Host-Guest System Based on Collagen-Like Triple-Helix Hybridization

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Host-Guest System Based on Collagen-Like Triple-Helix Hybridization

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A strategy inspired by tweezer receptors has been employed to develop a new host-guest system. The hybridization into a collagen-like triple helix is the driving force for the recognition that occurs with high affinity and selectivity. Several systems have been screened to find the best host-guest pair and this strategy may be implemented for tag fused protein recongnition.

The design and synthesis of molecular receptors that recognize specific peptide sequence is highly attractive since it can provide insights into the principles that govern molecular recognition processes. Moreover, it can be useful for many biological applications such as the development of synthetic molecules able to inhibit the effect of natural compounds or to disrupt protein-protein interactions.[1] The development of selective sensors is a field that involves molecular recognition principles and requires a smart rational design. In most cases, molecular receptors functionalized with fluorophore are metal complexes, metal-ligand interaction being potentially a versatile driving-force for peptide recognition since it can work effectively under physiological aqueous conditions.[2] However, lack of selectivity can be observed due to presence of many charged species in cell media.[3] Another approach involving molecular recognition of peptides consists of recognizing isolated peptides by forming specific structures such as coiled coil[4] and leucine zipper[5] or using tweezer receptors.[6,7] Tweezer receptors, made of two peptidic strands attached to a template, can bind selectively peptides through the formation of β sheet-like interactions, i.e. hydrophobic interactions and hydrogen-bonding. In most cases, the pre-organization afforded by the template lowers the entropy penalty associated with the folding event and thus increases the host-guest affinity. Furthermore, depending on the nature of the template, additional interactions can be introduced to increase the selectivity and/or the affinity of the host molecule toward the guest peptide.[7] However, for water soluble molecular tweezer/peptide pairs, the affinities are noticeably lower than in organic solvents, which limits their bio-applications.[8]

In this work, we describe a new strategy inspired by tweezer receptors, where the two peptidic moieties attached to a scaffold are designed to hybridize with the guest peptide into a collagen-like triple helical structure. The hybridization should afford not only an additional driving-force for the binding event but also a way to tune the selectivity of the host molecule toward the guest peptide depending on the sequence. Templates have already been used to nucleate triple helices but as far as we know, this is the first time that only two of the three strands are linked to the template.[9] Thus, a new strategy is required to characterize the formation of the expected triple helices.

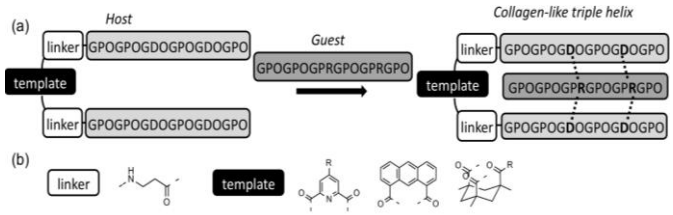

Figure 1. (a) Schematic representation of the new host-guest system leading to the formation of a heterodimeric collagen-like triple helical structure. The dashed lines show interstrand electrostatic interactions between the carboxylates of host and the guanidiniums of the guest. (b) Structure of the linker and templates studied.

The peptide sequence is designed by analogy with that from collagen with a repetitive triad G-X-Y, (G = glycine), G-P-O being the most stabilizing tripeptide unit for triple helix formation (P = proline, O = hydroxyproline) (Figure 1).[10] In addition, to promote interstrand interactions, aspartic acids (D) and arginines (R) have been introduced in some triads in X and Y position of the host peptides and guest peptide respectively.[11] By this way, it was expected that charge repulsions could occur into the homo-oligomers, thus decreasing the stability of these structures. In host molecules, two identical peptides built on the collagen model were directly assembled on templates or *via* linkers. Templates were chosen to orientate the attached peptides in the same direction in order to nucleate the triple helix formation. This has been achieved using 2,6-diamidopyridine. Thanks to the pseudo conjugation of this moiety, (i) electrostatic repulsions between the endocyclic nitrogen and aromatic carbonyls and (ii) hydrogen bounding between this nitrogen and amide

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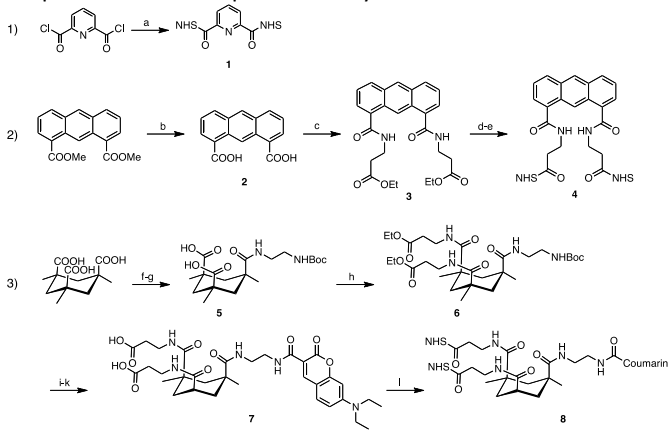
protons favour an anti conformation of the aryl-amide bounds. Alternatively, templates derived from 1,8-diamidoanthracene and bearing β-alanines as linkers were also studied. Finally, a scaffold derived from cis,cis-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (Kemp's triacid, KTA) was synthesized with β-alanines. In this case, the "chair" conformation with the three functional groups in axial position, parallel to each other, can facilitate interactions of the two peptides chains (Figure1). In addition to their propensity to pre-organize the peptides, these templates can also be functionalized with a fluorophore at the opposite side of the peptides. The templates bearing linkers were synthesized in solution as activated esters as described in Scheme 1. The final coupling between these succidimyl esters (compounds **1**, **4**, **8**, scheme 1) and the deprotected peptides synthesized by conventional solid phase peptide synthesis were accomplished in solution. The crudes were purified by HPLC and the Table 1 summarizes the peptides studied. In templated collagen-like triple helices, the intrinsic propensity of the template to orientate the peptides to the same direction in addition with a subtle balance between steric hindrance and flexibility afforded by the linker to allow the required one-residue stagger, are key parameters involved in their further stabilization. However, the difficulty to estimate the contribution of these parameters led us to screen the three templates for peptides having six triads. Circular dichroism (CD) was used in order to observe the polyproline II helix signature and to measure the melting temperature of the triple helices by thermal denaturation.



Scheme 1. Synthesis of the ready-to-use templates. a) NHS, DIEA, THF, 12h, r.t., 84%. b) LiOH, THF/MeOH/H_2O, r.t., 4h, 90%. c) H-βAla-OEt, PyBOP, DIEA, DMF, 12h, r.t., 88%. d) LiOH, THF/MeOH/H_2O, r.t., 4h, 90%. e) NHS, WSCI•HCl, DIEA, DMF, r.t., 12h, 68%. f) Sublimation, 90%. g) $H_2N(CH_2)_2NHBoc$, DIEA, DMF, 6h, r.t., 88%. h) H-βAla-OEt, HBTU, HOBt, DIEA, DMF, 12h, r.t., 56%. i) LiOH, THF/H_2O 1:1, r.t., 1h, 98%. j) TFA/DCM 1:1, r.t., 30 min, 82%. k) DMACA-SE, DIEA, DMF, r.t., 12h, 45%. l) NHS, WSCI•HCl, DIEA, DMF, r.t., 12h, 84%.

Entry	Sequence	Abbreviation
1	Ac-$(GPO)_2$-G**D**O-GPO-G**D**O-GPO-NH_2	AcD-6o
2	Ac-$(GPO)_2$-GP**R**-GPO-GP**R**-GPO-W-NH_2	AcR-6o
3	Ac-GP**R**-$(GPO)_2$-GP**R**-GPO-GP**R**-GPO-NH_2	AcR-7o
4	Ac-GP**R**-$(GPP)_2$-GP**R**-GPP-GP**R**-GPP-NH_2	AcR-7
5	Ac-PGG**R**PGPGPPGP**R**PGPP**R**PPG-NH_2	AcR-7s
6	Py-$[(GPO)_2$-G**D**O-GPO-G**D**O-GPO-$NH_2]_2$	R1-6
7	Anth-$[\beta Ala$-$(GPO)_2$-G**D**O-GPO-G**D**O-GPO-$NH_2]_2$	R2-6
8	KTA-Coum-$[\beta Ala$-$(GPO)_2$-G**D**O-GPO-G**D**O-GPO-$NH_2]_2$	R3-6
9	Anth-$[\beta Ala$-G**D**O-$(GPO)_2$-G**D**O-GPO-G**D**O-GPO-$NH_2]_2$	R2-7

Table 1. Sequences and abbreviations of the templated peptides and template free peptides. The charged amino acids are in bold character.

In absence of the guest, templated peptides show large negative peak at ca. 200 nm and a small positive peak at ca 225 nm characteristic of PPII conformation at 5°C (data not shown). R2-6 shows additional bands at 250 and 260 nm. When unfolding studies were performed by CD, host molecules themselves showed a single cooperative transition enabling the determination of melting temperatures (Tm) that depend on the template (Table 2, Figure S2). This was not observed for the free AcD-6 (Table 2, Figure S1) at the same concentration in peptide, which clearly demonstrates that templates strongly participate to the stabilization of the triple helical structures. From the three systems tested, the anthracene template led to the most stable self-assembled helical structure with a Tm of 27°C (Table 2). It is likely that, with the pyridine, the direct attachment of the peptides to the pyridine probably leads to a bulkier structure where the steric hindrance is greater and disfavour the formation of homo-oligomers (Tm of 23°C, Table 2).

Upon mixing the different hosts with the guest peptide AcR-6o in a 1:1 ratio, characteristic signals for collagen-like triple helices were observed by CD. Unfolding studies show in each case a single transition suggesting that only one species is formed in solution (Table 2, Figure 2, Figure S2). In all cases, the formed triple helix were found to be more stable than the 2:1 AcD-6/AcR-6o mixture of the template free peptides (Tm 20°C, Table 2, Figure S1 and S2) confirming the stabilization of the structures by the template effect. Complexes involving the three hosts (R1,2,3-6) exhibit melting temperatures slightly higher than the host homo-oligomers triple helices (Table 2). Among the factors that can explain the stabilization of the heterodimeric triple helices, electrostatic interactions may contribute to some extent.

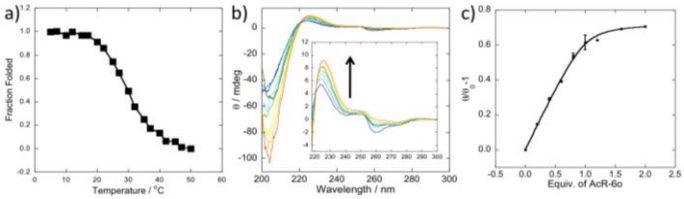


Figure 2. Thermal denaturation of R2-6/AcR-6o 1:1 mixture (a) and titration experiment monitored by circular dichroism (b) and (c). The titration was performed at 20°C at 5 μM in phosphate buffer (10 mM, pH = 7.2) and (c) shows the titration curve monitored at 225 nm. The inset of (b) shows a zoom of the monitored band at 225 nm. Error bars represent the standard deviation.

Peptides	Melting temperature T_m*	ΔT_m	Isobestic point (nm)	Apparent Binding constant (M^{-1})
AcD-6o	< 5°C			
AcR-6o	< 5°C			
AcD-7o	< 5°C			
AcR-7o	< 5°C			
AcR-7	< 5°C			
AcD-6o/AcR-6o 2:1	20°C			
AcD-7o/AcR-7o 2:1	30°C			
R1-6	23°C	4°C		
R1-6/AcR-6o 1:1	27°C		222	$3.1 \pm 2.0\ 10^{5}$ [a]
R2-6	27°C	3°C		
R2-6/AcR-6o 1:1	30°C		221	$5.8 \pm 2.5\ 10^{6}$ [a]
R3-6	25°C			
R3-6/AcR-6o 1:1	27°C	2°C	219	$1.1 \pm 0.6\ 10^{5}$ [a] $1.0 \pm 0.4\ 10^{5}$ [b]
R2-7	30°C			
R2-7/AcR-7o 1:1	40°C	10°C	218	$7.5 \pm 2.5\ 10^{6}$ [a] $8.5 \pm 4.8\ 10^{6}$ [b]

Table 2. Summary of the parameters of the template free peptides, the templated peptides and mixtures with guest peptides. For binding constants, host concentrations were 5 µM in 10 mM phosphate buffer (pH 7.2) excepted for R3-6 (21 µM) and R2-7 (1 µM). * Melting temperatures are given with an estimated error of 2°C. [a] Apparent binding constant measured by CD. [b] Apparent binding constant measured by fluorescence spectroscopy (SI).

The difference (ΔTm) between the melting temperatures of host homo-oligomers and host-guest heterodimers is in the 2-4°C range, with the most stabilizing effect of the hybridization into heterodimer for R1-6 (ΔTm = 4°C) (Table 2). In that case, the small distance between the two peptidic strands probably enhances electrostatic repulsions in homo-oligomers, whereas in the heterodimer they may be shielded by the presence of the positively charged guest.In order to confirm the formation of the 1:1 host-guest complexes, titrations by CD and fluorescence were performed. Most importantly, this provides an estimation of the apparent binding constant associated to the triple helix formation. For CD and fluorescence experiments, host and guest peptides were mixed at several ratios, preheated and slowly cooled down to allow heterodimers formation (see SI). Because all hosts fold into triple helices, with a characteristic PPII signal (at ca. 200 and 225 nm), changes in the Cotton effect at 225 nm were monitored by CD upon increasing guest concentration (from 0 to 2 equivalents) to observe the equilibrium shift towards the expected host-guest complexes. The CD signal at 225 nm increased significantly and non-linearly with saturation after one equivalent and a clear isobestic point, suggesting that the binding event was effectively driven by host-guest hybridization into triple helix (Figure 2 and S2). The same trend was observed by fluorescence for R3-6 upon irradiation of the coumarin moiety: fluorescence intensity increases non-linearly, revealing changes in coumarin environment and thus modifications of the initial triple helices (Figure S7). The titration curves obtained were successfully fitted to 1:1 stoichiometry models confirming the formation of 1:1 host-guest complexes. These results are in agreement with thermal denaturation experiments where the melting temperatures of the host-guest mixtures were higher than the hosts triple helices. Binding affinities obtained for R1-6, R2-6 and R3-6 show the same trend as the melting temperatures: R1-6/AcR-6o and R3-6/AcR-6o (Tm = 27°C) have similar binding constant (~10^5 M^{-1}), whereas R2-6/AcR-6o (Tm = 30°C) has a higher binding constant (~ 10^6 M^{-1}) (Table 2).

From the experiments described above, it appears clearly that the anthracene template (R2-6) is the best to induce the formation of a stable host-guest triple helix. Therefore, we further focused on this system. A host peptide R2-7 having seven triads instead of six and its corresponding guest peptides AcR-7o were synthesized. GDO and GPR triads were added at the N-terminus of the host and guest peptides respectively, to induce additional electrostatic interactions that could destabilize the host self-assembled triple helices (repulsions) but would stabilize the host-guest triple helices (attractions). Thermal unfolding studies using CD showed again single transitions for R2-7 and R2-7/AcR-7o (1:1), with melting temperatures of 30°C and 40°C respectively (Figure 3a and S4). Addition of the GDO triad increased moderately the stability of the host triple helix (with Tm increasing from 27°C for R2-6 to 30°C for R2-7) whereas the host-guest triple helix was much more stabilized (from 30°C for R2-6/AcR-6o to 40°C for R2-7/AcR-7o). This observation confirms that electrostatic interactions between negatively charged host residues and positively charged guest residues may play a crucial role for the triple helix stabilization. Titration experiments performed by CD at 20°C showed the same trend that of R2-6/AcR-6o complex: upon addition of AcR-7o, a non-linear increase of the signal at 225 nm is observed (Figure 3b,c) and a clear isobestic point is again observed. Indeed, the curve fitting for a 1:1 complex leads to a binding constant which is 1.5 fold stronger that the R2-6/AcR-6o system. This was confirmed by a titration performed by fluorescence upon excitation of the anthracene at 360 nm (Figure 3d,e). To extend the scope of the recognition to genetically encodable sequences, non-encodable hydroxyprolines have been substituted by prolines in the guest sequence leading to the peptide AcR-7. Titrations by CD, fluorescence and UV spectroscopies were performed (Figure S5 and S6). From the fitting of the curves, it appears that AcR-7 binds R2-7 less tightly than AcR-7o does, but substituting hydroxyprolines for prolines does not affect the binding constant to a large extent. Consequently, genetically encodable sequence can be efficiently recognized by this strategy. Finally the selectivity of the recognition was assessed with a guest peptide having a randomized sequence AcR-7s; bearing the same residues as AcR-7 but at different positions. The titrations of R2-7 with AcR-7s by CD, UV and fluorescence did not show saturation curves and weak I/I_0 signals were observed upon addition of AcR-7s (Figure S6), suggesting that the guest peptide is not recognized. For this peptide, the repetitive triad GXY is no more present which more likely prevents the formation of the expected triple helix. Thus, this system leads to high selectivity for peptide sequences having a GXY repetitive triad and a high content of imino residues at X and Y positions. In this new host-guest system based on triple-helix hybridization, it has been shown that the structure of

both template and linkers are important to avoid getting too stable triple helices made of self-assembled host peptides and to rather favour the formation of stable heterodimeric (host-guest) triple helices.

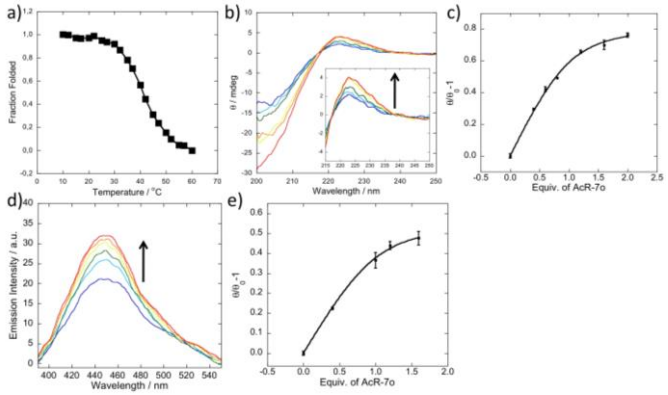


Figure 3. Caracterisation of the host peptide R2-7. a) Thermal denaturation R2-7/AcR-7o 1:1 mixture (100/100 µM) monitored by CD at 225 nm. b) CD spectra of a 1 µM solution of R2-7 upon addition of increasing concentration of AcR-7o revealing the formation of a 1:1 host/guest complex with a clear isobestic point observed at 218 nm and at 20°C. The inset shows a zoom of the monitored band. c) Titration curve monitored at 225 nm. d) Fluorescence spectra of a 1 µM solution of R2-7 upon addition of increasing concentration of AcR-7o. λ_{exc} = 360 nm, $slit_{exc}$ = $slit_{em}$ = 15 nm. e) Titration curve monitored at 450 nm. Error bars represent the standard deviation.

It appeared that 1,8-anthracene dicarboxylic acid with β-alanine as linkers allowed obtaining the most stable heterodimeric triple helices. Titration experiments confirmed the formation of 1:1 complexes, and allowed us to estimate for the first time the apparent binding constant involved in the hybridization into triple helix. More interestingly this strategy can be extended to recognize peptides having an encodable GPP repetitive triad, which opens the opportunity to apply this strategy for tag-fused protein recognition and in particular in bio-imaging experiments since a fluorophore can be added to the template.

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