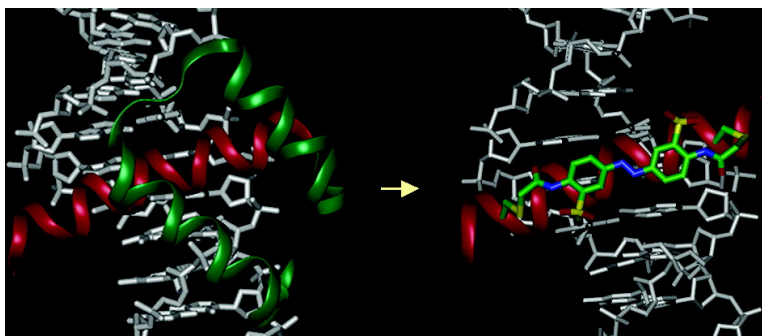


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Photocontrol of DNA Binding Specificity of a Miniature Engrailed Homeodomain

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Abstract: Control of DNA binding of HDH-3, a 18-residue polypeptide based on the recognition helix of the Q50K engrailed homeodomain, has been achieved. HDH-3 was linked to an azobenzene cross-linker through two cysteine residues in an $i, i + 11$ spacing. For the thermodynamically stable trans configuration of the cross-linker, the dark-adapted peptide (dad-HDH-3) adopted a mainly α -helical structure as judged by circular dichroism (CD) spectroscopy. After irradiation with light of 360 nm, the helical content of the peptide (irrad-HDH-3) was reduced significantly and the CD spectrum of the irradiated peptide resembled that of the largely unstructured, unalkylated peptide. Despite lacking helices-1 and -2 and the N-terminal arm of Q50K engrailed, dad-HDH-3 bound to its natural DNA target sequence TAATCC (QRE) with high affinity ($K_D = 7.5 \pm 1.3$ nM). The binding affinity for the mutant DNA sequence, TAATTA (ERE), was reduced significantly ($K_D = 140 \pm 11$ nM). Unlike irrad-HDH-3, which like the unalkylated parent peptide displayed only marginal DNA binding specificity, dad-HDH-3 specified base pairs 5 and 6 of QRE with an accuracy rivaling that of the intact wild-type Q50K engrailed homeodomain, making dad-HDH-3 the most specific designed DNA binding miniature homeodomain reported to date. Moreover, DNA binding affinity and specificity of HDH-3 could be controlled externally by irradiation with light.

Introduction

Many DNA binding proteins rely on α -helices to bind to their target sequences. The specificity of DNA binding proteins and the stability of their recognition α -helices are intimately linked. High affinity and selectivity of the binding reactions are often dependent on the presence of the remainder of the protein, which increases the stability of the recognition helix and restricts the conformational flexibility of the residues that make specific contacts with the nucleobases. Recently, several examples of active miniature proteins have been described in which functionally important residues were introduced into small, natural polypeptides of high intrinsic stability,^{1–3} leading to significant levels of binding specificity.^{4–6} The relatively stable pancreatic polypeptide has been used to produce catalytically active

miniature enzymes and to present several of the DNA contacting residues of the homeodomain protein engrailed in a miniature DNA binding protein.

Homeodomains are the DNA binding regions of a highly conserved family of transcription factors involved in the development of eukaryotic organisms. In addition to some interactions between their N-terminal arm and the minor groove, DNA binding by homeodomains relies on interaction between helix-3 within a helix-turn-helix motif and the major groove of DNA (Figure 1). Residue 50 within helix-3 of the engrailed homeodomain has been shown to be particularly important for the affinity and specificity of DNA binding and is responsible for distinguishing binding sites of the form TAATNN. While the wild-type protein binds to ERE sequences (TAATTA),⁷ the Q50K mutant recognizes QRE sites (TAATCC) with increased affinity and specificity.^{8–10} Introduction of the DNA binding

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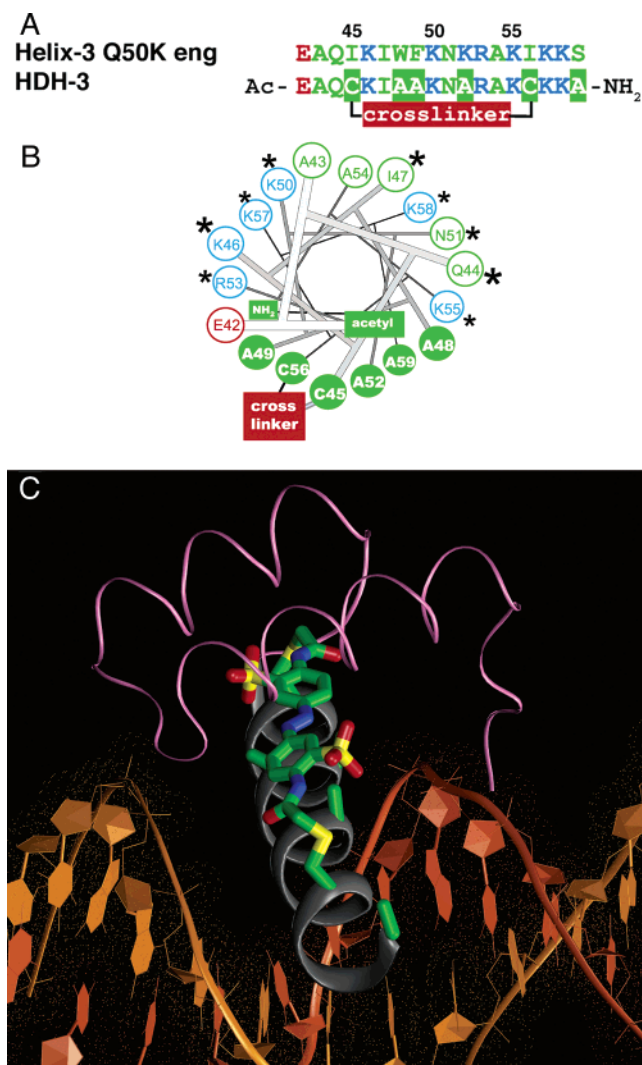


Figure 1. (A) Alignment of helix-3 of Q50K engrailed homeodomain and of alkylated HDH-3. (B) Helical wheel of alkylated HDH-3. The residues that contact DNA according to the crystal structure of the DNA complex of Q50K engrailed (pdb 2hdd) are marked with an asterisk.¹⁹ Charged residues are marked by a blue or red circle, while residues in the vicinity of the cross-linker that have been substituted to avoid steric clash are marked by filled green circles. (C) Model of the DNA complex of dad-HDH-3 generated from the X-ray structure of Q50K engrailed and viewed from the C-terminal end of the recognition helix. The backbone of the helix-turn-helix motif of engrailed that is absent in HDH-3 and replaced by the azobenzene cross-linker is shown in pink. The cross-linker is shown in a stick model in its trans configuration.

residues of helix-3 of the Q50K engrailed homeodomain into pancreatic polypeptide led to a miniature protein with significant DNA binding specificity despite the absence of the N-terminal arm.⁶

For many applications control of the properties of miniature proteins is desirable. We have previously described strategies for the modulation of the activities of miniature enzymes and of the DNA binding protein MyoD in response to changes of the redox conditions. The high stability of the neurotoxic peptide apamin was used to stabilize an α -helix through the formation of two disulfide bonds to hold functional residues in reactive conformations.^{11,12} This method is limited to in vitro applica-

tions, because the redox conditions in vivo cannot be controlled easily. Previous work had shown that the light-induced isomerization of azobenzene-based cross-linkers can be used to switch peptides between their α -helical and random coil-like conformations.^{13,14} Here we describe the incorporation of an azobenzene cross-linker into an 18-residue polypeptide containing the DNA binding residues of Q50K engrailed that binds to DNA with nanomolar affinities and specificities rivaling those of the full-length Q50K engrailed protein. Unlike for the natural protein, the DNA binding properties of this peptide could be controlled by light-induced isomerization of the cross-linker.

Experimental Section

Synthesis and Purification of HDH-3. HDH-3 (Ac-EAQCKIAAKNARAKCKKA-NH₂), acetylated at the N-terminal end and amidated at the C-terminus, was synthesized by solid phase peptide synthesis using standard protocols for Fmoc chemistry. The crude peptide was dissolved in acetonitrile:water (50:50) and purified on a reversed phase HPLC column (Luna 10 μ m C₁₈). Its experimentally determined mass (1973.0) corresponded well with the calculated mass of 1972.1. Peptide concentrations were determined by measuring the UV absorptions at 210, 215, and 220 nm.¹⁵

Alkylation of HDH-3 with Photoisomerizable Cross-Linker 3,3'-Bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene. The photoisomerizable cross-linker 3,3'-bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene was synthesized as previously described.¹⁶ All reactions involving the cross-linker were performed in the dark. Prior to alkylation, HDH-3 was completely reduced by incubation of a 0.5 mM solution in 50 mM Tris-Cl (pH 8.3) with tris(carboxyethyl)phosphine (TCEP) (2 mM) at 4 °C for 15 min and 3,3'-bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene (2 mM), dissolved in 50 mM Tris-Cl (pH 8.3), added to the reaction mixture in three aliquots of 333.3 μ L with gaps of 20 min. After the final addition the reaction was stirred at 4 °C for 16 h.

Cross-linked HDH-3 was purified by reversed phase HPLC on a Luna 10 μ m C₁₈ column using a linear gradient over 60 min from 0 to 100% acetonitrile:water (60:40; 0.05% TFA) with a flow rate of 3 mL min⁻¹. The alkylated peptide eluted after 21.4 min. MALDI-TOF mass spectrometry confirmed the calculated mass of 2425.8. Peptide concentrations of alkylated HDH-3 were determined using a molar extinction coefficient at 363 nm of 24 000 M⁻¹ cm⁻¹ for the dark-adapted azobenzene.

Photoisomerization of Alkylated HDH-3. Photoisomerization of dark-adapted (dad) HDH-3 was achieved by irradiating a thermostated controlled solution of the peptide (2 μ M) 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, immediately after irradiation by HPLC monitoring the elution at 315 nm (an isosbestic point) and measuring the peak area.

UV-Vis Spectroscopy. Absorption experiments were carried out using a Shimadzu UV-2401PC UV-vis spectrophotometer with a

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cuvette of 1 cm path length at 15 °C. Peptide concentrations were 2 μM in 5 mM potassium phosphate (pH 8.0).

Oligonucleotides. All oligonucleotides were synthesized by Alta Bioscience (University of Birmingham, UK). Annealing of complementary single-stranded oligonucleotides was carried out by heating the mixture to 95 °C 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 $\text{M}^{-1} \text{cm}^{-1}$ at 494 nm for fluorescein.

The full sequences of double-stranded oligonucleotides were the following:

QRE: 5'-CGCAGTGTAAATCCCCTCGAC-3'
3'-GCGTCACATTAGGGGAGCTG-5'

ERE: 5'-CGCAGTGTAAATTACCTCGAC-3'
3'-GCGTCACATTAATGGAGCTG-5'

MCK-S: 5'-CAGGCAGCAGGTGTTGG-3'
3'-GTCCGTCGTCACAACC-5'

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jasco J810 spectropolarimeter with a Jasco Peltier-type thermostat. Spectra were analyzed using Spectra Manager software (Jasco). A cuvette (Hellma) with path length 1 mm was used for all experiments. The resolution used was 0.2 nm, bandwidth was 2 nm, response time was 4 s, and scan time was 50 mdeg min^{-1} . Peptide samples (10–20 μM) were dissolved in 5 mM potassium phosphate (pH 8.0). Experiments with unalkylated HDH-3 also contained 0.5 mM dithiothreitol (DTT) to prevent oxidation of the unalkylated peptide. The CD spectra of alkylated HDH-3 were not affected by the presence of DTT, which was therefore omitted. Mean residue ellipticities, $[\Theta]_r$, were calculated from the measured ellipticities according to the equation

$$[\Theta]_r = \Theta / (10ncl)$$

where n is the number of backbone amide bonds, c is the concentration (M) of the peptide, and l is the path length (cm).

DNA Binding Experiments Using Fluorescence Anisotropy Measurements. Fluorescence anisotropy measurements were performed on 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 (Hellma). The assay buffer was 5 mM Tris-HCl (pH 7.9), 150 mM NaCl, 6 mM MgCl_2 , and 15% glycerol (the buffer contained 2.5 mM TCEP for measurements with unalkylated HDH-3). Defined volumes of 0.2–2.5 μL of a 5–20 μM stock solution of the peptides were added successively to 0.3–2 nM fluorescein labeled DNA in a total volume of 1 mL. The change of anisotropy was carefully monitored to ensure that the binding reaction had reached equilibrium, which occurred in less than 5 min. To determine K_D values for irradiated (irrad) HDH-3, a stock solution of dad-HDH-3 was irradiated as described above, prior to titration into the fluorescence cuvette.

The G factor (ratio of sensitivities of the monochromator for horizontally and vertically polarized light) was calculated for each measurement using the equation¹⁷

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

where I_{\parallel} and I_{\perp} are the intensities of the fluorescent emissions in parallel and perpendicular planes, respectively, to the excitation plane. The G

factor value was always close to 1 (1.033 ± 0.01). Values for fluorescence anisotropy (A) were then determined from the equation¹⁸

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

For each anisotropy value 10 measurements were taken and averaged using an integration time of 5 s.

The experimental fluorescence anisotropy data was fitted using the equation

$$A_{\text{theory}} = A_{\text{free}}(1 - \Phi_{\text{theory}}) + A_{\text{bound}}\Phi_{\text{theory}}$$

The Langmuir equation was used to produce values for Φ , the proportion of bound DNA:

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

where $[P]$ is the peptide concentration and K_D is the dissociation constant of the complex (both in nM). Fitting was performed using the nonlinear fit procedure within the Sigmaplot program. In practice, it was found that individual runs showed a wide variation in A_{free} and A_{bound} , so each run was separately fitted. For each fit K_D , A_{free} and A_{bound} were varied so as to make A_{theory} match the set of experimentally measured values A_{expt} for the run. This allowed each run to produce a separate value for K_D . The values were averaged to give an overall value for the dissociation constant. After these individual fits, data from separate experiments were conveniently combined by converting the experimentally measured anisotropy A_{expt} to an equivalent Φ value, using the values for A_{free} and A_{bound} from each fit.

$$\Phi_{\text{equivalent}} = (A_{\text{expt}} - A_{\text{free}}) / (A_{\text{bound}} - A_{\text{free}})$$

In practice, only the complexes of dad-HDH-3 and irrad-HDH-3 with QRE could be fitted to the simple Langmuir relation ($n = 1$). The binding curves for all other cases showed low degrees of binding up to 50 nM followed by a steep increase, suggesting cooperativity in binding. Good fits were obtained assuming that four peptides bound DNA simultaneously:

$$\Phi_{\text{theory}} = 1 / (1 + K_D^4 / [P]^4)$$

Results and Discussion

To reversibly control the stability of helix-3 of the Q50K engrailed homeodomain and hence its DNA binding specificity in the absence of the remainder of the protein, we replaced Ile 45 and Ile 56, two residues located on the face of the recognition helix opposite the DNA interface, with cysteines (Figure 1). Molecular modeling indicated that alkylation of the cysteines with the azobenzene derived photoisomerizable cross-linker 3,3'-bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene¹⁶ should not interfere with DNA binding. The cross-linker was well away from Gln 44, Lys 46, Ile 47, Trp 48, Lys 50, Asn 51, Arg 53, Lys 55, Lys 57, and Lys 58, which contact the major groove of the DNA target (Figure 1).^{8–10,19} In this $i, i + 11$ spacing of the cysteine residues, the trans configuration of the cross-linker has previously been shown to stabilize the α -helical conformation of peptides.¹⁴ The bulky Trp 48, Phe 49, and Lys 52 of Q50K engrailed, which are located on the same side of the helix as the cross-linker, were replaced with alanines to avoid steric conflict, and the C-terminal serine was substituted by the helix

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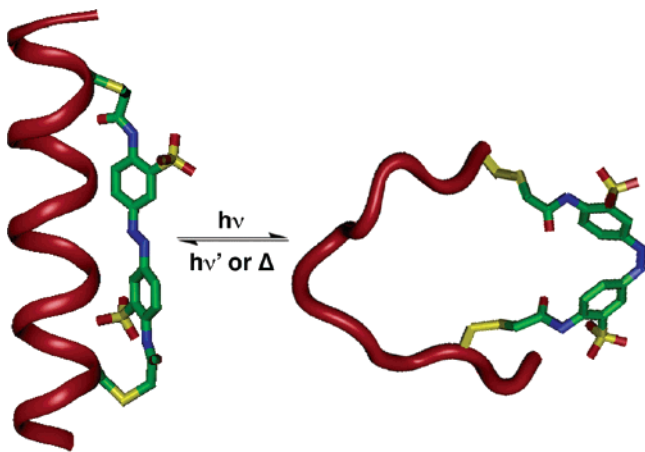


Figure 2. Sketch of the isomerization between *trans*- and *cis*-HDH-3.

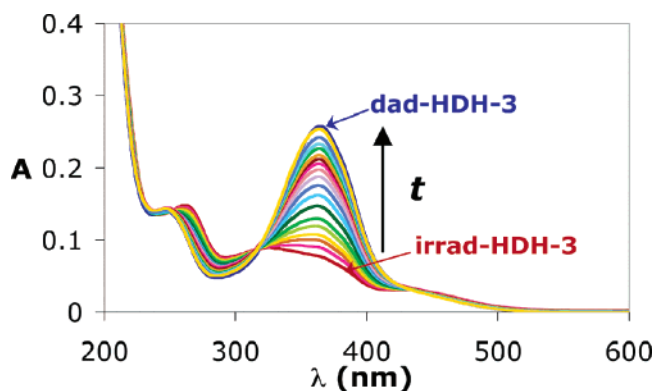


Figure 3. Absorption spectra of the isomerization of alkylated HDH-3. The spectrum of dad-HDH-3 is in dark blue. The spectrum immediately after irradiation with 360 nm light is shown in red. The intermediate spectra are those for the thermal reversion of irradiated HDH-3 after 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 360, 420, 540, and 1470 min.

stabilizing alanine.²⁰ The resulting 18-residue polypeptide, HDH-3, was synthesized as the C-terminal amide by solid phase peptide synthesis using standard Fmoc chemistry. For optimal helix stability, the N-terminal amine of HDH-3 was acetylated. After complete reduction, HDH-3 was alkylated in the dark with 3,3'-bis(sulfo)-4,4'-bis(chloroacetamido)azobenzene at the cysteine residues. MALDI-TOF mass spectrometry indicated a molecular mass for alkylated HDH-3 of 2426.0, which by comparison with the calculated mass of 2425.8 confirmed the intramolecular cross-linkage.

Absorption spectroscopy was used to characterize the conformation of alkylated HDH-3 in the *trans* and the *cis* configurations of the cross-linker. The *cis* configuration of azobenzene cross-linkers, which is formed upon irradiation with UV light of a wavelength of approximately 360 nm, is thermally labile and reverts to the thermodynamically more stable *trans* form (Figure 2). The UV-vis spectrum of dark-adapted HDH-3 (dad-HDH-3), where the azobenzene cross-linker is in the *trans* configuration, displayed the strong maximum at 363 nm typical for amide-substituted *trans*-azobenzene π - π^* transitions (Figure 3).²¹ The spectrum was similar to that recorded previously for azobenzene cross-linked peptides.¹⁴ Upon irradiation with light

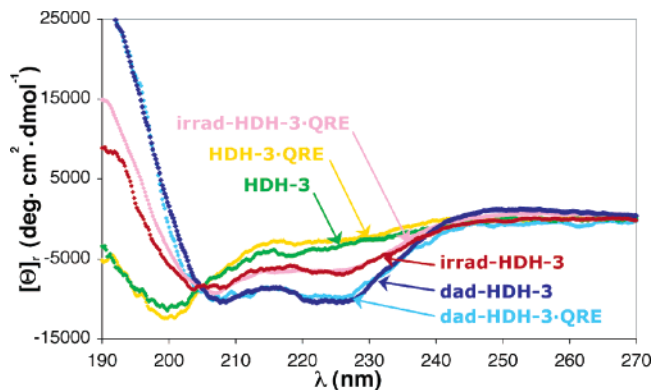


Figure 4. CD spectra of unalkylated HDH-3 (green), irradiated HDH-3 (red), and dad-HDH-3 (dark blue) as well as their QRE complexes shown in yellow, pink, and light blue, respectively ($T = 15\text{ }^{\circ}\text{C}$).

of 360 nm, the absorption maximum at 363 nm disappeared and a less intense maximum was observed at 262 nm. Irradiated HDH-3 (irrad-HDH-3) then reverted in a nonphotochemical process to dad-HDH-3. This process, which showed isosbestic points at 250, 315, and 434 nm, occurred with a half-life of approximately 150 min at 15 °C. The percentage of isomerization of irradiated material was determined by HPLC separation of the irradiated material and monitoring of the elution profile at 315 nm. Seventy-five percent of the irradiated material was found to be in the *cis* configuration, which is a value typically achieved by photoisomerization of *trans*-azobenzenes.^{14,21,22}

CD spectroscopy revealed significant structural differences in the conformations of the un-cross-linked peptide and irradiated and dad-HDH-3 (Figure 4). In its un-cross-linked form, HDH-3 displayed the CD spectrum of a mostly unstructured protein. A minimum was observed at 200 nm and the mean residue ellipticity at 222 nm, $[\Theta]_{r,222}$, was only $\sim -3600\text{ deg cm}^2\text{ dmol}^{-1}$. For the *trans* configuration of the cross-linker, on the other hand, the CD spectrum revealed significant amounts of α -helical structure with a mean residue ellipticity at 222 nm of $-12\,500\text{ deg cm}^2\text{ dmol}^{-1}$. Only marginal changes of the CD spectrum were observed between 2 and 30 °C, as had been observed previously for the *trans* configuration of a cross-linked model peptide.¹³ Irradiation of dad-HDH-3 with light of 360 nm led to a significant increase in $[\Theta]_{r,222}$ to $-5800\text{ deg cm}^2\text{ dmol}^{-1}$, a value close to that found for the un-cross-linked peptide. It is interesting to compare the helix change observed here to that found in previous work for FK-11X,¹⁴ a peptide where the $i, i + 11$ spacing of the cysteine residues was shown to produce helix stabilization for the *trans* form of the cross-linker but destabilization for the *cis* configuration. The degree of helix stabilization for the *trans* configuration is smaller in dad-HDH-3, but this is not surprising considering that forcing HDH-3 into an α -helical conformation involves lining up a row of four positively charged residues. The thermal reversion from the light-induced state to the *trans* configuration occurred with half-lives of 372 and 480 min at 15 and 5 °C. This was significantly longer than had been observed by UV-vis spectroscopy. The two measurements monitor different physical events: *cis*-*trans* isomerization of the linker (UV-vis) and folding of the helix (CD). The discrepancy in half-lives is

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Table 1. DNA Binding Parameters for HDH-3, dad-HDH-3, and irrad-HDH-3

	K_D (nM)			specificity	
	QRE	ERE	MCK-S	K_D^{ERE}/K_D^{QRE}	K_D^{MCK-S}/K_D^{QRE}
dad-HDH-3	7.5 ± 1.3^a	140 ± 11^b	>6000	19	>900
irrad-HDH-3	140 ± 25^a	160 ± 15^b	>6000	1.1	>75
HDH-3	200 ± 11^b	175 ± 16^b	nd	0.9	

^a Data fitted with single binding site model. ^b Data fitted with four binding site model.

therefore strongly suggestive of a two-step process, in which the cis–trans isomerization of the azobenzene cross-linker is followed by a slower change of the peptide conformation. The conformation of un-cross-linked HDH-3 did not change significantly on DNA binding according to CD spectroscopy (Figure 4). The peptide clearly did not adopt the α -helical structure observed for helix-3 in the crystal structure of the complex of intact engrailed with DNA,¹⁹ but remained largely unfolded. In the absence of the stabilization of the conformation of helix-3 provided by helices-1 and -2, no well-defined α -helical structure was observed in the QRE complex of HDH-3. dad-HDH-3, on the other hand, was largely α -helical even in the absence of DNA due to the stabilization provided by the cross-linker. CD spectroscopy indicated that its structure did not change on DNA binding, suggesting that in the DNA complex the recognition helix adopted a structure similar to that observed for helix-3 in the DNA complex of the intact engrailed protein (Figure 4).

Fluorescence anisotropy measurements^{23–25} were carried out to quantify the DNA binding affinities of the un-cross-linked peptide and of dad- and irrad-HDH-3 for sequences containing a QRE binding site (TAATCC), the highest affinity target of the Q50K engrailed homeodomain.⁹ This method is premised on the differences in the rates of rotational and fluorescence anisotropies of the free fluorescein-labeled oligonucleotide and its protein complex. dad-HDH-3 bound tightly to its target site with an equilibrium dissociation constant of 7.5 ± 1.3 nM at 25 °C (Table 1). The best fit of the experimental data was obtained for the expected binding mode where one peptide bound to one DNA molecule (Figure 5). The QRE complex of dad-HDH-3 was more than 200 times more stable than the QRE complex of the isolated helix-3 of engrailed.⁶ Since the replacement of four residues in helix-3 with alanines resulted in only an 8.5-fold increase in the DNA binding affinity (Table 1 and ref 6), the reduced dissociation constant of dad-HDH-3 was mainly due to the increased stability of the α -helix of dad-HDH-3. On the other hand, full-length engrailed homeodomain binds to QRE approximately 1000 times tighter than dad-HDH-3.¹⁰ However, the high affinity binding of dad-HDH-3 was achieved despite the absence of three DNA contacting residues from the N-terminal arm. In addition, helices-1 and -2 of the engrailed homeodomain, which pack tightly against the hydrophobic face of helix-3, were missing. These helices are central for the stability of helix-3, and in their absence only inefficient

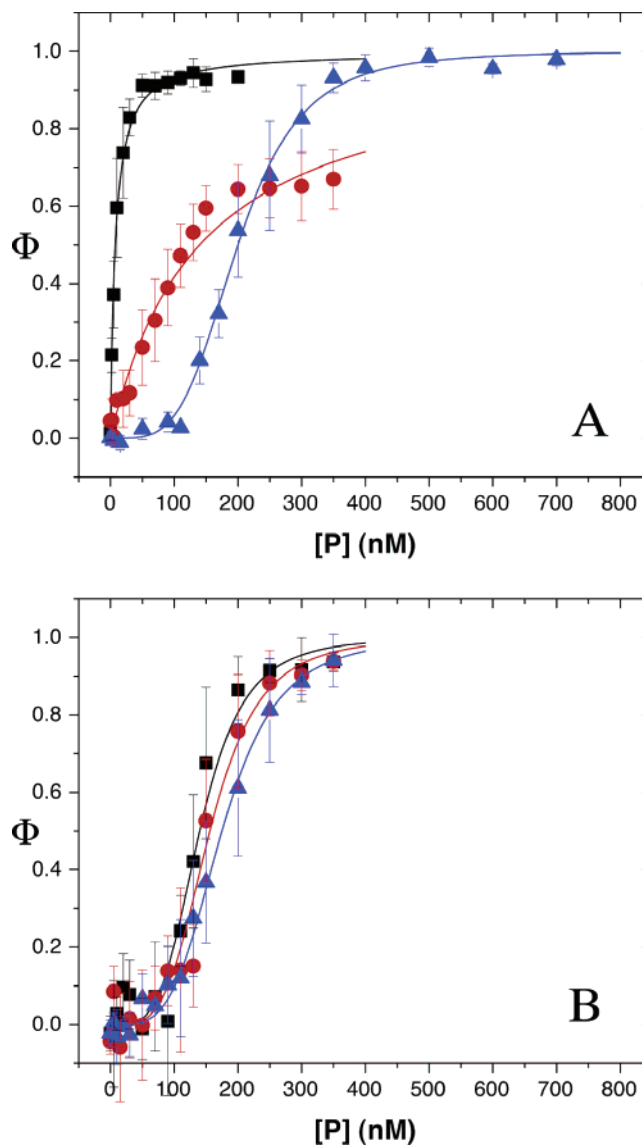


Figure 5. Binding curves for un-cross-linked HDH-3 (blue), irrad-HDH-3 (red), and dad-HDH-3 (black) to QRE (A) and ERE (B) DNA target sequences.

DNA binding is observed.⁶ In dad-HDH-3, the role of helices-1 and -2 was therefore effectively accomplished by the cross-linker in its trans configuration.

The complex between dad-HDH-3 and QRE was more than 25 times more stable than the QRE complex of the un-cross-linked peptide ($K_D = 200 \pm 11$ nM). Interestingly, the experimental fluorescence anisotropy data obtained for binding of the un-cross-linked peptide to QRE could not be fitted to the Langmuir equation, assuming a simple 1:1 binding mode. The binding curve showed only a low degree of binding up to 50 nM followed by a relatively steep increase, suggesting cooperative binding (Figure 5). The best fit to the experimental

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data was obtained under the assumption that four un-cross-linked HDH-3 molecules bound QRE simultaneously in a low affinity binding mode that was clearly different from the high affinity binding mode observed for dad-HDH-3. The stability of the QRE complex of irradi-HDH-3 was reduced almost 20-fold ($K_D = 140 \pm 25$ nM) relative to dad-HDH-3. Geometric constraints imposed by the cis configuration of the cross-linker, which makes up 75% of irradi-HDH-3, appeared to prevent the formation of an α -helix between the two cysteine residues, leading to an altered binding mechanism with reduced affinity when compared to dad-HDH-3.

The DNA binding specificities of the peptides were examined by measuring the dissociation constants of their complexes with oligonucleotides containing the natural target of wild-type engrailed (ERE)^{9,10} and the completely unrelated MCK-S sequences.²⁶ No binding to MCK-S could be detected for concentrations up to 6 μ M for irradi- and dad-HDH-3 (Table 1), indicating that these peptides discriminated fully between the typical DNA targets of homeodomain proteins and noncognate sequences.

When ERE was used as the DNA target, both un-cross-linked HDH-3 and irradi-HDH-3 displayed virtually no specificity. The K_D of the complex of un-cross-linked peptide with ERE was 175 ± 16 nM, identical within error to that measured for QRE binding (200 ± 11 nM) (Table 1). Similarly, the dissociation constants for the QRE and ERE complexes of irradi-HDH-3 were identical.

In strong contrast to these weakly binding peptides, dad-HDH-3 displayed significant amounts of DNA binding specificity and successfully discriminated between QRE and ERE. The binding affinity for ERE ($K_D = 140 \pm 11$ nM) was significantly

reduced when compared to QRE (Table 1), leading to a DNA binding specificity of 19 ($K_D^{\text{ERE}}/K_D^{\text{QRE}}$). dad-HDH-3 therefore specified base pairs 5 and 6 with an accuracy rivaling that of the intact Q50K engrailed homeodomain ($K_D^{\text{ERE}}/K_D^{\text{QRE}} = 36$), making dad-HDH-3 the most specific designed miniature homeodomain protein reported to date. These results demonstrated that high DNA binding specificity could be achieved in the absence of the N-terminal arm and helices-1 and -2 of the engrailed homeodomain by stabilizing helix-3 with an azobenzene cross-linker. ERE binding of both irradi- and dad-HDH-3 appeared to follow the same low affinity binding mechanism observed for the un-cross-linked peptide, which was best described by a cooperative four binding site model (Figure 5).

In summary, alkylating with an azobenzene derived cross-linker two cysteine residues in an $i, i + 11$ spacing within a peptide comprising the recognition α -helix of the Q50K engrailed homeodomain led to DNA binding with high affinity and specificity when the cross-linker was in the trans configuration. The remarkable sequence specificity of dad-HDH-3 was achieved with only 18 amino acid residues and was most likely a consequence of the cross-linker induced preorganization of its recognition helix, which compensated for the loss of the tertiary interactions used by the wild-type protein to stabilize helix-3.¹⁹ Moreover, unlike for the natural transcription factor, the specificity could be controlled externally by irradiation with light, opening the way to studies of cellular processes through reversible photocontrol of transcription.

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Supporting Information Available: Coordinates of the model of the DNA complex of dad-HDH-3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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