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Protein Design and Folding: Template Trapping of Self-assembled Helical Bundles

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Abstract: An experimental system is described, permitting a detailed and systematic analysis of the factors governing self-assembly of amphipathic helices, e.g. to a four-helical bundle, a subject of major relevance for tertiary structure formation, protein folding and design. Following the Template Assembled Synthetic Proteins (TASP) approach, helices of different packing potential are competitively assembled in solution with a preformed two-helix TASP molecule, and after equilibration are covalently attached ('template trapping') via chemoselective thioether formation. The quantitative analysis of the individual TASP molecules by high performance liquid chromatography (HPLC) and electrospray mass spectrometry (ES-MS) allows the delineation of the role of complementary packing in helix bundle formation. The procedure established represents a general tool for the experimental verification of modern concepts in molecular recognition.

Keywords: complementary packing; protein design; protein folding; self-assembly; Template Assembled Synthetic Proteins; template trapping

INTRODUCTION

Protein *de novo* design aims to construct novel polypeptide chains which fold into a predetermined three-dimensional structure [1–4]. Whereas the major principles governing helical structure formation are understood well enough to allow fairly accurate predictions [5], the interaction of energetic factors, such as the hydrophobic effect, van der Waals packing, or Coulombic interactions determining the assembly of secondary-structure elements into characteristic folding units or tertiary structures are still to be elucidated on the molecular level, and represent challenging research topics in protein folding and design.

Approaches for the experimental verification of our present understanding of helical self-assembly and folding are, therefore, of fundamental importance. In this context, Harbury *et al.* [6] have studied the dimer–trimer–tetramer equilibrium perturbed by substitutions in the coiled-coil heptad sequence, and Kazmierski *et al.* [7] have applied crosslinking experiments to elucidate the dimerization behavior of coiled coil proteins. Kamtekar *et al.* [8] have shown a high rate of helix-bundle formation with random hydrophobic/polar patterning in the helix sequences. Several other groups have studied the assembly of helices induced by direct metal ion binding [9,10], or more indirectly, with the help of prosthetic groups [11]. In contrast to these three- or four-helical protein bundles, Fujita *et al.* have extended the self-assembly of polypeptides to the generation of helical monolayers [12,13]. Ghadiri *et al.* showed that by self-assembly of amphipathic building blocks, systems capable of self-replication can be generated [14].

Abbreviations: Abu, α-amino-butyric acid; ESI-MS, electrospray ionization mass spectrometry; RP-HPLC, reversed phase high performance liquid chromatography; TASP, Template Assembled Synthetic Proteins; TFE, 2,2,2-trifluoroethanol.

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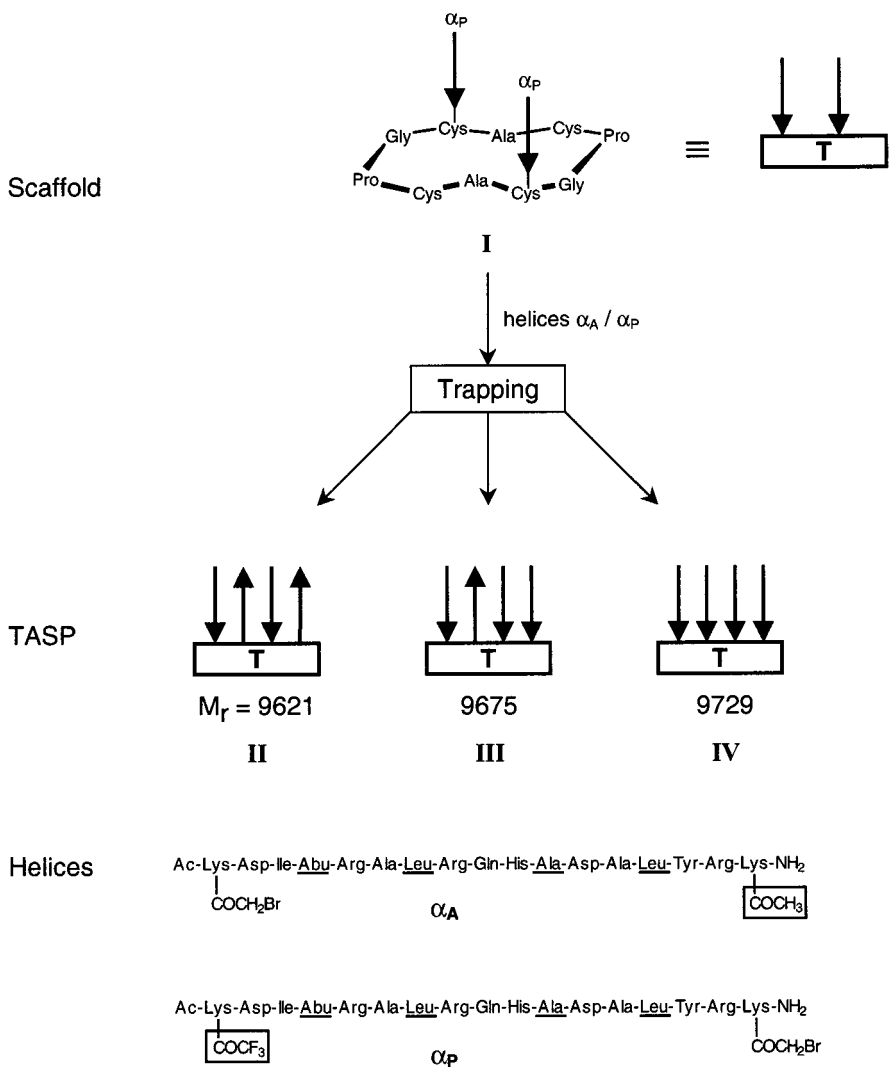
Despite these very substantial contributions, such experiments have often addressed special cases, and generally demonstrate the successful assembly of particular designed sequences without quantitative comparisons. An experimental system permitting a detailed and systematic analysis of the factors governing the formation of tertiary structure has not yet been elaborated.

MATERIALS AND METHODS

Synthesis of Building Blocks, α_A, α_P and Scaffold I (Scheme 1)

Helices α_P and α_A were prepared using Fmoc-based solid phase methodology [15,16] on a Rink-Amid MBHA resin applying HBTU activation; α_A was obtained after pNO_2Z deprotection of Ac-Lys(pNO_2Z)-Asp(O*t*Bu)-Ile-Abu-Arg(Pmc)-Ala-Leu-Arg(Pmc)-Gln-(Trt)-His(Trt)-Ala-Asp(O*t*Bu)-Ala-Leu-Tyr(*t*Bu)-Arg-(Pmc)-Lys(Ac)-resin with 2 M $SnCl_2$ in DMF and coupling with 2 equivalents of $BrCH_2COOSu$ in DMF/DIPEA; α_P was obtained after Dde deprotection (hydrazine) of Ac-Lys($COCF_3$)-Asp(O*t*Bu)-Ile-Abu-Arg(Pmc)-Ala-Leu-Arg(Pmc)-Gln(Trt)-His(Trt)-Ala-Asp(O*t*Bu)-Ala-Leu-Tyr(*t*Bu)-Arg(Pmc)-Lys(Dde)-resin and coupling with two equivalents of $BrCH_2COOSu$ in DMF/DIPEA. All peptides were purified by reversed phase high-performance liquid chromatography (RP-HPLC), and their integrity was confirmed by electrospray ionization mass spectrometry (ESI-MS). The cyclic template with two



Scheme 1 Building blocks and reaction scheme of the competitive trapping experiments (see also Plate 1). In the two sequences, core side-chains are underlined and mass markers are boxed.

different orthogonal protecting groups (Trt, Acm) was obtained as described earlier [17,18]. **I** was prepared by thioether formation [19] of template T with helix α_P and characterized by RP-HPLC and ESI-MS.

Trapping Reaction

The trapping experiments were performed in a degassed solvent mixture of 50% TFE in 0.1 M sodium phosphate, pH 8 under argon. The scaffold **I** (0.14 mM) and the corresponding quantities of helices (see Table 1) were preassembled at pH 4.5 for 30 min in 270 μL TFE/H_2O. The trapping reaction was started by addition of 30 μL 1 M sodium phosphate, pH 8 and monitored by RP-HPLC. After 30 h, no changes in the reaction profile were observed.

RESULTS AND DISCUSSION

In the present paper, we describe a general approach for accessing the structural and energetic factors governing the self-assembly process of peptides exhibiting secondary structures in solution by applying an extension of the template concept [3] in protein *de novo* design. Following the Template Assembled Synthetic Proteins (TASP) approach for the construction of artificial folding units, such as four-α-helical bundles [20–22], topological templates (i.e., regioselectively addressable cyclic peptides with two different protecting groups on the template) [17], are used to attach covalently pre-assembled helical bundles from solution. This procedure is called 'template trapping'. The competitive trapping of helical peptides on template molecules and subsequent product analysis allow the systematic investigation of factors, such as the role of the helix-macrodipole, internal packing or Coulombic interactions in four-helix bundle formation, providing valuable information for the *de novo* design of proteins [17].

Preliminary results from trapping experiments have shown that the helix macrodipol of sequentially identical helices did not significantly contribute to the energetic factors directing the parallel or antiparallel arrangement of neighbouring helices in a four-helix bundle [22]. Based on these results, we focus in the present study on the role of internal packing in the self-assembly of amphipathic helices. For this purpose, we start from a four-helical bundle TASP molecule called SymROP (Plate 1), consisting of four identical, antiparallel helices representing an idealized 222 symmetrical structure [23] derived from ROP (Repressor of Primer) protein [24]. ROP is characterized by an alternation of intercalating large and small residues in the hydrophobic core of the bundle along the helix axes, in positions a and d of the heptad motif [24]. The smaller side-chains (Ala, Cys, Thr, or Abu) pack between the tightly-spaced helix pairs (the monomers, in ROP), while the Leu or larger side-chains pack between the wider spaced helix pairs (between monomers in ROP) [23,25]. In Plate 1, the packing between two-helical TASP **I** and the competing helices α_A and α_P is illustrated by showing the first-order effect of alternating side-chain size.

Table 1 Distribution of the Trapped TASP Molecules **II–IV** in the Competitive Self-assembly of Three Different Helix Proportions (α_A, α_P) with Scaffold **I** (see Scheme 1 and Plate 1)

Proportion of helices α_A:α_P	1:1[a]		2:1[b]		1:2[c]	
TASP	st	ex	st	ex	st	ex
II	25	53	44	67	12	33
III	50	41	44	29	44	50
IV	25	6	12	4	44	17

[a] 1:1 ratio: 0.26 mM α_A, 0.26 mM α_P, and 0.13 mM **I**.
[b] 2:1 ratio: 0.26 mM α_A, 0.13 mM α_P, and 0.13 mM **I**.
[c] 1:2 ratio: 0.13 mM α_A, 0.26 mM α_P, and 0.13 mM **I**; 0.1 M sodium phosphate buffer, pH 8; 50% TFE.
st, statistically expected distribution (%) of products without preference for parallel or antiparallel orientation; ex, experimentally determined product distribution (%) (error limits ± 4%).

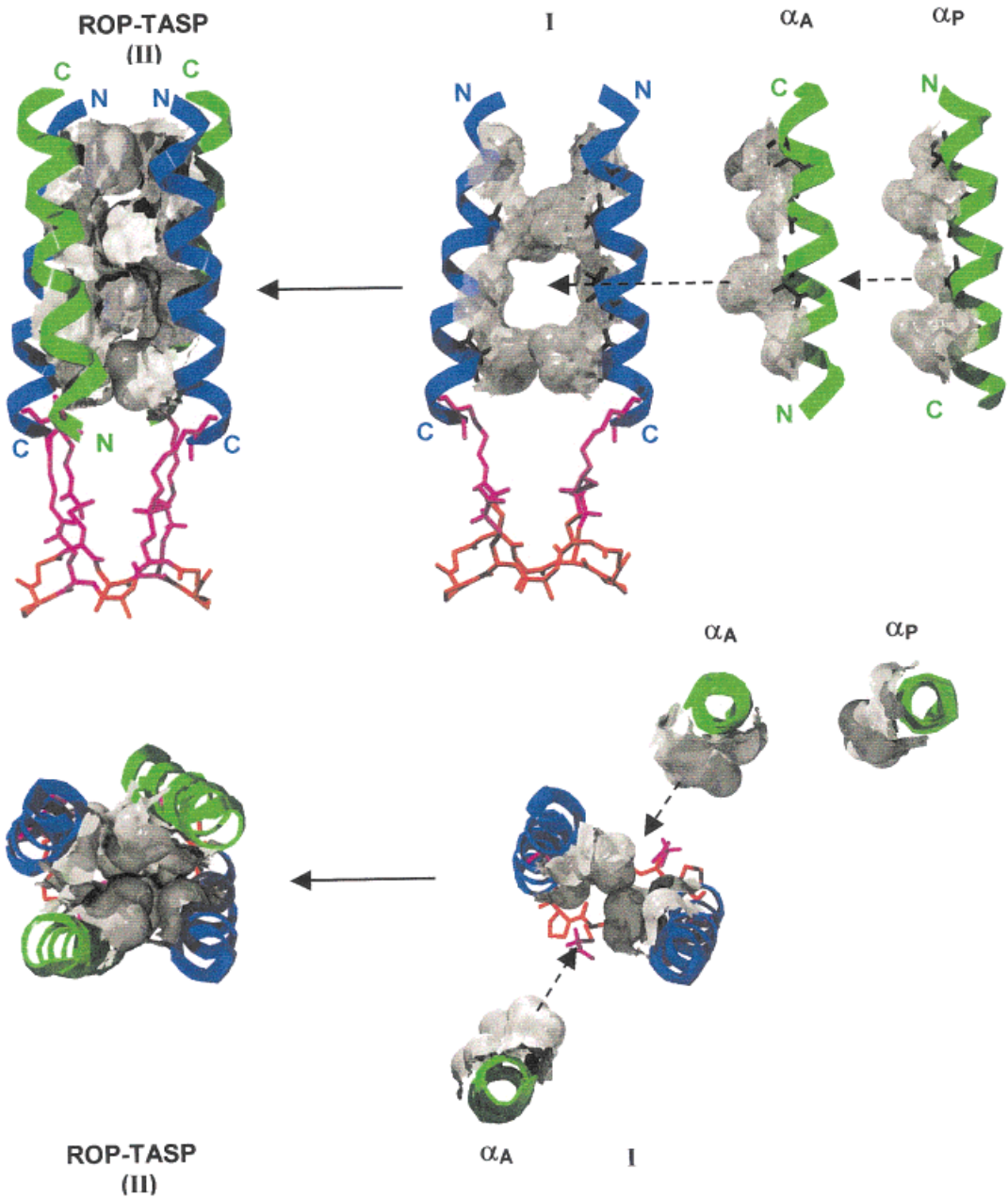


Plate 1 Ribbon representation of the association process of helices to the scaffold TASP **I** representing the complementary packing. Top: side view of the antiparallel ROP-TASP **II** (left) obtained after association of helix α_A to scaffold **I**. Bottom: top view of **II** and the competitive association of α_P and α_A with **I**. Helices of **II** are represented as blue ribbons, helices α_P and α_A as green ribbons. Surfaces of core side-chains are shown to highlight the complementarity of packing.

Optimal complementary packing can only be achieved in the antiparallel orientation of the helices, thus preferentially trapping two helices of type α_A. The parallel arrangement of the four helices (trapping of two α_P) would result in an energetically less favourable increase of total volume and of solvent-accessible surface. The effect of interhelical salt bridges was reduced by design in order to delineate the impact of core packing on the bundle formation; hydrophobicity is, of course, the same in both orientations. Furthermore, special attention was given to the optimization of the helix–helix interfaces [23] using all-atom contact analysis [26]. In order to experimentally verify the hypothesis, that side-chain packing matters to the energetics of helix assembly, we use for the study of helix self-association a starting TASP molecule as scaffold (**I**) which disposes two parallel helices (α_P) in diagonal arrangement on the template molecule (Scheme 1). The competitive assembly of helices α_A and α_P to the other two open attachment sites on this scaffold may result in a completely parallel (two helices of type α_P, **IV** in Scheme 1), antiparallel ($2\alpha_A$, **II**), or mixed ($1\alpha_A$, $1\alpha_P$, **III**) mutual arrangement in the final four-helical bundle TASP. As a major element of the trapping experiment, the individual helices α_A and α_P of identical amino acid sequence dispose their chemoselectively reactive group (α-bromoacetyl) either at the *N*- (α_A) or *C*-terminal (α_P) chain end, allowing either *N*- or *C*-terminal trapping of the helices on scaffold **I** (which has two reactive thiol functions) via thioether formation [19] (Scheme 1). In order to identify the individual four-helical bundle TASP of type **II–IV** by mass spectrometry after high performance liquid chromatography (HPLC) separation, the sequentially identical helices α_A and α_P carry mass markers ($COCH_3$ for α_A and $COCF_3$ for α_P) at the terminus opposite to the attachment site (Scheme 1). For ensuring a high content of helicity during the trapping assay, the helix-inducing solvent 2,2,2-trifluoroethanol (TFE) [27] was added prior to the ligation process, resulting in helicities higher than 70% (Figure 1) [28].

In a typical trapping experiment, the helix bundle was preassembled in solution by mixing the scaffold TASP molecule **I** with varying proportions of helices α_A and α_P (1:1, 2:1, and 1:2). The trapping reaction was then started by an induced change of pH, and its progress was monitored by RP-HPLC. The resulting mixture of TASP molecules was investigated by mass-spectrometry (ESI-MS) (Figure 2(A)) and quantitative HPLC evaluation (Table 1 and Figure 2(B)). The resulting distributions of the three TASP

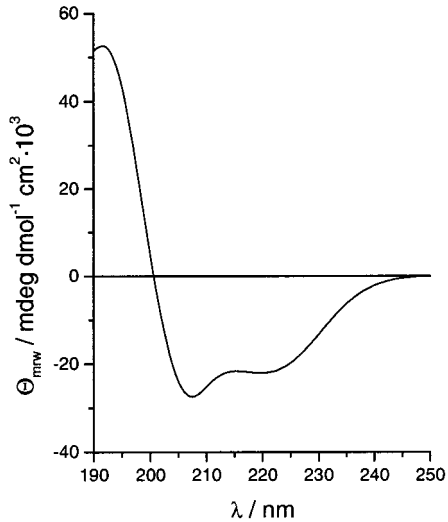


Figure 1 Far-UV CD spectra of helical peptides α_P and α_A (identical curves) in (Tris–HCl buffer 10 mM, pH 8; 50% TFE).

molecules, **II** (all-antiparallel bundle), **III** (partially antiparallel), and **IV** (all parallel) were compared with their statistical distributions, as expected in the absence of a preference for orientation (Table 1 and Figure 2(B)).

In all experiments, a strong preference for the antiparallel helix arrangement (TASP **II**) was observed. For example, the statistically expected value of 25% for the all-antiparallel arrangement of helices (**II**) is exceeded by a factor of two (53%), whereas the all-parallel arrangement (**II**) is found only to about one forth of the statistical value; there is nine times as much **II** as **IV**. Most notably, even a two-fold excess of helix α_P compared with α_A resulted in a 100% excess of the antiparallel arrangement **II** (33%) compared with **IV** (17%), although a four-fold excess of **IV** would be expected according to the statistical distribution. In contrast, a corresponding excess of the antiparallel helix α_A dramatically reduced the formation of all-parallel **IV** and 'mixed' TASP molecule **III** to about 30% and 60%, respectively, of the statistical values, with 16 times as much **II** as **IV**.

These pronounced preferences for an antiparallel assembly of helices in a four-helical bundle caused by favourable packing interactions are actually underestimated, owing to the irreversibility of the chemical ligation procedure. Using the unique functional site on each helix, the distribution of the trapped four-helix bundle TASP molecules is biased by unspecific (kinetically driven) ligations of

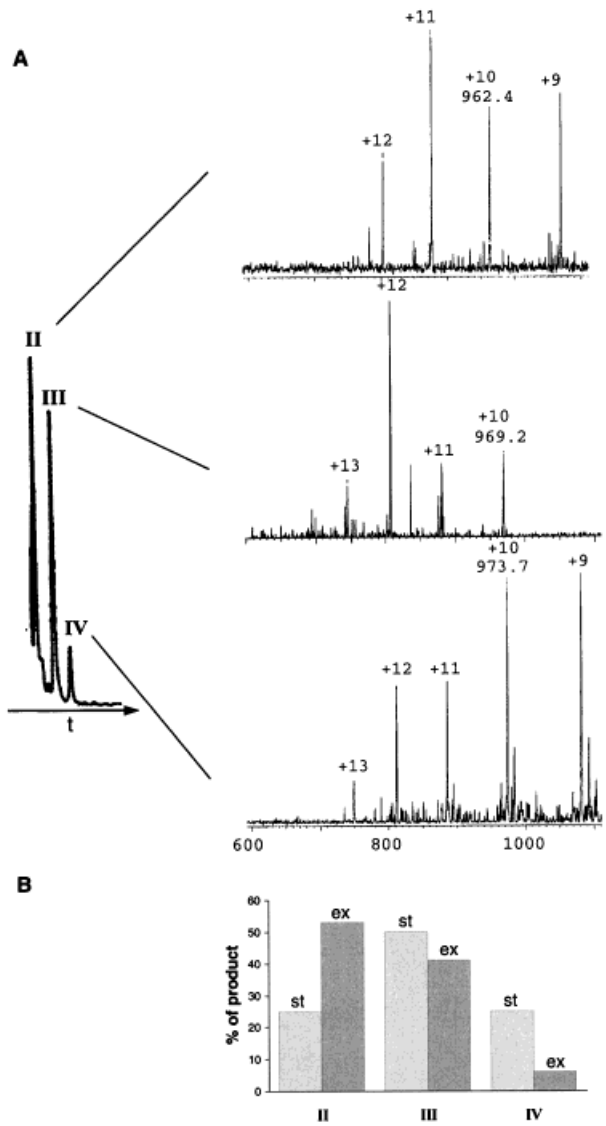


Figure 2 (A) RP-HPLC elution profile of the competitive trapping experiment applying identical helix proportions (Table 1[a]) and identification of product peaks by ESI-MS: **II** ($M_r = 9621.4$), **III** ($M_r = 9675.4$), and **IV** ($M_r = 9729.3$). (B) Statistical (st) and experimental (ex) proportions of TASP molecules **II–IV** in the competitive trapping experiment applying 1:1 ratio of helices α_A and α_P (Table 1[a]).

thermodynamically unfavourable (i.e. parallel) orientations of helices. Consequently, in a fully reversible self-assembly process reaching the thermodynamic equilibrium prior to the trapping process, the distribution is expected to be even further shifted to the benefit of the all-antiparallel helix orientation (**II**).

CONCLUSIONS

In view of our previous observations of a random assembly of structurally identical helices in four-helix bundle formation using a similar trapping system [22], the above data can only be rationalized by a dominance of packing effects, as expected from the computer-assisted design of the ROP-derived TASP molecule [23] showing the importance of geometrical complementary in protein folding and protein design.

In conclusion, the elaborated concept of competitive template-trapping represents a powerful tool for the experimental investigation of self-assembly in solution, applicable to any type of secondary and tertiary structure formation. Studies are in progress to combine this approach, with combinatorial methods for the elucidation of the factors governing self-assembly folding in supramolecular chemistry.

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