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Side-Chain Cross-Linked Short α-Helices That Behave like Original Proteins in Biomacromolecular Interactions

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Supporting Information

ABSTRACT: We explored the effect of α-helical stabilization upon the binding of short peptides to DNAs. The short peptides were designed according to the binding domains of DNA-binding proteins and were cross-linked between their side chains with diacetylenic or isophthalic cross-linking agents to keep stable α-helices. The binding abilities of the peptides to DNAs were evaluated by fluorescence resonance energy transfer analysis. When a cross-linked peptide based on the homeodomain of the transcription factor was titrated with a target DNA duplex, its dissociation constant (K_d) was calculated to be ∼0.5 nM. This value was the double-digit smaller than that of the corresponding non-cross-linked peptide. The cross-linked peptide showed high substrate specificity for DNAs at the same level as the original DNA-binding protein.

Protein−biomacromolecule (protein and DNA/RNA) interactions play a central role in numerous biological functions in organisms. Therefore, powerful inhibitors for the interactions are expected to bring breakthroughs in the resolution of intractable diseases and to be next-generation drugs. Such inhibitors are required to cover large contact areas upon the binding sites of the target macromolecules, and it is hard for conventional inhibitors with small molecular weights to accomplish this mission. Although protein-based inhibitors promise high inhibition efficiency, there are serious problems with their use as practical inhibitors with respect to molecular size, cell permeability, chemical modification, folding, large-scale synthesis, etc.

In view of these difficulties, artificial helical foldamers or stable α-helices of short peptides are considered to be potent candidates for the above mission because of the importance of α-helices in protein−biomacromolecule interactions.[1] Several artificial helical foldamers have been developed on the basis of, for example, pyridyl pyridinone, benzourea, and β-peptide skeletons.[2,3] However, their unnatural bodies not only need many synthetic efforts but also complicate the faithful replication of α-helical architectures. Indeed, the β-peptide-based foldamers tend to adopt 14-helices, implying that the relative positions and directions of their side chains differ from those of the α-helices seen in their original proteins. On the other hand, stable α-helices of short peptides are divided into three classes: miniature proteins,[4] hydrogen-bonding surrogates (HBSs),[5] and side-chain cross-linked helices.[6] Miniature proteins consist of only natural amino acids, including avian pancreatic polypeptide or Trp-cage as a helix stabilizer.[4] Although the stabilization method can be applied to a variety of native proteins, miniature proteins readily suffer from degradation with protease. HBS α-helices show relatively high binding affinities for target macromolecules and resistance to protease. Recently, the inhibition of a transcription coactivator interaction by using an HBS α-helix has been reported.[5a] However, the HBS helices need much higher binding affinity to mature into practical inhibitors. We previously reported a general method for stabilizing α-helices of short peptides by using a variety of cross-linking agents.[6a] This methodology can copy α-helix domains out as faithfully as possible and result in high α-helicities of the "isolated" peptides. Therefore, such copies are expected to be practical inhibitors for biomacromolecular interactions. In the present work, we synthesized short helical peptides based on the binding domains of DNA-binding proteins and examined their strong affinities to the target DNAs.

We chose homeodomains of transcription factors in eukaryotic genes as a helix-domain source among various DNA-binding proteins because the homeodomain and its variants have been comprehensively studied for their binding properties.[7] One of the variants, QK50, has been reported to strongly bind to a specific DNA duplex, QRE, with a dissociation constant (K_d) of ∼0.01 nM.[7c] We designed the sequence of peptide **qk-1** according to the crystal structure of the complex of QK50 and QRE (Figure 1a). The original sequence of the binding domain in QK50 is peptide **qk-2**. Two ornithine (Orn) residues were placed at the i and $i + 11$ positions on **qk-1** for a cross-linking reaction in place of two Ile residues on **qk-2**. On the basis of the crystal structure of the complex of QK50 and QRE, their side chains should point in the direction opposite to those of the binding residues. Therefore, the resulting cross-linkers were expected not only to reproduce the original bindings but also to have little direct influence upon the bindings. Furthermore, three Ala residues were introduced into **qk-1** instead of Trp, Phe, and Lys to simplify the sequence because their bulky side chains make little contribution to the association with QRE. For fluorescence resonance energy transfer (FRET) analysis, the amino termini of the peptides were modified with a Tokyo Green (TG) derivative,[8] while one strand of the duplex QRE was labeled with TAMRA at its 5′ end; "**A**" is used as the abbreviation of the labeled duplex (Figure 1b,c). The emission property of the TG fluorophore is similar to that of fluorescein, a FRET donor against TAMRA. Among a series of cross-linking agents previously reported by us, the diacetylenic molecule **1**[9] was used to afford a cross-linked peptide **qk-1^1** because of its high ability for stabilizing the α-helices of short peptides at the i and $i + 11$ cross-linking positions (Figure 1d).

The helical contents of the peptide **qk-1** and the cross-linked peptide **qk-1^1** were calculated to be ∼20 and 60%, respectively,

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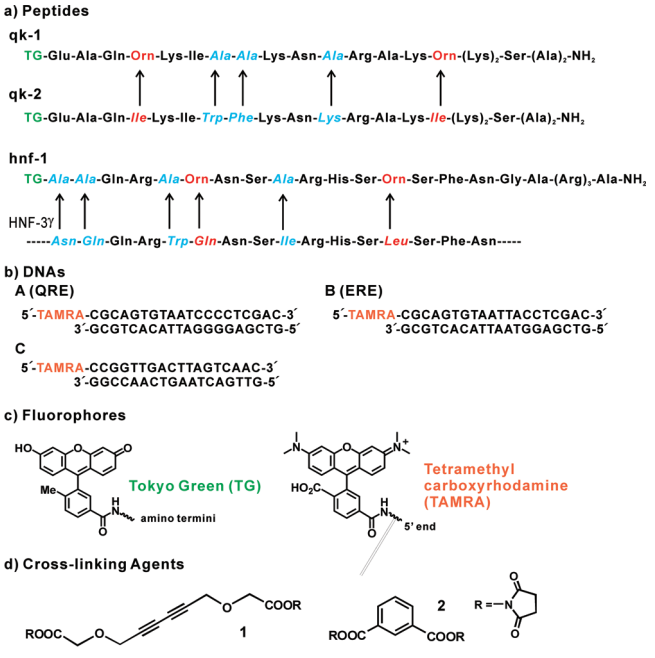

Figure 1. (a) Sequences of peptides **qk-1**, **qk-2**, and **hnf-1** and of HNF-3γ. The arrows indicate residues changed from the original sequences. (b) Sequences of DNAs **A**−**C**. (c) Chemical structures of Tokyo Green (TG) and tetramethylcarboxyrhodamine (TAMRA). (d) The diacetylenic and isophthalic cross-linking agents **1** and **2**.

from the mean residue ellipticity at 222 nm (Figure S1 in the Supporting Information).[6c,9] This relatively high value for **qk-1^1** reveals that the TG modification scarcely disturbed the α-helical structure.[6c] This finding suggests that a variety of functional groups can be introduced at the N-termini of cross-linked peptides without damaging the α-helical stabilization.

Cross-linked **qk-1^1** was titrated with duplex **A** (QRE) at 25 °C by fluorescence spectroscopy. When duplex **A** was added to a solution of **qk-1^1** under irradiation with excitation light at 500 nm, the fluorescence intensity from the TG fluorophore at 520 nm decreased (Figure 2a).[10] Although the fluorescence decrease was also observed from the titration experiment involving the combination of non-cross-linked **qk-1** and duplex **A** (Figure S2), the tendency was much more sluggish than that observed in the case of **qk-1^1**. This finding indicates that the helical **qk-1^1** binds to duplex **A** much more strongly than **qk-1** does. To quantitatively compare the binding affinities of **qk-1^1**, **qk-1**, and the original **qk-2** for duplex **A**, their K_d values were estimated from their binding isotherms according to a 1:1 binding mode by nonlinear analysis (Figure 2b), and the values are presented in Table 1. The K_d values for **qk-1^1**, **qk-1**, and **qk-2** were <1.0, 30 ± 5, and 23 ± 4 nM, respectively. Unfortunately, it was difficult to estimate exactly the binding affinity of **qk-1^1** for duplex **A** at an NaCl concentration of 150 mM because of the low limit of detection. Thus, additional titration experiments were performed at NaCl concentrations of 300 and 500 mM, where the binding affinity was reduced as a result of electrostatic interactions. In the presence of 300 (500) mM NaCl, the K_d values of **qk-1^1** and **qk-1** changed to 3 ± 1 (87 ± 5) and 165 ± 13 (>2500) nM, respectively, and $\Delta G_{\text{qk-1^1}} - \Delta G_{\text{qk-1}} \approx 4$ kJ/mol at both NaCl concentrations (Figure S3). Since the difference in ΔG values could be considered independent of the salt concentration, the K_d value of **qk-1^1** in the presence of 150 mM

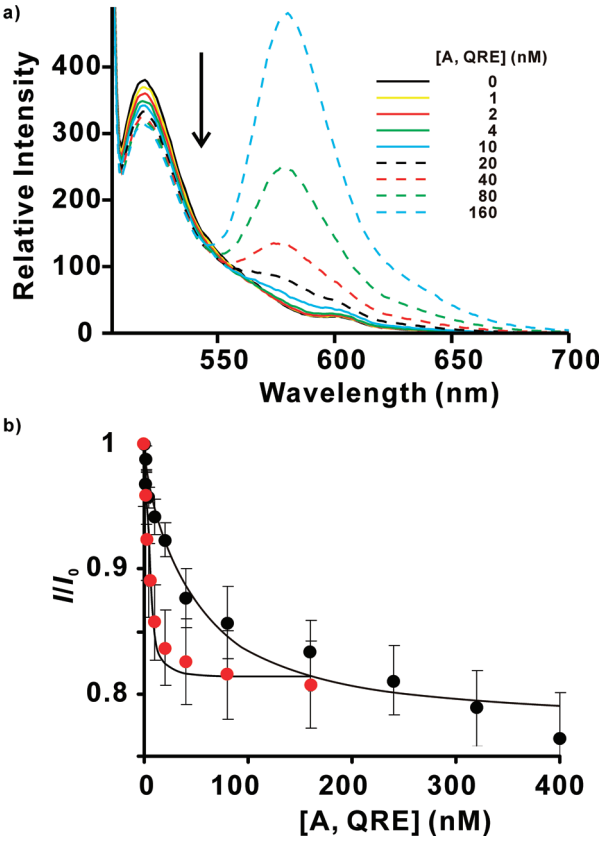

Figure 2. (a) Fluorescence spectra of **qk-1^1** (10 nM) titrated with duplex **A** (QRE) (0−160 nM) in a 10 mM Tris-HCl buffer containing 150 mM NaCl and 6 mM $MgCl_2$. (b) Isotherm curves for the fluorescence titrations of **qk-1^1** (red) and **qk-1** (black) in the presence of 150 mM NaCl. The observation wavelengths were 520 and 518 nm for **qk-1^1** and **qk-1**, respectively.

Table 1. Dissociation Constants (in units of nM) for Peptides with the DNAs QRE and ERE at various NaCl concentrations (in mM) Obtained from the FRET Analysis

	QRE			ERE
	150	300	500	150
qk-1^1	~0.5	3 ± 1	87 ± 5	11 ± 3
qk-1	30 ± 5	165 ± 13	>2500	34 ± 4
qk-2	23 ± 4	–	–	–

NaCl could be recalculated as ~0.5 nM. This value is much smaller than the values of 7.5 and 17 nM in the systems of Woolley's side-chain cross-linked α-helix[7a] and Schepartz's miniature protein,[7b] respectively.

The K_d values were also determined by surface plasmon resonance (SPR) analysis (Figure S4).[11] The K_d values of nonlabeled **qk-1^1**, **qk-1**, and **qk-2** for nonlabeled duplex **A** were calculated to be ~70, 6000, and 1000 nM, respectively, from their sensorgrams. Although the order of the binding affinities, **qk-1^1** > **qk-2** > **qk-1**, was the same as that observed in the FRET analysis, the K_d values were ~100 times as large as those in the FRET analysis at [NaCl] = 150 mM. The high values might have large errors because the molecular weights of 2100−2400 made it hard to obtain strong signals and good signal-to-noise ratios in the SPR analysis. On the other hand, the K_d values were close to those (87 and >2500 nM for **qk-1^1** and

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qk-1, respectively) at the NaCl concentration of 500 mM in the FRET analysis. As shown in Figure S3b, the isotherm curves accompanying small error bars afforded the relatively accurate K_d values at the NaCl concentration of 500 mM. Thus, the K_d value of ~0.5 nM for the cross-linked **qk-1^1** in the FRET analysis at [NaCl] = 150 mM expresses its real binding affinity.

To support the effect of the cross-linkage on the binding affinity, the melting temperatures (T_m) of nonlabeled duplex **A** (QRE) (1 μM) were measured in the absence and presence of nonlabeled **qk-1^1** and **qk-1**. The T_m value was 69.0 °C in the absence of the peptides. The value increased to 70.8 °C in the presence of 3 equiv of **qk-1^1** but scarcely changed upon addition of **qk-1**. This T_m increase also certifies the effect of the α-helical stabilization upon the interaction between the cross-linked peptide and the duplex.

To examine the specificity of the cross-linked **qk-1^1**, fluorescence titration experiments were carried out with another DNA duplex having a different sequence, **B** (ERE), which is known to bind QK50 more weakly than QRE (Figure 1b).[7c] The K_d of **qk-1^1** for duplex **B** was ~11 nM (Figure S5), and the $K_{d(\mathbf{A}:QRE)}/K_{d(\mathbf{B}:ERE)}$ ratio was 22, which is close to that for the original protein QK50 (34).[7c] On the other hand, the non-cross-linked **qk-1** exhibited no DNA specificity; the K_d for duplex **B** was ~34 nM.

To show the generality of our cross-linking method, the DNA-binding protein HNF-3γ was selected as another example.[12] As shown in Figure 1a, the sequence of a peptide **hnf-1** was arranged on the basis of the original sequence of HNF-3γ. Four Ala residues were introduced into **hnf-1** for the same reason as for **qk-1** mentioned above. Two Orn residues were positioned at the i and $i + 7$ on **hnf-1** for the cross-linking reaction in place of the Gln and Leu residues in the original sequence. The isophthalic cross-linker **2** can effectively stabilize α-helices of short peptides at the i and $i + 7$ cross-linking positions.[6a] Furthermore, the sequence of **hnf-1** includes an extra Arg-repeated moiety at the C-terminus. The original HNF-3γ was reported to bind to a target DNA duplex through the α-helical and the Arg-repeated moieties; the duplex labeled with TAMRA is abbreviated as "**C**" (Figure 1b). Therefore, the Arg-repeated sequence was added to the C-terminus of **hnf-1**. The helical contents of the peptide **hnf-1** and the cross-linked **hnf-1^2** were calculated to be ~20 and 70%, respectively (Figure S6).[6c,9] In the titration experiments involving the peptides and duplex **C**, a binding enhancement by the α-helical stabilization similar to that seen in the above case was observed. The K_d value changed from 570 nM for the non-cross-linked **hnf-1** to 4 nM for the cross-linked **hnf-1^2** (Figure S7). Thus, our strategy proved to be applicable for emulating various DNA-binding proteins.

Finally, we explored the resistance property of the cross-linked peptides against digestive enzymes. The cross-linked **qk-1^1** and the non-cross-linked **qk-1** (50 μM) were digested with trypsin (0.5 μM), and the digested peptides were analyzed by HPLC (Figure S8). The half-life for digestion ($\tau_{1/2}$) of **qk-1^1** was 65 min, while the $\tau_{1/2}$ value for **qk-1** was 15 min. Thus, the cross-linked peptides could somewhat obtain the resistance property against digestive enzymes because of the α-helical stabilization and/or the presence of the cross-linking moiety. This finding shows the possibility for future applications of the cross-linked peptides such as to in vivo use.

In conclusion, cross-linked peptides with stable α-helices were found to work well, like the original proteins. Their binding affinities for DNAs were strong (sub-nanomolar level), and their substrate specificities were comparable to those of the original proteins. Furthermore, in the presence of digestive enzymes, the cross-linked peptides survived much longer than native peptides. These results have encouraged us to extend our strategy to protein−protein interactions related to intractable diseases.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S8 and all experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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labeling. The decrease in the fluorescence intensity resulting from the addition of the labeled **A** was due not to quenching by nucleobase residues such as guanine but to FRET from TG to TAMRA.

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Supporting Information

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Contents:

General Methods and Materials. MALDI-TOF mass spectra were recorded with 2,5-dihydroxy benzoic acid as a matrix. Reagents were purchased from commercial sources and were used without further purification. The cross-linking and fluorescent labeling agents for peptides, **1**[1], **2**[2], and the TG derivative[3] were synthesized according to the previously published procedures.

Synthesis of TG-labeled, cross-linked peptide. All of peptides were synthesized by an automated peptide synthesizer using the standard Fmoc chemistry. Pre-labeled peptides were constructed on an Fmoc-NH-SAL resin (capacity 0.65 mmol/g). After the automated synthesis, the *N*-terminal amino groups of the peptides were labeled with the TG fluorophore on the resin by treating with the TG derivative[3] (5 equiv), HBTU (10 equiv), HOBt (10 equiv.) and *N*,*N*'-diisopropylethyl amine (10% v/v) in DMF over 8 h at room temperature. Prior to cross-linking reaction, the protecting groups of the Orn residues, Dde (= 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) were selectively removed with hydrazine monohydrate (3% v/v) in DMF in 1 h×2 at room temperature. The deprotected peptides were cross-linked with **1**[1] (3 equiv) over 12 h at room temperature on the resin. Deprotection of other amino acid residues was performed in parallel with peptide cleavage from the resin by treating with the TFA-ethanedithiol-thioanisole (18 : 1 : 1) over 90 min at room temperature. The cross-linking reaction of **hnf-1** with **2**[2] was carried out as follows. To a solution of the isolated **hnf-1** in EtOH (0.5 mL; 1.0×10^{-4} M) was added **2** in DMSO (0.5 mL; 5.0×10^{-4} M). The reaction mixture was incubated at 45 °C in a thermo-mixer for 8 h. Nonlabeled peptides were prepared according to the procedure previously published by us.[2]

Peptide Purification. All of peptides were purified by reversed-phase HPLC (column; COSMOSIL $5C_{18}$ MS-2 nacalai tesque, 10 × 150 mm) and were eluted with a mixed solvent between a 0.1% TFA buffer and CH_3CN including 0.1% TFA by using the following linear gradients of CH_3CN at a flow rate of 2.0 mL/min: 5–45% (0–40 min) for **qk-1**, 10–50% (0–40 min) for **qk-1^1**, 20–60% (0–40 min) for **qk-2**, 15–55% (0–40 min) for **hnf-1**, and 18–28% (0–60 min) for **hnf-1^2**. The isolated fractions of the peptides were identified with a MALDI-TOF MS spectrometer.

qk-1: TG-labeled: *m/z*: calcd for $C_{111}H_{180}N_{33}O_{29}$ ($[M+H]^+$) 2439.36, found 2439.38; nonlabeled: *m/z*: calcd for $C_{92}H_{170}N_{33}O_{26}$ ($[M+H]^+$) 2153.30, found 2153.30.

qk-1^1: TG-labeled: *m/z*: calcd for $C_{121}H_{186}N_{33}O_{33}$ ($[M+H]^+$) 2629.39, found 2629.49; nonlabeled: *m/z*: calcd for $C_{102}H_{176}N_{33}O_{30}$ ($[M+H]^+$) 2343.33, found 2343.36.

qk-2: TG-labeled: *m/z*: calcd for $C_{130}H_{198}N_{33}O_{29}$ ($[M+H]^+$) 2685.50, found 2685.53.

hnf-1: TG-labeled: *m/z*: calcd for $C_{118}H_{180}N_{45}O_{32}$ ($[M+H]^+$) 2739.38, found 2739.96.

hnf-1^2: TG-labeled: *m/z*: calcd for $C_{126}H_{182}N_{45}O_{34}$ ($[M+H]^+$) 2869.39, found 2869.68.

Fluorescence titration experiment. To a peptide solution ([**qk-1**] = [**qk-1^1**] = 10 nM, [**qk-2**] = 30 nM, [**hnf-1**] = 100 nM, and [**hnf-1^2**] = 20 nM) in a 10 mM Tris-HCl buffer containing 150–500 mM NaCl and 6 mM $MgCl_2$ was added a DNA duplex solution at 25 °C. The mixture was stirred at 25 °C until no more change of the fluorescence spectrum occurred (ca. 15 min). Each excitation wavelength was 500 for **qk-1**, **qk-1^1**, and **qk-2** and 496 nm for **hnf-1** and **hnf-1^2**. The fluorescence spectra were recorded from 450 to 700 nm at every addition of the DNA solution. The dissociation constants of peptide-DNA complexes were calculated by a curve-fitting method applying to plots of fluorescence intensities from the TG fluorophore.

SPR measurement. The BIAcore system (BIAcore, Uppsala, Sweden) was utilized for exploring the bindings of peptides to DNAs. DNAs were immobilized onto a sensor chip CM5 that possesses dextran-carboxylic acids on its surface using a running buffer (pH 7.4) consisting of 10 mM HEPES and 150 mM NaCl at 25 ºC. After the activation of the carboxy groups on the sensor chip with 200 mM *N*-[3-(Dimethylamino)propyl]-*N*'-ethylcarbodiimide hydrochloride and 50 mM *N*-Hydroxysuccinimide for 7 min, 3'-amino modified ODN dissolved in a 10 mM HEPES buffer (pH 7.4) including 0.6 mM hexadecyltrimethylammonium bromide was injected into the flow cell. The complementary ODN dissolved in a 10 mM HEPES buffer (pH 7.4) including 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20 was injected into the flow cell for 6 min to afford duplex-modified sensor chips.

In the binding analysis of peptides, as a running buffer was used a 10 mM HEPES buffer (pH 7.4) including 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20. Peptides (250 nM) dissolved in the running buffer were injected into the flow cell for 2 min at 25 ºC.

Binding analysis for SPR measurement. The dissociation constants of peptides for DNAs were determined according to the published procedure.[4] From the sensorgrams of peptides, the association and dissociation rate constants (k_{ass} and k_{dis}) were determined to give the dissociation constant $K_d = k_{dis}/k_{ass}$.

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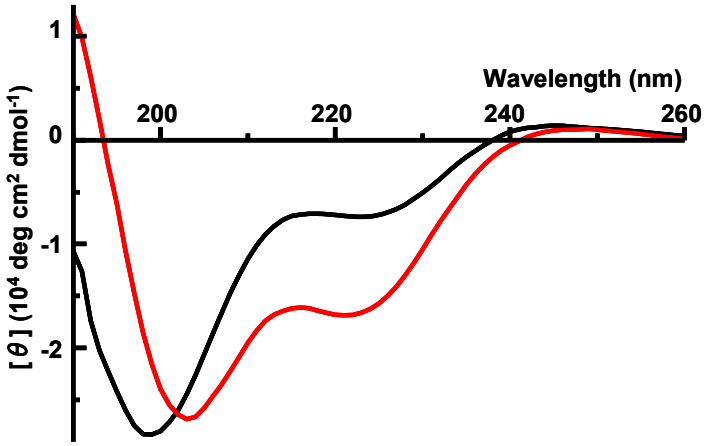

Figure S1. CD spectra of **qk-1** (black) and **qk-1^1** (red) dissolved in a 100 mM phosphate buffer (pH 6.6) at 5 ºC.

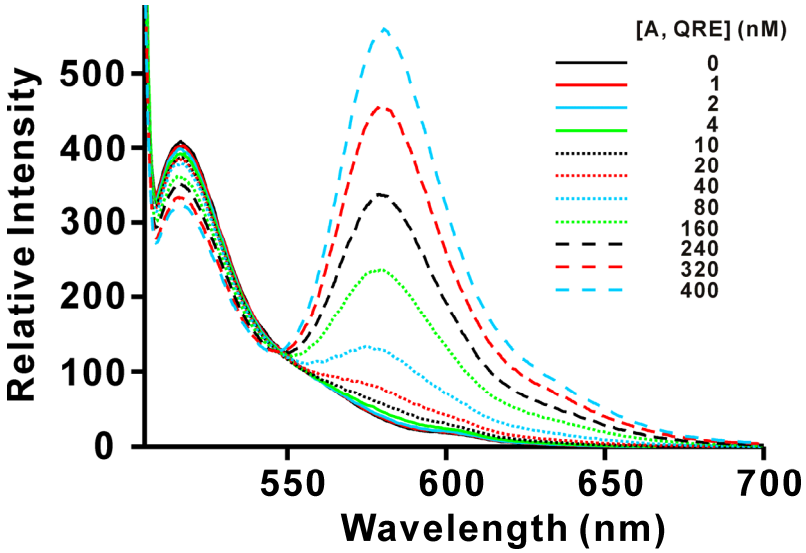

Figure S2. Fluorescence spectra of **qk-1** (10 nM) titrated with duplex **A** (QRE) (0–400 nM) in a 10 mM Tris-HCl buffer containing 150 mM NaCl and 6 mM $MgCl_2$.

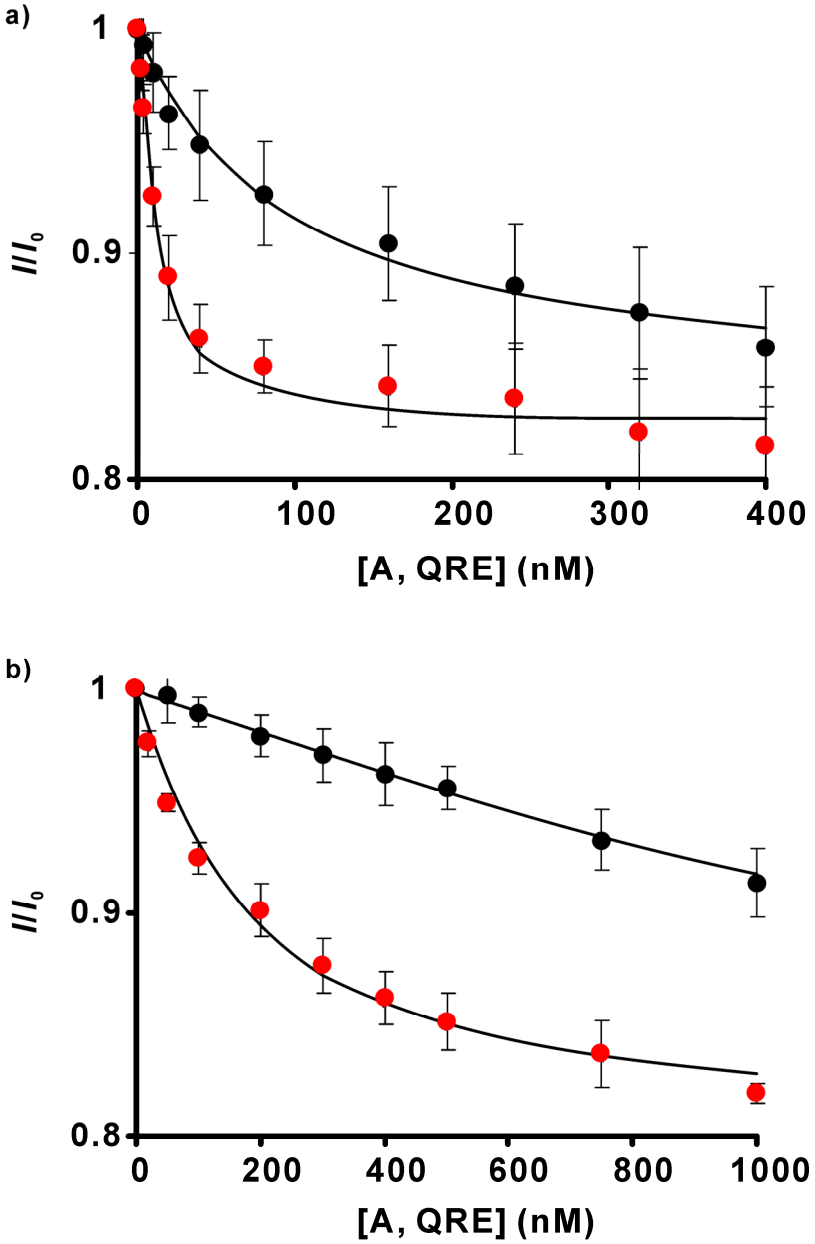

Figure S3. Isotherm curves for the fluorescence titration of **qk-1^1** (red) or **qk-1** (black) with duplex **A** (QRE) in the presence of NaCl of a) 300 mM ([**qk-1^1**] = [**qk-1**] = 20 nM) and b) 500 mM ([**qk-1^1**] = [**qk-1**] = 100 nM): the observation wavelengths were 520 and 518 nm for **qk-1^1** and **qk-1**, respectively.

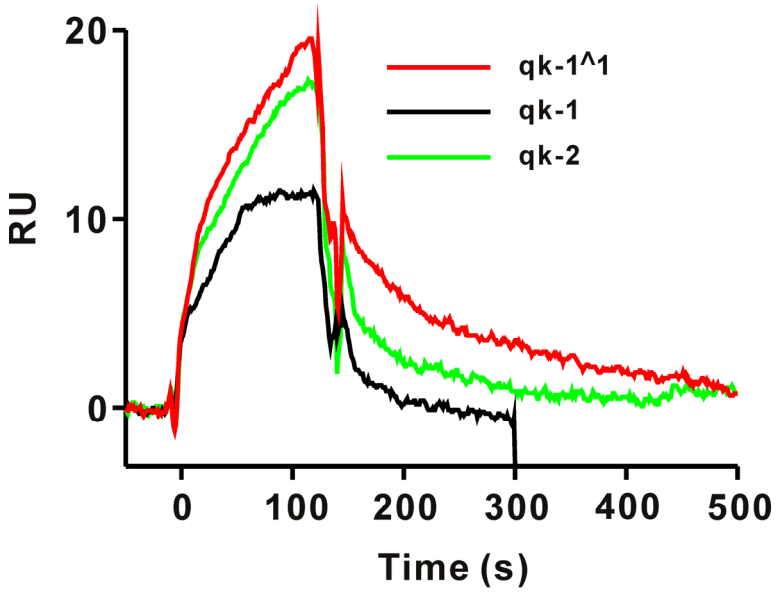

Figure S4. SPR sensorgrams of nonlabeled **qk-1^1** (red), **qk-1** (black), and **qk-2** (green) for nonlabeled duplex **A** (QRE) immobilized onto a sensor chip CM5: their peptide concentrations were 250 nM.

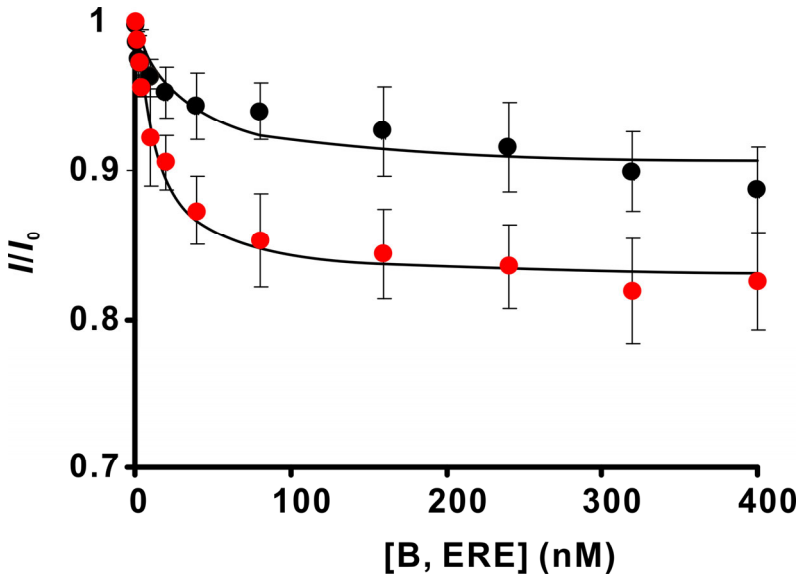

Figure S5. Isotherm curves for the fluorescence titration of **qk-1^1** (10 nM, red) or **qk-1** (10 nM, black) with duplex **B** (ERE) in the presence of 150 mM NaCl: the observation wavelengths were 520 and 518 nm for **qk-1^1** and **qk-1**, respectively.

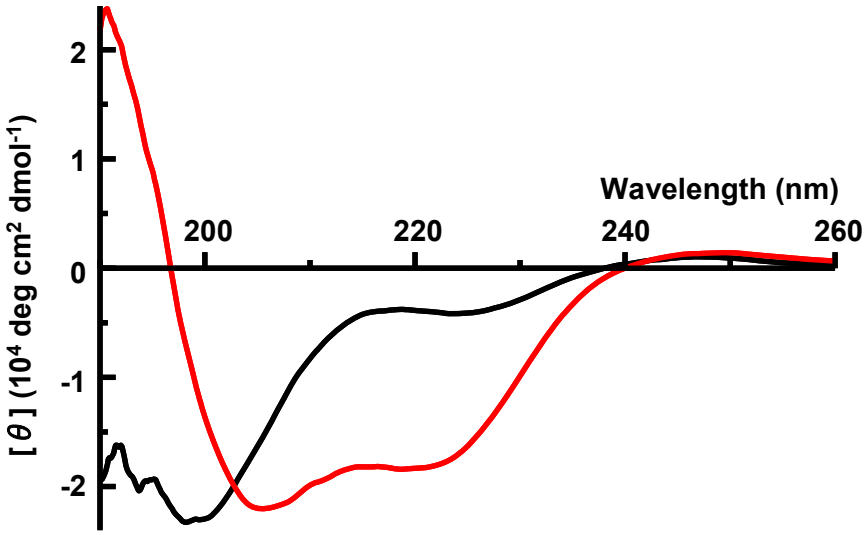

Figure S6. CD spectra of **hnf-1** (black) and **hnf-1^2** (red) dissolved in a 100 mM phosphate buffer (pH 6.6) at 5 ºC.

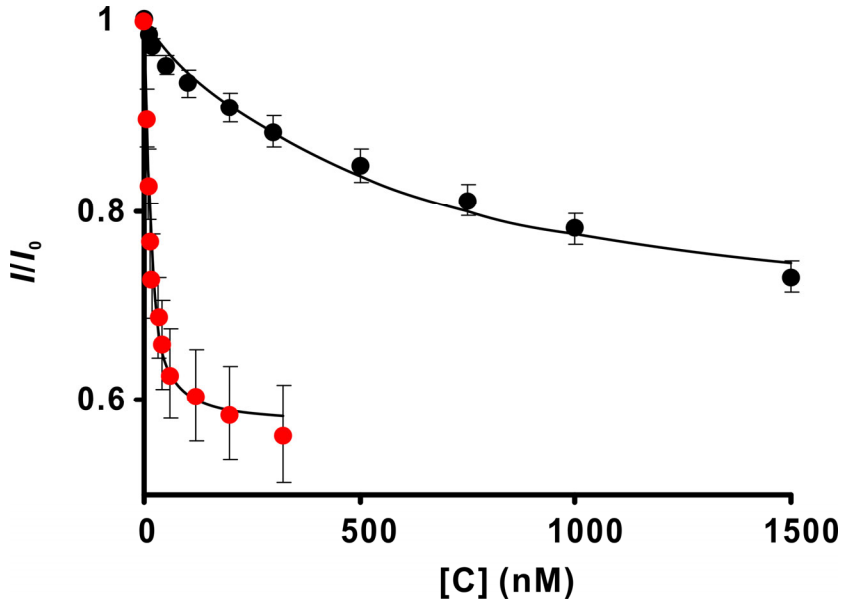

Figure S7. Isotherm curves for the fluorescence titration of **hnf-1^2** (20 nM, red) and **hnf-1** (100 nM, black) with duplex **C** in the presence of 150 mM NaCl: the observation wavelengths were 518 and 516 nm for **hnf-1^2** and **hnf-1**, respectively.

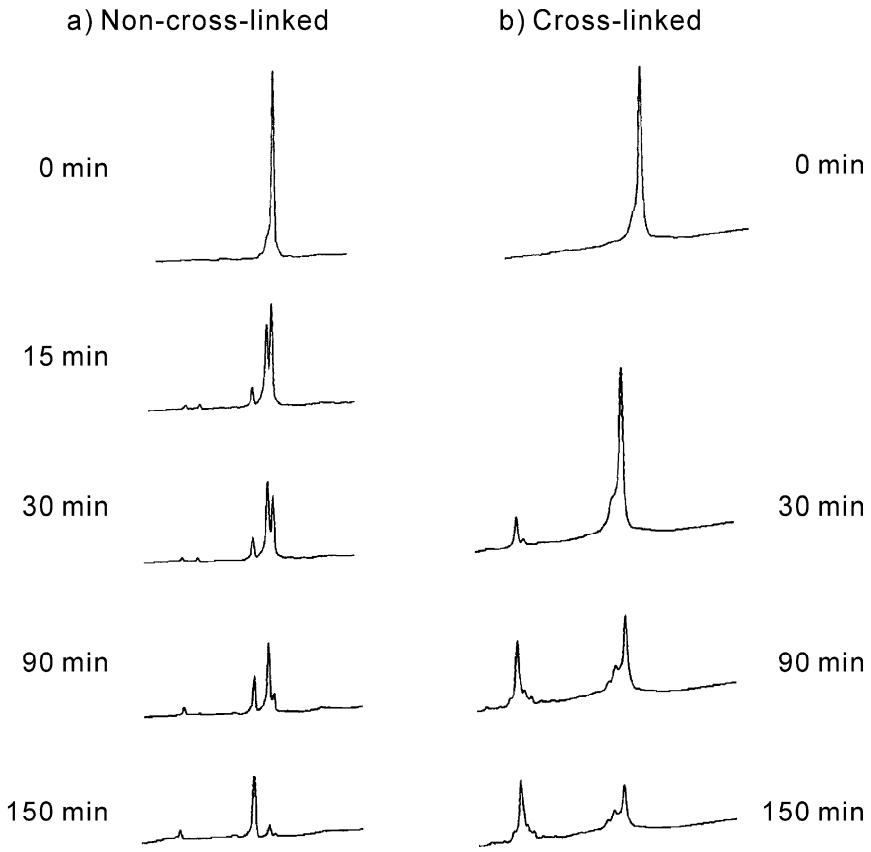

Figure S8. HPLC profiles of a) nonlabeled **qk-1** (50 μM) and b) nonlabeled **qk-1^1** (50 μM) after enzyme digestion with trypsin (0.5 μM) from 0 to 150 min.