2) family.[8] MyoD belongs to a family of transcription factors that rely on a basic helix-loop-helix (bHLH) domain for DNA binding. MyoD dimerizes through the HLH domain and contacts the major groove of the DNA target sequences through its N-terminal recognition α helix. The production of MyoD in a wide variety of cell types, including fibroblasts and myoblasts, activates a cascade of genes that eventually results in cellular differentiation and the production of muscle cells.[9] The physiological activity of MyoD depends on the presence of DNA sequences that contain the symmetrical core motif CANNTG (E-box) that is found in the promoters and enhancers of many muscle specific genes, such as the creatine kinase enhancer.[10] In stark contrast to its high physiological specificity, MyoD displays only limited DNA-binding specificity in vitro.[11] The specificity of transcriptional activation that is needed to explain the physiological specificity of MyoD is therefore most likely achieved through cooperative interactions with other components of the transcriptional machinery such as MEF-2C.[8]

The identification of mechanisms to control the DNA-binding properties of transcription factors such as MyoD would permit the regulation of their activity. Previously, we have shown that stabilization of the DNA-recognition helix of MyoD, through two disulfide bonds from an N-terminally fused apamin extension, led to a tenfold increase in the DNA-binding specificity.[12] However, due to the lability of disulfide bonds in cells, the application of such apamin-stabilized recognition helices is limited. Furthermore, it would be desirable to control the properties of DNA-binding proteins in response to external signals such as light.

Previous work had shown significant stabilization of the α-helical conformation of peptides linked through cysteine residues in an i, $i+7$ spacing, to azobenzene-derived cross-linkers in their *cis* configuration (Figure 1a).[13] Met 116 and Ser 123, which are located on the water-exposed face of the recognition helix of MyoD, were therefore replaced with cysteine residues (Figure 1b). MyoD-bHLH-M116C-S123C was alkylated with 3,3′-bis(sulfo)-4,4′-bis(chloroacetamido)-azobenzene to generate photoMyoD (Figure 1c).[14] The i, $i+7$ spacing avoided unfavourable steric interactions between residues in the helix and the cross-linker. Furthermore, this spacing maximized the conformational differences in the *cis* and *trans* configurations of the cross-linker of the residues that make specific contacts to the nucleobases, namely Arg 111, Thr 115, and Glu 118.[15]

UV/Vis spectroscopy was used to follow the isomerization of photoMyoD. When the cross-linker was in its thermally stable *trans* configuration, the spectrum showed the strong maximum at 363 nm typical for amide-substituted azobenzene π–π* transitions (Figure 2).[16] However, irradiation with light at a wavelength of 360 nm led to the loss of that absorption maximum. The percentage of isomerization was determined by HPLC separation of the irradiated material and through monitoring the elution profile at the isosbestic point of 315 nm. Of the irradiated material, 61 % was found to be in the *cis* configuration, which is a value typically achieved by photoisomerization of *trans* azobenzenes.[13,17] The thermal reversion of irradiated photoMyoD to the *trans* configuration occurred with a half life of 193 min at 15 °C.

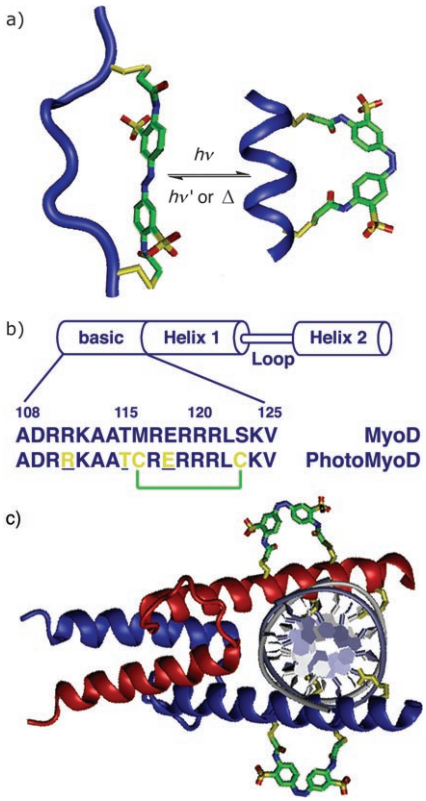

Figure 1. a) Sketch of the proposed structures of the basic regions of *trans* and *cis* photoMyoD indicating the conformational switch induced by the isomerization of the azobenzene cross-linker. b) Primary amino acid sequence of the basic region of MyoD and of photoMyoD. The residues that make specific contacts to the nucleobases in the X-ray crystal structure of the DNA complex of MyoD-bHLH[15] are underlined. c) Model of the DNA complex of photoMyoD with the cross-linker in its *cis* configuration. The DNA is complexed by the recognition helices of the two bHLH subunits of the dimeric protein. The cross-linker is located on the water-exposed face of the helices. The coordinates were from the crystal structure of MyoD-bHLH and DNA.[15]

CD spectroscopy revealed that the unalkylated MyoD-bHLH-M116C-S123C displayed only approximately 10 % helicity as judged from the mean residue ellipticity at 222 nm ($[\Theta]_{r,222} = -3658 \pm 232$ deg cm^2 dmol^{-1}) (Figure 3). The unalkylated form therefore existed predominantly in a random coil conformation like the parent MyoD-bHLH domain, which adopts an α-helical conformation only upon DNA binding.[11] The mean residue ellipticity of dark-adapted photoMyoD, in which the cross-linker is in its *trans* configuration, was similar to that of the non-cross-linked protein ($[\Theta]_{r,222} = -3328 \pm 345$ deg cm^2 dmol^{-1}). However, upon irra-

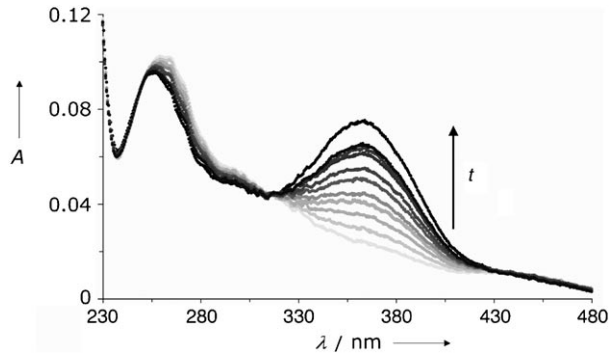

Figure 2. UV/Vis absorption spectra showing the photoisomerization of dark-adapted photoMyoD to the light-induced state. Black line (top) is the spectrum of dark-adapted photoMyoD (2 μM in 5 mM potassium phosphate buffer, pH 8.0, 15 °C). Light gray line (bottom) is the spectrum obtained immediately after exposure to 360 nm light. The intermediate curves show the spectra at varying times after photoisomerization (30, 60, 100, 135, 200, 260, 370, 430, 490 min). The sample was kept in the dark at 15 °C throughout the experiment.

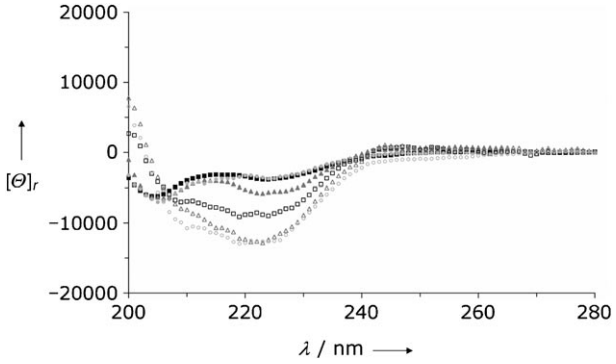

Figure 3. CD spectra showing DNA binding of MyoD-bHLH-M116C-S123C and photoMyoD. The protein concentrations were 2 μM in potassium phosphate (5 mM, pH 8.0) at 5 °C. ■ dark-adapted photoMyoD, ● non-cross-linked MyoD-bHLH-M116C-S123C, ▲ irradiated photoMyoD, ○ non-cross-linked MyoD-bHLH-M116C-S123C plus MCK-S (1 equiv, 1 μM), □ dark-adapted photoMyoD plus MCK-S (1 equiv, 1 μM); △ irradiated photoMyoD plus MCK-S (1 equiv, 1 μM). Mean residue ellipticity, [Θ]r, is in units of deg cm^2 dmol^{-1}.

diation with light of wavelength 360 nm, the mean residue ellipticity was reduced to -5597 ± 346 deg cm^2 dmol^{-1}. Given that this irradiated state of photoMyoD contained 39 % of the *trans* conformer, the mean residue ellipticity of pure *cis* photoMyoD could be calculated as -7060 ± 611 deg cm^2 dmol^{-1}. This corresponded to 19 % helicity or approximately 11 amino acids of the bHLH domain. It was not surprising that little helix induction was seen outside the eight residues spanned by the cross-linker as even at high concentrations where bHLH domains are folded dimers, the basic domain remains unstructured in the absence of DNA most likely owing to the electrostatic interactions of its many positively charged residues.[18]

The DNA-binding reactions of dark-adapted and irradiated photoMyoD, as well as that of the non-cross-linked parent protein, were studied with CD spectroscopy by using an oligonucleotide (MCK-S) comprised of 17 base pairs of the IgH enhancer-like element of the muscle-specific creatine kinase enhancer with the central E-box sequence, CAGGTG. Like MyoD-bHLH,[11] the non-cross-linked protein underwent a transition from a mostly disordered conformation to a mainly α-helical structure upon the addition of MCK-S (Figure 3). The observed value for the mean residue ellipticity at 222 nm, $[\Theta]_{r,222} = -13030 \pm 228$ deg cm^2 dmol^{-1}, indicated that the introduction of the two cysteine residues did not alter the DNA-binding properties of MyoD. The CD spectrum of the MCK-S complex of dark-adapted photoMyoD was characterized by $[\Theta]_{r,222} = -8913 \pm 182$ deg cm^2 dmol^{-1}, which indicates a significant loss of α-helical character. The CD spectrum of the DNA complex of irradiated photoMyoD was of a similar shape and intensity as that of the DNA complex of the non-alkylated protein. The value for $[\Theta]_{r,222}$ was reduced to -12130 ± 205 deg cm^2 dmol^{-1}. When the solution of the MCK-S complex of dark-adapted photoMyoD was irradiated with 360-nm light, the resultant CD spectrum was virtually identical to that of a solution of irradiated photoMyoD and DNA (Figure 3). This therefore indicated that the presence of DNA did not prevent the isomerization process.

The apparent dissociation constants of the DNA complexes of dark-adapted and irradiated photoMyoD were determined by using measurements of the fluorescence anisotropy.[12,19–22] The addition of protein to a solution of fluorescently labeled, double-stranded oligonucleotides resulted in a saturable increase in the fluorescence anisotropy, indicating that the protein had bound to the DNA in solution (Figure 4). The titration curves were fit to the Langmuir isotherm $\Phi_{Fit} = 1/(1 + K_D^n/[P]^n)$. For complexes with MCK-S, the best results were obtained for a model of cooperative two-state binding of two protein monomers to one DNA duplex as is commonly observed for bHLH proteins.[11,18,23] These fits yielded the apparent dissociation constant (K_D) from which the concentration of the protein, at which half-maximal DNA binding occurs ($[P]_{1/2}$) was calculated (see Supporting Information).

The K_D value for the MCK-S complex of dark-adapted photoMyoD ($1.5 \pm 0.38 \times 10^{-14}$ M^2) was similar to that of non-alkylated MyoD-bHLH-M116C-S123C ($8.8 \pm 2.2 \times 10^{-15}$ M^2). The stability of the MCK-S complex of irradiated photoMyoD, however, was increased significantly, and its apparent dissociation constant was reduced by more than three orders of magnitude to $4.4 \pm 0.16 \times 10^{-18}$ M^2. To the best of our knowledge, this almost 60-fold decrease in $[P]_{1/2}$ is the biggest change in DNA affinity caused by covalent helix stabilization reported to date.

The DNA-binding specificities of dark-adapted and irradiated photoMyoD were measured by comparison of the apparent dissociation constants of their complexes with MCK-S and with the oligonucleotide NoE-box (same base composition as MCK-S but with a scrambled sequence and without an E-box).[12] The bHLH proteins MASH-1 and MyoD have been shown previously not to distinguish efficiently between MCK-S and NoE-box.[11,23–25] Similarly, the K_D values for binding of non-alkylated MyoD-bHLH-M116C-S123C to MCK-S and NoE-box ($5.9 \pm 1.0 \times 10^{-15}$ M^2) were identical (Figure 4 and Supporting Information) and in

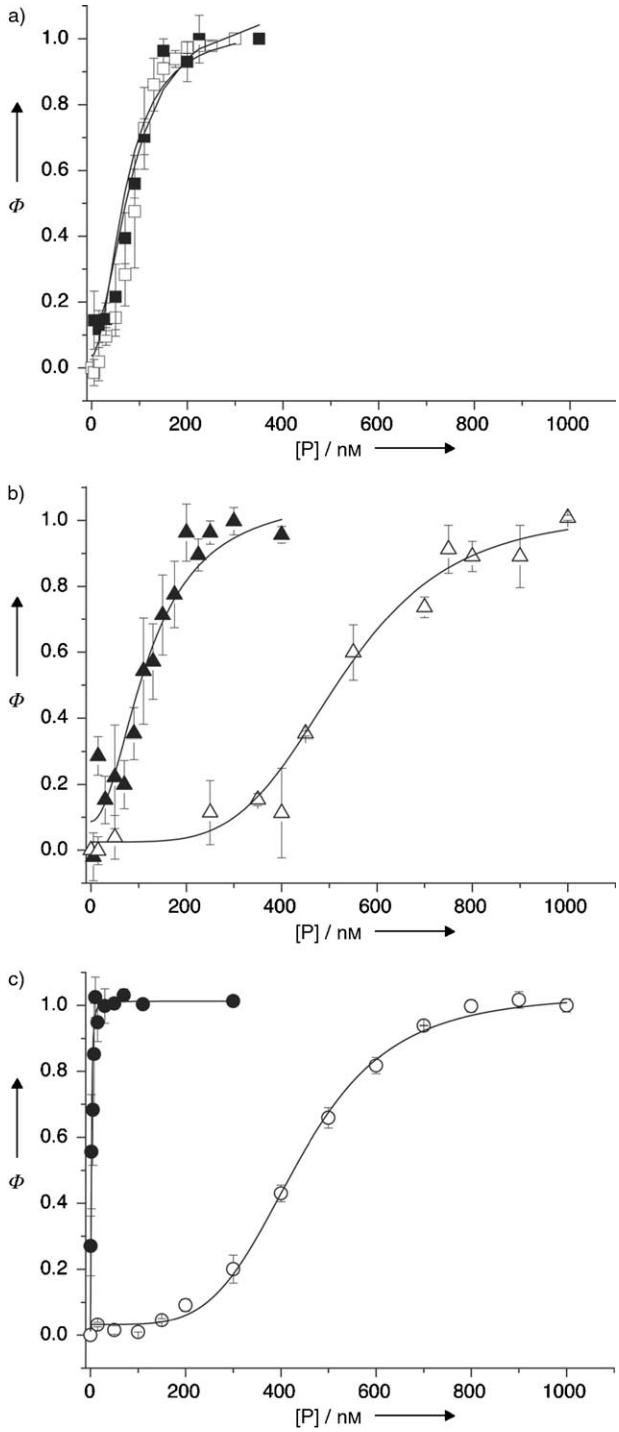

Figure 4. Characterization of DNA binding of non-cross-linked MyoD-bHLH-M116C-S123C and dark-adapted and irradiated photoMyoD measured by fluorescence anisotropy. Fraction (Φ) of bound MCK-S (solid symbols) and NoE-box (open symbols) in the complexes of a) MyoD-bHLH-M116C-S123C, b) dark-adapted and c) irradiated photoMyoD is plotted against the concentration of the free protein (P).

agreement with a previous study of MyoD-bHLH.[12] The association of MyoD-bHLH-M116C-S123C and NoE-box also displayed cooperative binding of two protein monomers per duplex DNA as had been observed for the MCK-S complex (Figure 4).

Dark-adapted photoMyoD showed only a slight increase in DNA-binding specificity. Although it bound MCK-S with comparable affinity to the non-alkylated parent protein MyoD-bHLH-M116C-S123C, the stability of its complex with NoE-box was reduced ($2.7 \pm 0.05 \times 10^{-13}$ M^2). In strong contrast, a reduction in affinity of irradiated photoMyoD for NoE-box ($1.9 \pm 0.06 \times 10^{-13}$ M^2) was accompanied by a dramatic increase in its affinity for MCK-S. The K_D value of the MCK-S complex of the irradiated protein ($4.4 \pm 0.16 \times 10^{-18}$ M^2) was reduced approximately 3400-fold relative to that of the dark-adapted state, whereas the K_D values of the NoE-box complexes of irradiated and dark-adapted photoMyoD ($2.7 \pm 0.05 \times 10^{-13}$ M^2) were similar.

At the high concentrations of irradiated and dark-adapted photoMyoD required to bind to the NoE-box duplex, the mechanism of DNA binding appeared to be altered when compared to the high-affinity binding reaction observed for binding to specific DNA. The best fit to the Langmuir isotherm for binding to noncognate DNA was obtained for binding orders of approximately 4.5 (Figure 4). This may be a consequence of interactions between the alkylated proteins at these elevated concentrations possibly through their aromatic cross-linkers.

The introduction of an azobenzene-derived photo-cross-linker into the DNA-recognition helix of the transcriptional activator MyoD produced a DNA-binding protein, the activity of which could be controlled by light pulses. In its irradiated state, in which the cross-linker was predominantly in the *cis* configuration, significant stabilization of the recognition helix and of the specific DNA complex was observed relative to the dark-adapted state. Because the affinity for heterologous DNA sequences was comparable for the dark-adapted and the irradiated proteins, photoactivation generated increases in the DNA-binding specificity of more than two orders of magnitude. Such photocontrolled DNA-binding proteins could serve as building blocks for the generation of reagents to control developmental processes and therapeutics. The activity of transcriptional activators is typically regulated through interaction with other protein domains, and these interfaces have proven difficult to target with small molecules. Reinforcing the native structure of DNA-recognition elements may be an alternative strategy to target these interactions and interfere with biological processes in a specific and controlled fashion.

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