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Supporting Information

69451 Weinheim, Germany

Photochemical regulation of DNA binding specificity of MyoD

Lucia Guerrero, Oliver S. Smart, Chris J. Weston, Darcy C. Burns, G. Andrew Woolley, Rudolf K. Allemann*

Experimental Details

Synthesis of MyoD-bHLH-M116C-S123C. The two cysteines were introduced in the cDNA for the bHLH domain of MyoD within the plasmid pJGetita using standard PCR techniques. This plasmid had been used previously for the production of the bHLH domain of MyoD.[1] To create MyoD-bHLH-M116C-S123C the primers M(M/S→C/C) (5'-AAGGCCGCCACCTGCCGCGAGCGCCGCCGCCTGTGCAAAGTGAAT-3'), which contains the codons for both cysteines and MyoD-BamHI (5′-AGCGGATCCTCATCAGTCGCGCAGCAGCTGCAG-3'), which contains the stop codon, were used (cysteine codons are underlined). The DNA sequence was verified using the dideoxy sequencing method. MyoD-bHLH-M116C-S123C was produced from this plasmid in BL21*(DE3) cells grown at 37 °C in LB-media containing 100 μg ml^{-1} ampicillin. At OD_{600} = 0.5, IPTG was added to a final concentration of 0.8 mM and the cells were harvested after 4 hrs by centrifugation.

Purification of MyoD-bHLH-M116C-S123C. For the purification of MyoD-bHLH-M116C-S123C, cells were gently thawed on ice, 3-4 ml of water per gram of wet cells were added followed by two volumes of lysis buffer (100 mM ammonium acetate, 100 mM sodium chloride, 100 mM 2-mercaptoethanol, pH 5.0) containing 0.5 mM phenylmethanesulfonyl fluoride. The suspension was sonicated for 20 minutes on ice

using a Sonicator W-37 (Heat Systems Ultrasonics Inc.) and the resulting lysate centrifuged at 6,800 g for 15 minutes at 4 °C. The pellet was resuspended in 20 ml of lysis buffer and recentrifuged. This step was repeated once more. MyoD-bHLH-M116C-S123C was solubilized by dissolving the pellet in 50 mM potassium phosphate (pH 8.0) containing 100 mM 2-mercaptoethanol. The suspension was sonicated for another 20 minutes and centrifuged at 17,500 g for 15 minutes. The supernatant was applied to CM-sepharose (20 ml, Pharmacia) equilibrated with 50 mM potassium phosphate (pH 8.0) containing 100 mM 2-mercaptoethanol at 4 °C. Bound protein was eluted with a NaCl gradient from 0 to 1.5 M with a flow rate of 3 ml min^{-1} over 67 min. MyoD-bHLH-M116C-S123C typically eluted at 300-400 mM NaCl. The eluate was dialysed twice against 50 mM potassium phosphate (pH 8.0) containing 100 mM 2-mercaptoethanol. Finally, the protein was further purified on a Resource™ S column (6 ml, Pharmacia) following the same procedure as for the CM column, but with a flow rate of 6 ml min^{-1} at 4 °C. The collected fractions containing MyoD-bHLH-M116C-S123C were pooled and dialysed against 2 changes of 2 l of 50 mM potassium phosphate (pH 8.0). The protein was homogenous as judged by SDS-PAGE. ESI-mass spectrometry showed a mass of 7012.0 in good agreement with the calculated mass of 7011.8. Protein concentration was determined by measuring the UV absorptions at 210, 215 and 220 nm. [2]

Alkylation of MyoD-bHLH-M116C-S123C with photoisomerisable crosslinker 3,3'-bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene. The photoisomerisable crosslinker 3,3'-bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene was synthesized as previously described.[3] All reactions involving the crosslinker were performed in the dark. Prior to alkylation, MyoD-bHLH-M116C-S123C was completely reduced by

incubation of a 0.1 mM solution in 50 mM Tris-Cl (pH 8.3) with tris-carboxyethyl phosphine (TCEP) (2 mM) for 15 minutes at 4 °C. 3,3'-Bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene (2 mM) dissolved in 50 mM Tris-Cl (pH 8.3) was added to MyoD-bHLH-M116C-S123C in three aliquots of 333.3 µl every 20 min. After the final addition the reaction was stirred overnight at 4 °C.

PhotoMyoD was purified by HPL-chromatography on a Luna 10 µ C_{18} column using a linear gradient over 40 minutes from 45 to 61 % acetonitrile:water (60:40; 0.05 % TFA) with a flow rate of 3 ml min^{-1}. Irradiated and dark-adapted PhotoMyoD eluted after 29.5 and 27.5 min, respectively. The mass of 7462.0 measured by ESI-MS confirmed the intramolecular crosslinking reaction (calculated mass 7461.5). Protein concentrations of PhotoMyoD were measured assuming an extinction coefficient of 24,000 M^{-1} cm^{-1} at 363 nm for the dark-adapted crosslinker.

Photoisomerization of dark-adapted PhotoMyoD was achieved by irradiating a thermostated solution of the protein (2 µM) in 5 mM potassium phosphate (pH 8.0) with a 250 W metal halide UV Light Point Source (UV-P 280) coupled to a 360 nm band pass filter. Photoisomerization was complete in less than 5 min as judged by the absence of further changes in the UV-Vis spectra. The percentage of isomerization was calculated by separating pure trans and cis isomers by HPLC immediately after irradiation. From the relative peak areas of the absorption at the isosbestic point at 315 nm were measured.[4]

UV-Vis experiments. UV-Visible absorption experiments were carried out using a Shimadzu UV-2401PC UV-Vis Recording Spectrophotometer in a 1 cm cuvette. Samples (2 µM) were dissolved in 5 mM potassium phosphate buffer (pH 8.0) and experiments were run at 15 ºC.

Oligonucleotides. All oligonucleotides were synthesized by Alta Bioscience (University of Birmingham). Annealing of complementary single-stranded oligonucleotides was carried out by heating the mixture to 95 ºC for 5 min followed by overnight cooling to room temperature. For fluorescence anisotropy experiments, one of the DNA strands was labeled at the 3'-end with acetamido-5-fluorescein. Concentrations of labeled oligonucleotides were determined assuming a molar extinction coefficient of 83,000 M^{-1} cm^{-1} at 494 nm.

The full sequences of double stranded oligonucleotides were (E-box underlined):

MCK-S 5'-CAGGCAG<u>CAGGTG</u>TTGG-3'

3'-GTCCGTC<u>GTCCAC</u>AACC-5'

NoEbox 5'-GGAGGCTTCTAGGACGG-3'

3'-CCTCCGAAGATCCTGCC-5'

CD Spectroscopy. CD spectra were measured in 0.5 cm cuvettes using a Jasco J810 spectropolarimeter. Protein concentrations were 2 μM in 5 mM potassium phosphate (pH 8.0). For measurements with MyoD-bHLH-M116C-S123C, 0.5 mM DTT was also added. The temperature-dependent denaturation scans were performed for temperatures ranging from 5 ºC to 95 ºC with rates of heating of 0.5 ºC min^{-1}. Mean residue ellipticities, $[\Theta]_r$, were calculated according to the equation:

$$[\Theta]_r = \Theta / (10 \cdot n \cdot c \cdot l),$$

where *n* is the number of backbone amide bonds, *c* is the concentration (M) and *l* is the pathlength (cm). The α-helical content was calculated following the equation:

100% helix [Θ] = -40,000 (n-4) / n.

DNA binding experiments using fluorescence anisotropy measurements.

Fluorescence anisotropy measurements were performed at 25 °C using a Perkin Elmer Luminescence Spectrometer LS50B arranged in L format (494 nm excitation; 525 nm emission). Titrations were performed in a 1 ml fluorescence quartz cuvette. The assay buffer was 5 mM Tris-Cl (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$, 15 % glycerol, containing 2.5 mM TCEP for measurements carried out with the unalkylated protein. Defined volumes of 0.5-2 µl of a stock solution of protein (2-20 µM) were added successively to 0.1-1 nM of fluorescein labelled DNA in a total volume of 1 ml. For the determination of K_D values of irradiated PhotoMyoD, the stock solutions of dark-adapted PhotoMyoD were irradiated prior to titration.

The G factor (ratio of sensitivities of the monochromator for horizontally and vertically polarized light) was calculated for each measurement using the equation:[5]

$$G = I_{\perp} / I_{||}$$

where $I_{||}$ and $I_{\perp}$ are the intensities of the fluorescent emissions in parallel and perpendicular planes to the excitation plane. The G factor value was always close to 1 (1.005 ± 0.008). Values for fluorescence anisotropy (A) were then determined from the equation:[6]

$$A = (I_{||} - GI_{\perp}) / (I_{||} + 2GI_{\perp})$$

For each anisotropy value ten measurements were taken and averaged using an integration time of 5 s.

Fluorescence data were expressed as:

$\Phi = (A-A_D) / (A_{PD}-A_D)$,

where *A* denotes the fluorescence anisotropy in the presence of the indicated concentration of protein, A_D is the fluorescence anisotropy of the solution containing only the fluorescently-labeled DNA, and A_{PD} is the fluorescence anisotropy at saturation.[6]

The data were fit to the Langmuir isotherm:

$\Phi_{???} = 1 / (1 + K_D^n / [P]^n)$.

The apparent dissociation constant for cis-PhotoMyoD was calculated from the dissociation constants of dark-adapted and irradiated PhotoMyoD according to the equation:

$\ln K_D^{cis} = -1/x \{(1-x) \ln K_D^{trans} - \ln K_D^{irradiated}\}$,

where x is the relative amount of cis-isomer in irradiated PhotoMyoD.

Table S1 DNA binding parameters for unalkylated MyoD-bHLH-M116C-S123C, dark-adapted and irradiated PhotoMyoD, and the theoretical cis-PhotoMyoD.

Protein	**MCK-S**		**NoEbox**		**Specificity** ($P_{1/2}^{NoEbox} / P_{1/2}^{MCK-S}$)
	$P_{1/2}$ (nM)	**K_D (M^2)**	**$P_{1/2}$ (nM)**	**K_D (M^2)**	
MyoD-bHLH-M116C-S123C	93.8 ± 11.8	8.8 ± 2.2 x 10^{-15}	77.4 ± 6.6	5.9 ± 1.0 x10^{-15}	0.83 ± 0.1
Dark-adapted-PhotoMyoD	121.4 ± 15.8	1.5 ± 0.38 x 10^{-14}	525.9 ± 5.2	2.7 ± 0.05 x10^{-13}	4.3 ± 0.6
irradiated-PhotoMyoD	2.1 ± 0.4	4.4 ± 0.16 x 10^{-18}	438.7 ± 6.6	1.9 ± 0.06 x10^{-13}	209 ± 40

References

[1] A. G. Künne, D. Meierhans, R. K. Allemann, *FEBS Lett* **1996**, *391*, 79.

[2] D. R. Wetlaufer, *Adv. Protein Chem.* **1962**, *17*, 303.

[3] Z. Zhang, D. C. Burns, J. R. Kumita, O. S. Smart, G. A. Woolley, *Bioconjug Chem* **2003**, *14*, 824.

[4] J. R. Kumita, O. S. Smart, G. A. Woolley, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 3803.

[5] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., Plenum Press, New York, **1999**.

[6] T. Heyduk, Y. Ma, H. Tang, R. H. Ebright, in *Meth. Enzymol., Vol. 274*, **1996**, pp. 492.