Control of Peptide Activity

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DNA-Controlled Reversible Switching of Peptide Conformation and Bioactivity**

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Conformational switching is a key biological process that modulates the function of proteins in protein–protein interaction networks. The importance of this process in cellular signaling and the prospect of interfering in signal transduction pathways at controlled time points have stimulated research in the design of switchable peptide conjugates.[1] In analogy to cellular signaling, the reception of an external stimulus is envisioned to change the conformational state of the peptide, and thus its biological activity. In many cases peptide modifications have been selected that undergo conformational reorganization upon changes of reagent, solvent, pH, metal-ion concentration, or temperature.[2] While of high interest to investigations of protein folding, these methods have limited utility as far as switching of bioactivity under cell-compatible conditions is concerned. Towards this end, grafting of photoisomerizable chromophores to specific sites of a peptide offers interesting opportunities.[1a,3] The completeness of photoswitching is an important issue which is difficult to achieve with the commonly employed azobenzene-based photoswitches.[4] DNA hybridization has recently been used to control the activity of an engineered protein.[5] Herein we present a novel concept of controlling the conformational state of a peptide ligand at physiological conditions. It will be shown that nucleic acid hybridization can act as a trigger to deliberately reversibly increase or decrease the affinity of a peptide to a protein involved in cellular signaling.

The peptide of interest is equipped with DNA-analogous noncomplementary peptide nucleic acid (PNA) arm segments, which will flank the C and the N termini (Figure 1).[6] Accordingly, addition of complementary DNA to PNA-peptide chimera **A** will lead to the formation of double-strand complexes **B**, **C**, and **D**. The double-helical segments in **B**, **C**, and **D** serve as constraints to limit the conformational flexibility of the embedded peptide. Depending on the DNA template, various geometries may be realized, from stabilization of loop conformations in **B** to enhancements of the proclivity to adopt extended conformations in **D**. According to this scheme DNA acts as a structural template that can either activate or deactivate PNA-peptide chimera **A**. An additional attractive feature of this concept is that it may prove feasible to use cell-endogenous mRNA as a stimulus of activation/deactivation processes.

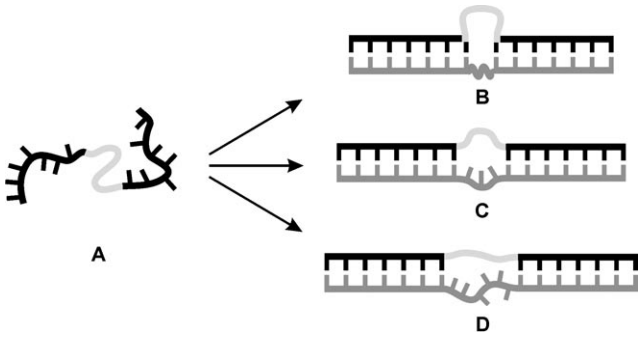

Figure 1. Hybridization-directed control of peptide conformation. The addition of complementary or partially complementary DNA to nucleic acid peptide chimera **A** results in the formation of duplexes **B**–**D**. Flexible regions are indicated by wavelike representation. (Cartoons illustrate possible conformations rather than defined structures.)

In a model study, we chose to explore switchable binding of a phosphopeptide to the Src-SH2 domain.[7–9] The tyrosine kinase activity of Src is regulated by its SH2 domain, which can bind an internal phosphotyrosine (pTyr) peptide segment.[10] Crystal structure analysis of Src-SH2 complexed with pTyr-containing peptides reveal that the peptide is bound in an extended conformation.[11] We expected that constraints that stabilize the extended conformation will lead to increases of binding activity. Decreases of binding activity were expected for cases in which the phosphopeptide is forced to accommodate a loop conformation.

The design of switchable Src-SH2 binders was guided by the crystal structure of the known high-affinity binder Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Tyr-Leu complexed to Src-SH2. This showed tight contacts to pTyr and isoleucine as well as to the glutamine Gln(−1) residue at the N terminus of the preferred recognition motif (underlined).[11] We therefore decided to explore conformational switching of the pentapeptide Gln-pTyr-Glu-Glu-Ile. The first step was to assess the stability of the chimera–DNA complexes. Three PNA-phosphopeptide-PNA chimeras featuring 7-mer (**1**), 8-mer (**2**), and 9-mer arm segments (**3**) were synthesized by linear solid-phase peptide synthesis (Figure 2, see also the Supporting Information). The PNA-phosphopeptide chimeras were hybridized with DNAs **4a**–**4i**, which were fully complemen-

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

H-Gly-Lys-gcgtata-Gln-pTyr-Glu-Glu-Ile-ccaatag-Lys-Gly-NH_2 (**1**)
H-Gly-Lys-cgcgtata-Gln-pTyr-Glu-Glu-Ile-ccaatagc-Lys-Gly-NH_2 (**2**)
H-Gly-Lys-tcgcgtata-Gln-pTyr-Glu-Glu-Ile-ccaatagca-Lys-Gly-NH_2 (**3**)

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        |||||||||                  |||||||||
3'-AGCGCATAT-          X        -GGTTATCGT-5' (4a-i)
   3'-GCATAT-          X        -GGTTAT-5' (5a)

a:X=-   c:X=GC    e:X=GACT    g:X=GACTGA
b:X=C   d:X=GAC   f:X=GACTG   h:X=GACTGGTA
i:X=GACTGGTAAT
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Figure 2. Chimera–DNA complexes used in this study. Lowercase letters indicate PNA bases, uppercase letters indicate DNA bases; amino acids are noted in three-letter code where pTyr refers to phosphotyrosine. Terminal lysine residues were included for solubility reasons.

tary to the 9-mer arm segments and contained none or additional 1–6, 8, and 10 nonpaired nucleotides opposite to the peptide part. All studied chimera–DNA complexes showed sigmoid melting curves, which indicates cooperative base-pairing (see Figure S1 in the Supporting Information). Two trends became apparent: first, thermal stability decreased with increasing number of nonpaired nucleotides (i.e. within one row, Table 1); and second, thermal stability increased with increasing length of the PNA arms (i.e. within one column, Table 1). The melting temperatures $T_M \geq 40\,^\circ C$ were considered sufficient to provide structural integrity in protein-binding experiments.

Table 1: Melting temperatures (in °C) of the complexes formed by the PNA-peptide-PNA chimeras (**1**–**3**) with the corresponding DNAs **4**.

	4a	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**4h**	**4i**
1	60	49	46	41	44	46	44	42	41
2	65	56	52	53	50	53	51	51	50
3	67	59	56	56	56	56	55	54	51

The affinity of PNA-phosphopeptide chimera **2** and its DNA complexes to the Src-SH2 domain was evaluated by means of a competition fluorescence polarization assay originally developed by Lynch and co-workers.[12] Briefly, the use of the fluorescently labeled binding peptide FAM-Gly-pTyr-Glu-Glu-Ile-Ala-NH_2 (**6**; $K_d = 0.24$ μM) allowed the determination of the peptide fraction bound to protein at different chimera concentrations. Noncomplexed chimera **2** inhibited binding of the reference peptide **6** with an IC_{50} value of 3.4 μM (Table 2). Addition of 3 equivalents of DNA **4a** lacking nonpaired nucleotides reduced the affinity to the SH2 protein domain, as evidenced by the increased IC_{50} value of 6.9 μM. All tested DNAs with more than one nonpaired nucleotide opposite to the peptide (**4c**–**4i**) conferred significant increases to the Src-SH2 affinity of phosphopeptide **2**. The maximum DNA-triggered affinity increase was reached with DNA **4g** ($IC_{50} = 0.5$ μM) bearing six nonpaired nucleotides opposite to the peptide part. The induced differences in IC_{50} values span more than one order of magnitude which should allow to switch from nearly no inhibition to nearly complete inhibition of binding.

Table 2: IC_{50} values (in μM) with estimated errors (± in μM) of chimera **2** and complexes with DNAs **4a**–**4i**.

	2	**2·4a**	**2·4b**	**2·4c**	**2·4d**	**2·4e**	**2·4f**	**2·4g**	**2·4h**	**2·4i**
IC_{50}	3.4	6.9	2.9	1.0	0.7	0.7	0.6	0.5	0.6	0.6
Error	0.4	1.0	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2

DNA-triggered switching was also feasible with the shorter PNA-phosphopeptide **1**. The extent of inhibition obtained under given conditions can be conveniently assessed by means of fractional inhibition ($f_i = 1 - (\text{fraction bound reference } \mathbf{6}) = \text{fraction bound } \mathbf{1} \text{ or complex } \mathbf{1{\cdot}4}$). Figure 3 shows that 1.25 μM chimera **1** inhibits 18% of the interaction between Src-SH2 (700 nM) and the FAM-labeled reference peptide **6** (20 nM). Addition of 1 equivalent of activating DNA **4g** released reference peptide **6** almost quantitatively into solution resulting in an increase of inhibition to 95%. In contrast, addition of deactivating DNA **4a** to **1** reduced fractional inhibition from 18% to 7%. Control experiments performed with the unlabeled peptide Ac-Gln-pTyr-Glu-Glu-Ile-NH_2 (**7**) as competitive binder revealed that neither DNA **4a** nor DNA **4g** directly acted as an allosteric regulator on Src-SH2 (see Figure S5 in the Supporting Information).

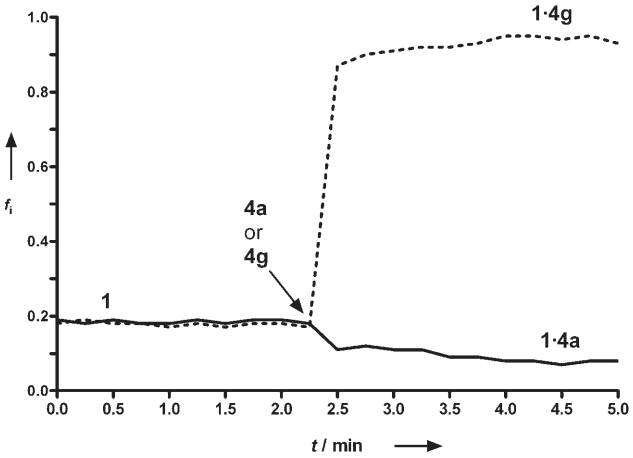

Figure 3. Switching of fractional inhibition (f_i) of the binding of peptide **6** to Src-SH2 mediated by chimera **1** through addition of DNAs **4a** (solid line) and **4g** (dashed line). The arrow indicates the point of DNA addition. The experiments were reproduced with DNA featuring a long overhang. The f_i values were calculated from fluorescence anisotropy of **6** measured at excitation and emission wavelengths of 485 nm and 525 nm, respectively. Conditions: 1.25 μM **1** in buffer (20 mM NaH_2PO_4, 100 mM NaCl, 2 mM 1,4-dithiothreitol (DTT), pH 7.4, 0.1% bovine serum albumin (BSA)) containing 20 nM **6** and 700 nM GST-Src-SH2.

It was the chief aim of this study to explore whether DNA hybridization allows for reversibility of switching. We reckoned that strand-exchange reactions could provide for the required reorganization of chimera–DNA complexes. One example is shown in Figure 4. The complex of chimera **1** with DNA **5a** was designed to have low Src-SH2 affinity as a result of the omission of unpaired nucleotides. As expected, **1·5a** proved inefficient in inhibiting protein binding of reference peptide **6** at a concentration of 1.25 μM. The deactivating DNA **5a** in complex **1·5a** was fashioned to allow replacement by activating DNA **4g**. This strand exchange was expected to be fueled by the formation of two additional stable base pairs in duplex **1·4g**. Indeed, addition of DNA **4g** to complex **1·5a** led to a dramatic increase of the binding affinity of chimera **1**.

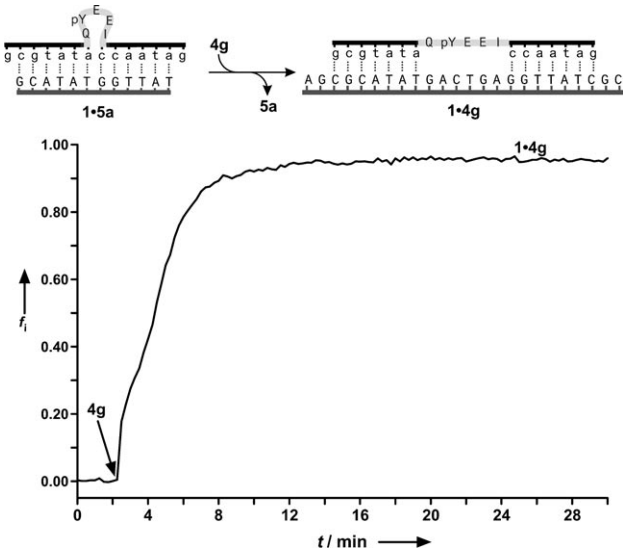

Figure 4. Activating low-activity peptide conjugate **1**·**5a** by strand exchange to furnish high-activity conjugate **1**·**4g**. The experiment was reproduced with DNA featuring a long overhang. Fractional inhibition (f_i) values were calculated as described in Figure 3. Conditions: 1.25 μM **1**, complexed with 2 μM **5a** in buffer (20 mM NaH_2PO_4, 100 mM NaCl, 2 mM DTT, pH 7.4, 0.1% BSA) containing 20 nM **6** and 700 nM GST-Src-SH2, addition of 10 μM **4g** after 135 s.

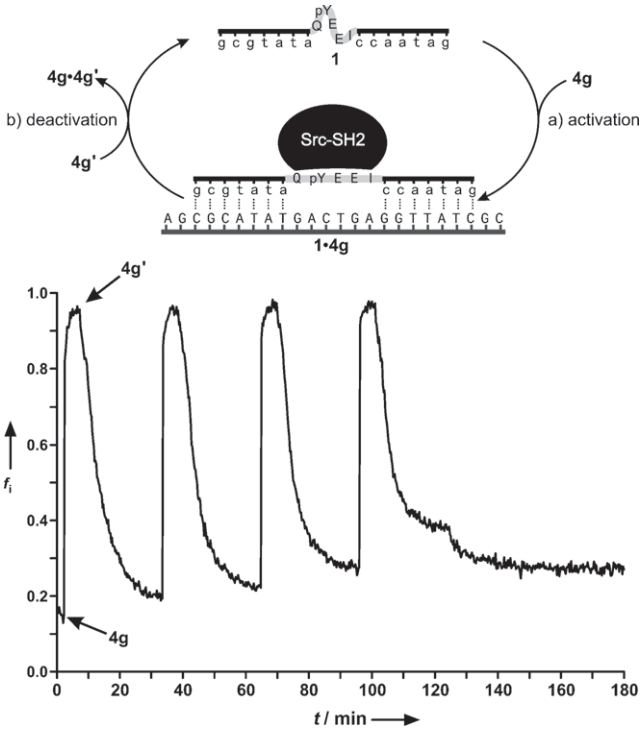

Figure 5. Reversible switching of peptide activity by alternate addition of DNAs **4g** and **4g′** to chimera **1**. Fractional inhibition (f_i) values were calculated as described in Figure 3. Conditions: 1.25 μM **1** in buffer (20 mM NaH_2PO_4, 100 mM NaCl, 2 mM DTT, pH 7.4, 0.1% BSA) containing 20 nM **6** and 700 nM GST-Src-SH2 followed by alternate addition of increasing amounts of DNAs **4g** and **4g′** starting with 1.25 μM **4g** ending up with 20 μM **4g′**. To complete switching additional 5 μM **4g′** was added after 125 min.

DNA **4g** served as a powerful activator, and strand exchange resulted in an increase of inhibition to 96%.

An example of the reversible switching of peptide activity is illustrated in Figure 5. First chimera **1** was treated with DNA **4g**. The resulting complex **1**·**4g** had a high binding affinity for Src-SH2 and hence conferred high fractional inhibition ($f_i = 0.96$). The terminal overhang by four nucleotides and the six nonpaired internal nucleobases in complex **1**·**4g** were targeted by DNA **4g′**, which is fully complementary to **4g**. Thus, addition of **4g′** to **1**·**4g** released **1** while **4g** was captured in complex **4g**·**4g′**.[13] Accordingly, fractional inhibition returned to a low value ($f_i = 0.20$). The two switching processes a) activation upon addition of **4g** and b) deactivation upon addition of **4g′** could be repeated several times. The rapidly occurring on-switching process (< 2 min) involves very fast hybridization (expected to occur within milliseconds)[14] and the displacement of the protein-bound peptide **6** which is in a dynamic on–off equilibrium.[15] In contrast, off switching requires more time (≈25 min) owing to a comparably slow strand-exchange reaction. Figure 5 shows four subsequent switching events and even more are possible (see Figure S6 in the Supporting Information).

There are many examples in which DNA serves to control conformational states of DNA- and RNA-based materials.[16] For example, DNA hybridization has been used to open and close DNA tweezers,[13] to direct DNA walking,[17] and to regulate the activity of aptamers, ribozymes,[18] and DNA-binding proteins.[19] Recently, DNA hybridization has been employed to control the activity of DNA–protein conjugates through mechanical tension.[5] The proteins guanylate kinase and protein kinase A required modification, site-directed mutagenesis, and bivalent attachment of a 60mer DNA control element. Reversibility of DNA-induced switching has not been demonstrated. Rather than changing the conformational state of an engineered protein, we chose to reversibly increase or decrease the activity of a peptide as ligand of a wild-type protein. Such an approach should allow applications in cell biology. We showed for the first time that DNA can trigger both decreases and increases of the protein-binding affinity of PNA-peptide conjugates such as **1** and **2**. Decreases induced by DNA **4a** may be caused by forcing the peptide in **1**·**4a** to accommodate a loop conformation not favored by Src-SH2. The enhancements of binding affinity observed upon addition of DNA such as **4g** to PNA-peptide chimeras can be explained by considering the tendencies to adopt the required extended conformation. Single-stranded chimeras are flexible molecules that can also exist in linear conformations. The IC_{50} value of 3.4 μM of chimera **2** is lower than that of the unlabeled pentapeptide Ac-Gln-pTyr-Glu-Glu-Ile-NH_2 (**7**; $IC_{50} = 5.5$ μM). Thus, negative effects of the PNA appendices in chimera **2** on the binding affinity of the peptide part can be excluded. Hybridization of the PNA arms restricts the conformational freedom of the peptide, which in activated complexes such as **1**·**4g** and **2**·**4g** may adopt the required extended conformation more readily than in single-stranded chimera. However, the single-stranded segments in these complexes are still flexible, and it can be imagined that more rigid double- or triple-helical spacers confer even higher binding activities. To explore the activating effect of the single-stranded DNA moiety we studied binding of chimera **3**

with the PNA arm segments complexed to "unconnected" DNA oligomers 5′-TGCTATTGG-3′ and 5′-TATACGCGA-3′. The IC_{50} value of 1.0 μM suggests that double-strand formation of each arm results in an increase of binding activity albeit not to the extent observed with "connected" DNA **4g**. It can be speculated that coulomb repulsion between the negatively charged duplex ends may provide an alternative but less efficient means to enhance the population of molecules adopting an extended conformation.[20]

The magnitude and the speed of the DNA-controlled changes of binding affinity should be sufficient to control protein–protein interactions involved in cellular signal transduction networks. Once chimeras such as **1** (or DNA complexes such as **1**·**5a**) have been brought into a cell (e.g. by microinjection, electroporation, or transfection), we envision intracellular RNA-triggered activation and deactivation of the peptide activity as an interesting opportunity to keep a signaling protein such as Src under the control of a particular endogenous RNA molecule. However, prior to such studies, we will investigate the ability of nucleic acid–peptide chimera such as **1** to regulate enzymatic activity in cell lysates.

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[20] One may also take into account that the PNA-phosphopeptide chimeras acquire negatively charged phosphodiester groups upon DNA hybridization. Multiphosphorylated peptides have been reported to interact with SH2 proteins: T. Gilmer, M. Rodriguez, S. Jordan, R. Crosby, K. Alligood, M. Green, M. Kimery, C. Wagner, D. Kinder, P. Charifson, *J. Biol. Chem.* **1994**, *269*, 31711 – 31719. However, the introduction of phosphodiester groups upon annealing of DNA **4a** and **5a** reduced the peptide-binding activity, which is difficult to understand if conformational effects were absent. It is conceivable that DNA hybridization can exert both conformational and charge effects and that both can be utilized to switch the binding activity of PNA-peptide chimeras.

