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## Exploiting anthracene photodimerization within peptides: light induced sequence-selective DNA binding†

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**The unprecedented use of anthracene photodimerization within a protein or peptide system is explored through its incorporation into a DNA-binding peptide, derived from the GCN4 transcription factor. This study demonstrates an effective and dynamic interplay between a photoreaction and a peptide–DNA assembly, with each process able to exert control over the other.**

The active form of many proteins arises from the formation of higher oligomeric states such as dimers.<sup>1</sup> Therefore much effort has been directed towards controlling the assembly of polypeptide subunits, with a view to using this as a tool to regulate biological processes.<sup>2</sup> Of particular interest is the use of light to control oligomerization due to the non-invasive and localized nature of this stimulus. Furthermore, it requires no external chemical additives, and varying the irradiation wavelength and intensity provides a handle for control. These advantages are the motivation behind the photo-induced crosslinking of unmodified proteins (PICUP) technique.<sup>3</sup> However, this technique is non-specific and indiscriminately crosslinks all proteins within the irradiated area. An alternative approach is to react chemical cross linkers with amino acid side chains. Several photoactivated examples based on azides are commercially available,<sup>4</sup> and some amino acid derivatives have also been reported.<sup>5</sup> However, these techniques, as with PICUP, do not allow for site-selective photocrosslinking between peptide subunits, which hitherto has yet to be demonstrated.

Anthracene is a fluorophore known to undergo an intramolecular  $[4\pi_s+4\pi_s]$  cycloaddition reaction, yielding two photodimers, head-to-head or head-to-tail.<sup>6</sup> Its photodimerization properties have been utilized extensively in the small molecule

and supramolecular community,<sup>7</sup> to allow for the photo-release of small molecules<sup>7a</sup> and the formation of gels,<sup>7d</sup> amongst other applications. However, despite its irradiation wavelengths being biologically compatible, the transition of anthracene photodimerization to the biological sciences has been limited to just a few examples involving DNA<sup>8</sup> and phospholipids,<sup>9</sup> and its effect on protein and peptide systems has remained unexplored.<sup>10</sup> Here we address this issue in order to demonstrate the unprecedented example of the formation of a light-activated specific crosslink between two peptide subunits.

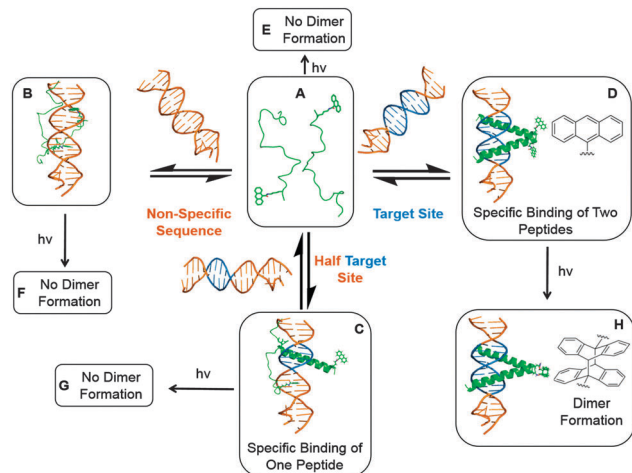
In order to perform this study, we selected a peptide that comprises a central component of the GCN4 yeast transcription factor, a well-studied DNA binding protein. The active form of the protein is a homodimer<sup>11</sup> with the monomer displaying a weaker affinity for target DNA.<sup>12</sup> The attraction of this system is that by controlling the oligomerization process (in this case using light), the strength of DNA binding, and therefore the transcription process, can also be potentially controlled, allowing for genes to be up- or down-regulated through an external stimulus. A number of groups have focused on perturbing GCN4's native dimerization unit, the leucine zipper, by altering the protein folding within this region.<sup>13</sup> A light-triggered example was reported by Woolley and co-workers, who introduced an azobenzene into the leucine zipper to control DNA binding through *trans-cis* isomerization.<sup>14</sup>

An alternative approach (similar to that used in this work) involves covalently linking two much shorter peptides that retain only the GCN4 residues directly responsible for sequence-selective DNA binding (the basic region). The first reported example of this approach simply linked the two DNA binding domains at the C-terminus *via* a disulphide bond.<sup>15</sup> Since then, more sophisticated examples have been developed,<sup>16</sup> including one by Mascareñas and co-workers, who employed an azobenzene unit to connect the two GCN4 domains together and then control their alignment and their subsequent DNA binding affinity through photo-isomerization.<sup>17</sup> Whereas these literature examples demonstrate photocontrol of DNA binding through intramolecular light-induced conformational changes (photoisomerization), our goal was to establish whether DNA binding could control, as well as be controlled by, the

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† Electronic supplementary information (ESI) available: Acknowledgements; materials and methods; peptide synthesis scheme; analytical HPLC and MS for peptide and DNA; absorbance tracked irradiation of peptide; fitted DNA peptide binding curves; circular dichroism tracked irradiation of peptide; summary of percentage changes in absorbance, fluorescence and folding upon irradiation; SDS PAGE tracked irradiation; Gel EMSA of peptide irradiated in the absence of DNA. See DOI: 10.1039/c5cc01618e



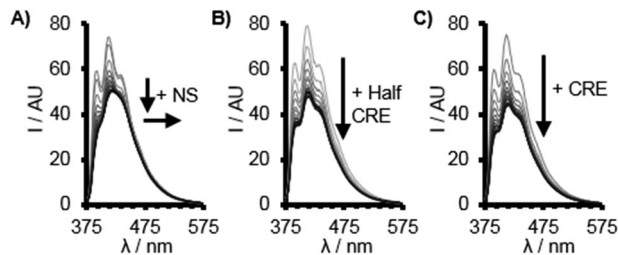


**Fig. 1** Cartoon illustrating sequence-selective photodimerization of the anthracene tagged peptide. Peptide (shown unbound in A) displays weak binding to a non-specific sequence of DNA (B); in the presence of DNA containing half a target site, one peptide binds through specific but weak interactions (C); and in the presence of DNA containing the full target site, specific, though weak, binding of two peptides is observed (D); peptide irradiated alone displays no photodimer formation (E); neither does peptide irradiated in the presence of either non-specific DNA (F) or DNA containing half a target site (G); however, when irradiated in the presence of DNA containing the full target site, due to the preorganization afforded by the target site, anthracene photodimer formation is observed, and is accompanied by an increased binding affinity (H).

unprecedented light-induced formation of a covalent bond between two peptide molecules (photodimerization), as shown in Fig. 1.

In this study, the essential residues for DNA binding within the GCN4 transcription factor<sup>18</sup> were retained, along with the linker region, which has previously been reported to enhance the stability of the resulting DNA complex.<sup>19</sup> A methyltrityl (Mtt) protected lysine was introduced towards the C-terminus during solid-phase peptide synthesis (SPPS), selectively deprotected and subsequently coupled with anthracene carboxylic acid (Scheme S1, ESI†). The remaining residues were coupled to yield the final peptide Ac-ALKRARNTAARRSRARKLQRMKQK(anthracene)-G-NH<sub>2</sub>, which was purified by C18 RP-HPLC and characterized as previously reported (Fig. S1 and S2, ESI†).<sup>20</sup>

Binding to DNA duplexes was initially tracked by monitoring changes in fluorescence. One duplex contains the CRE target site (CRE), a binding site of native GCN4 in which two  $\alpha$ -helices bind either side of the target site; the second duplex only presents half of the CRE target site (half CRE); and the third duplex, a non-specific sequence (NS) which contains the same number of G-C and A-T base-pairs. DNA was titrated into solutions of the anthracene tagged peptide, with the resulting fluorescence spectra found to be DNA strand dependent (Fig. 2). Upon addition of NS DNA, a reduction in fluorescence intensity and evidence of a small bathochromic shift are observed, which could be consistent with intercalation of the anthracene.<sup>21</sup> However, upon addition of half CRE DNA, a greater reduction in the fluorescence intensity was observed, with no significant bathochromic shift, possibly indicating a different binding mode.<sup>22</sup> The addition of the CRE DNA gave a further decrease in the fluorescence signal. Data from titrations of NS and CRE DNA into peptide monitored by UV-Vis and fluorescence could not be

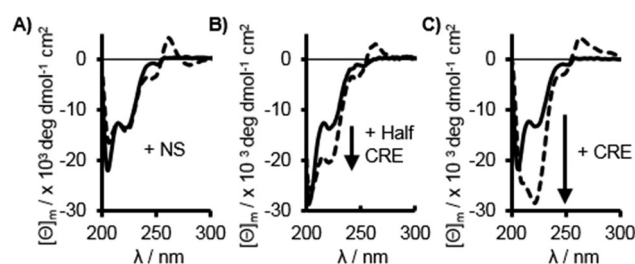


**Fig. 2** Fluorescence spectra of 2  $\mu$ M solutions of anthracene tagged peptide in the presence of increasing concentrations (0–3  $\mu$ M) of duplex (A) NS, (B) half CRE or (C) CRE DNA, in 10 mM sodium phosphate buffer pH 7 and 100 mM NaCl at 298 K  $\lambda_{\text{ex}} = 325$  nm.

unambiguously fitted to a single binding model, suggesting that other binding modes (*e.g.* intercalation) were also making a contribution in the case of CRE DNA. The  $K_D$  values are in the low micromolar range (see Fig. S4, ESI†), consistent with previously reported data for similar peptide monomers.<sup>12,23</sup>

The secondary structure of the anthracene tagged peptide, in the absence and presence of the DNA duplexes, was investigated using circular dichroism (CD) (Fig. 3). The peptide alone displayed a signal characteristic of a poorly folded peptide ( $38 \pm 1\%$  folded) which showed little change upon addition of NS DNA ( $40 \pm 2\%$  folded), see Fig. 3A. In contrast, a substantial increase in the  $\alpha$ -helical content of the peptide was observed upon the addition of either half CRE or CRE DNA. Upon addition of half CRE DNA, only one peptide is proposed to fold into an  $\alpha$ -helix (see Fig. 1C), and this leads to an increase in folding ( $57 \pm 2\%$  folded). In contrast when CRE DNA is present, two peptides can bind as  $\alpha$ -helices (Fig. 1D), resulting in a greater increase in folding ( $75 \pm 1\%$  folded). The folding of monomer peptides in the presence of target DNA sites is consistent with previous findings<sup>12,24</sup> and the hypothesis that the peptide binds primarily to the target DNA site *via*  $\alpha$ -helices (see Fig. 1C and D). These findings indicate that the peptide sequence used within this study is sufficient to maintain sequence selectivity. In all cases positive and negative signals observed above 245 nm are attributed to induced anthracene signals,<sup>25</sup> and changes to the structure of DNA caused by peptide binding.<sup>26</sup>

The CD spectra are consistent with the anthracene tagged peptide showing some folding in the presence of half CRE, and an even greater extent of folding in the presence of CRE DNA. However, given various reports of dimerized (*i.e.* covalently linked or



**Fig. 3** CD spectra of 2  $\mu$ M anthracene tagged peptide in the absence (solid) and presence (dashed) of 1  $\mu$ M duplex (A) NS, (B) half CRE or (C) CRE DNA, in 10 mM sodium phosphate buffer pH 7 and 100 mM NaCl at 298 K. Difference spectra corrected for DNA contribution.



through assembly) GCN4 units displaying enhanced DNA binding,<sup>12</sup> the peptide samples were irradiated for 10 minutes ( $\lambda_{\text{ex}} = 365$  nm), in order to assess whether light could cause such an effect through anthracene photodimerization. Studies were carried out in the absence and presence of NS, half CRE and CRE DNA, and in each case, the relative percentage changes in both the absorbance and fluorescence spectra were monitored. Photoirradiation of the peptide for 10 minutes in the absence of DNA led to only a small reduction ( $7 \pm 2\%$ ) in the fluorescence signal (solid vs. dotted lines, Fig. 4A). Similar reductions in the fluorescence signal were observed following photoirradiation in the presence of NS DNA ( $8 \pm 3\%$ ) (dashed vs. dotted lines, Fig. 4B) and half CRE DNA ( $4 \pm 3\%$ ) (dashed vs. dotted line, Fig. 4C). However, photoirradiation in the presence of CRE DNA was accompanied by a much greater reduction ( $20 \pm 1\%$ ) (dashed vs. dotted lines, Fig. 4D). Similar decreases were also seen in the absorbance spectra (Fig. S5 and Table S2, ESI<sup>†</sup>). Complementary CD studies showed no notable change in folding upon irradiation of the anthracene tagged peptide in the absence and presence of NS and half CRE DNA. However, an increase in peptide folding,  $75 \pm 1\% \rightarrow 84 \pm 1\%$ , was observed upon irradiation in the presence of CRE DNA (Fig. S6 and Table S2, ESI<sup>†</sup>). Taken together, these findings are consistent with photodimer formation only being notable in the presence of CRE DNA, which highlights how crucial the preorganization of two peptide strands *via* sequence selective binding is for templating and controlling the photo-induced behavior of this system.

The irradiated samples were analyzed by sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) experiments. Those samples irradiated both in the absence and presence of NS and half CRE DNA gave bands consistent with species of molecular weights corresponding to unirradiated anthracene tagged peptide. However, irradiation of the anthracene tagged peptide in the presence of CRE DNA led to the appearance of a new band of higher molecular weight (lane 6, Fig. 5). This species was found

to display near identical mobility through the gel as a similar covalently linked peptide dimer (GCN4bd1)<sub>2</sub>Pyr (lane 2, Fig. 5).<sup>24</sup> This data gives further evidence that the desired peptide photodimer only forms upon photoirradiation in the presence of CRE DNA, and not when the peptide is irradiated alone or in the presence of NS or half CRE DNA, at these low micromolar concentrations.

Irradiation experiments performed in the absence or presence of CRE and NS DNA, with aliquots taken over the course of 0–30 or 0–90 minutes irradiation, indicated no evidence of photodimer formation prior to irradiation, no photodimer formation in the presence of NS DNA following longer irradiation times, and no noticeable change in the amount of photodimer formed in the presence of CRE DNA after the first 15 minutes (Fig. S7, ESI<sup>†</sup>).

Though these findings all support the premise that DNA binding is sequence specific and that photodimer formation only occurs in the presence of DNA containing the full CRE target site, the hypothesis that the latter is associated with increased DNA affinity required verification through a gel electrophoretic mobility shift assay (gel EMSA), see Fig. 6. This shows that the unirradiated anthracene tagged peptide displays a weak affinity for all DNA duplexes (Fig. 6A), with only a faint band observed for the intact peptide–CRE DNA complex. Some affinity for all three DNA sequences is to be expected due to the formation of favorable non-specific electrostatic interactions between the positively charged peptide and the negatively charged DNA, as well as anthracene intercalation (*vide supra*), with some smearing in the gel consistent with such multiple binding modes. However, upon photoirradiation ( $\lambda_{\text{ex}} = 365$  nm, 10 minutes), a more intense band for the intact peptide–CRE DNA complex could be successfully distinguished, consistent with stronger binding of peptide dimers to CRE DNA, as has been previously reported for similar systems.<sup>13</sup> In the cases of NS and half CRE DNA, the gel appears similar to that obtained prior to irradiation, consistent with both weaker binding and the lack of photodimer formation (Fig. 6B). These results reveal a dynamic interdependent relationship, in which the CRE target site is required for preorganization of two peptides monomers in order to promote photodimerization, which is in turn accompanied by an increase in binding affinity.

In order to verify that photodimer formation only occurs in the presence of the CRE DNA target site, a similar gel EMSA was run in which a solution of the anthracene tagged peptide was irradiated alone prior to the addition of CRE DNA (Fig. S8, ESI<sup>†</sup>). This gel was similar to that recorded for the unirradiated sample

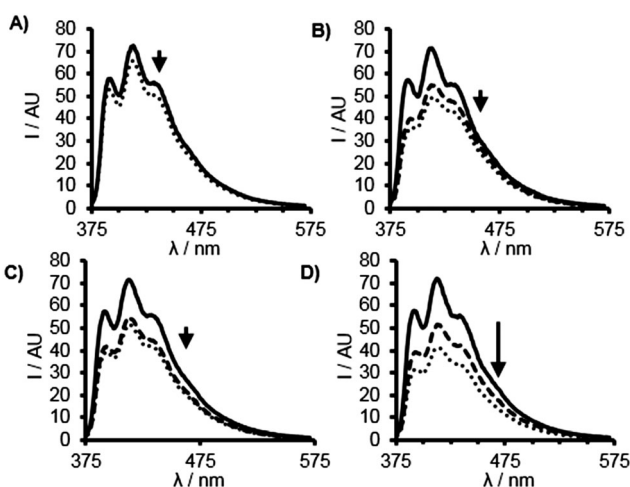


Fig. 4 Fluorescence spectra of 2  $\mu\text{M}$  solutions of anthracene tagged peptide (solid), with 1  $\mu\text{M}$  duplex DNA, where applicable (dashed), and after 10 minute irradiation with 365 nm light (dotted) in the absence (A) and the presence of NS (B), half CRE (C) and CRE (D) DNA, in 10 mM sodium phosphate buffer pH 7 and 100 mM NaCl at 298 K  $\lambda_{\text{ex}} = 325$  nm.

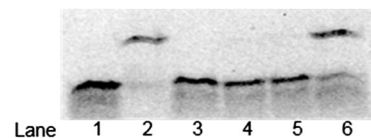


Fig. 5 SDS PAGE tracking photodimer formation following excitation at 365 nm for 10 min. Lane 1 – unirradiated anthracene tagged peptide control, 2 – (GCN4bd1)<sub>2</sub>Pyr covalent dimer control, 3 – irradiated peptide in the absence of DNA, 4 – irradiated peptide in the presence of NS DNA, 5 – irradiated peptide in the presence of half CRE DNA, 6 – irradiated peptide in the presence of CRE DNA. Visualized using Coomassie brilliant blue R-250 protein stain.



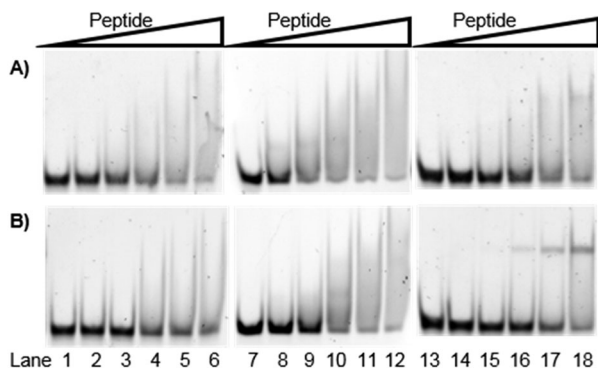


Fig. 6 Gel EMSA recorded for lanes 1–6 NS DNA (100 nM), lanes 7–12 half CRE DNA (100 nM) and lanes 13–18 CRE DNA (100 nM), in the presence of increasing concentrations 0, 100, 200, 400, 600 and 1000 nM anthracene tagged peptide; recorded for (A) unirradiated, or (B) irradiated samples. DNA visualized using SybrGold.

(see Fig. 6A), supporting the hypothesis that the photodimer only forms once templated by the CRE target site.

In summary, this study for the first time utilizes the photodimerization properties of anthracene in a peptide sequence, which has allowed us to demonstrate specific, light-controlled peptide dimerization through the formation of an intermolecular photocrosslink. This has been achieved by using the DNA binding domain of the GCN4 transcription factor, which has served to preorganize the two anthracene tagged peptides. Importantly, anthracene photodimerization only occurs in the presence of the correct DNA sequence, with stronger DNA binding then observed upon formation of the photocrosslink. The prospect of introducing a photocrosslink at a particular site within a peptide sequence makes this anthracene photodimerization approach particularly attractive. However the need for both anthracene tagged units to be preorganized through templation before photodimerization can occur, should serve to make the process very specific and controllable. We ultimately envisage exploring how light can be used to manipulate, amongst others, the transcription process itself, and to extend this approach to additional systems for the photoregulation of other important biological processes.

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