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STRATEGIES FOR THE DE NOVO DESIGN OF PROTEINS

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Summary

Over the last years, we have developed strategies for the construction of artificial proteins based on amphiphilic secondary structure building blocks designed to fold into predetermined tertiary structures.

In this progress report, we summarize the results obtained in the design and synthesis of secondary structure building blocks and their assembly to linear polypeptides with a propensity to fold into a bab-chain topology. Also, our new concept for the construction of artificial proteins with a nonnatural chain assembly (Template Assembled Synthetic Proteins, TASP) is exemplified for two prototypes of this new generation of macromolecules.

Introduction

The construction of new proteins is a challenging goal in present peptide and protein chemistry. The natural protein sequences selected during evolution are able to fold into tightly packed structures with optimized intramolecular interactions, a situation which can be hardly met by a "de novo" design of proteins. Moreover, the formation of a defined tertiary structure seems to be an exceptional feature of selected amino acid sequences. The arbitrary creation of primary sequences would unequivocally result in polypeptides with an unordered conformation. On the other hand, X-ray diffraction studies on a large number of proteins have revealed only a limited set of folding topologies[1] pointing to the degeneracy of the folding code. In other words, the occurrence of a specific structure is not bound to an unique amino acid sequence.

The question of how and why proteins are able to fold[2] cannot yet be answered in detail despite the enormous amount of work done on this subject. It is generally accepted that the primary sequence contains all the necessary information for the folding process and that the native conformation in a given environment represents a minimum of the Gibbs free energy of the whole system (including the solvent)[3], although it is not clear whether it is a local or global one. The folding process itself is thought to occur along a kinetically determined pathway, starting with the formation, induced by short-range interactions, of fluctuating secondary structure nucleation sites such as helices, β–structures and β–bends[1,3-4]. In a subsequent phase secondary structure blocks are assembled to structures of higher order due to middle- and long-range interactions. Finally, a slow optimization of the intramolecular packing interactions takes place. The key role attributed to secondary structure elements in the folding process has special implications for the design of artificial proteins.

We have aimed at designing and synthesizing peptides having the potential to adopt stable secondary structures, for their use in linear structures of higher order[5] (Fig. 1). The following aspects are of particular importance : (i) artificial isolated folding units lack any stabilization by long-range interactions of the kind found in natural proteins, so that the intrinsic stability of the secondary structure blocks is a prerequisite; (ii) the occurrence of secondary structure blocks along a polypeptide chain is not a sufficient condition for intramolecular folding, mainly because this process brings about a high loss of chain entropy that has to be compensated. The additional driving force needed for intramolecular folding can be provided by the use of amphiphilic secondary structure blocks[5-6]. Through the still imperfectly

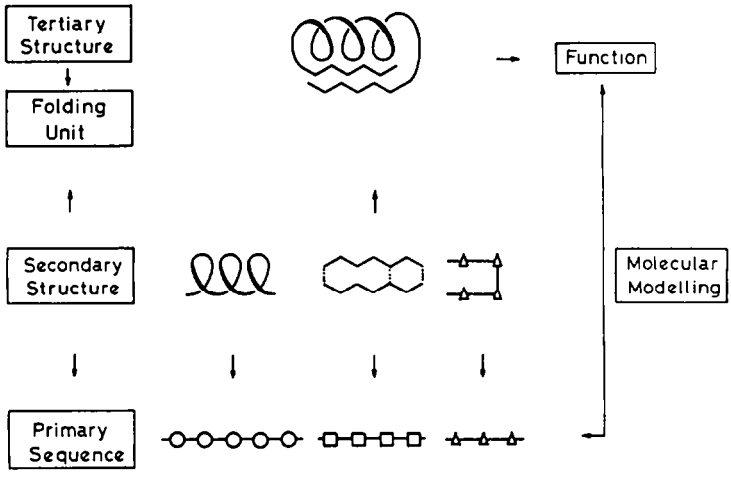

Figure 1 General strategy for the construction of polypeptides with a propensity to fold into a tertiary structure. Distinct folding units are obtained by the specific assembly of the secondary structure blocks.

understood "hydrophobic interaction"[7], these interact to form an energetically favourable structure with a hydrophobic core and a hydrophilic surface.

Despite some encouraging results[8-10], "de novo" design of proteins along these lines appears in certain cases to be limited by poor solubility of the target molecules, due to the competition between intramolecular interactions (process of folding) and intermolecular aggregation of the amphiphilic peptide segments. To overcome this intrinsic problem, we have recently proposed an alternative strategy for the construction of tertiary structures[11]. Instead of matching the folding mechanism of linear polypeptide chains (Fig. 2, left), we select nonnatural branched chain architectures that have a strongly enhanced propensity for intramolecular folding (Fig. 2, right). Oligopeptides having the potential to form amphiphilic helices and β–sheets are assembled on a multifunctional carrier molecule (template) which directs the peptide blocks to a predetermined arrangement. The spatial constraints of these branched macromolecules result in a higher volume density compared to an unfolded linear polypeptide chain of equal number of residues and act as a major driving force for intramolecular folding of the template attached amphiphilic peptide blocks to a globular structure in which the hydrophobic residues have minimal contact with the solvent (water).

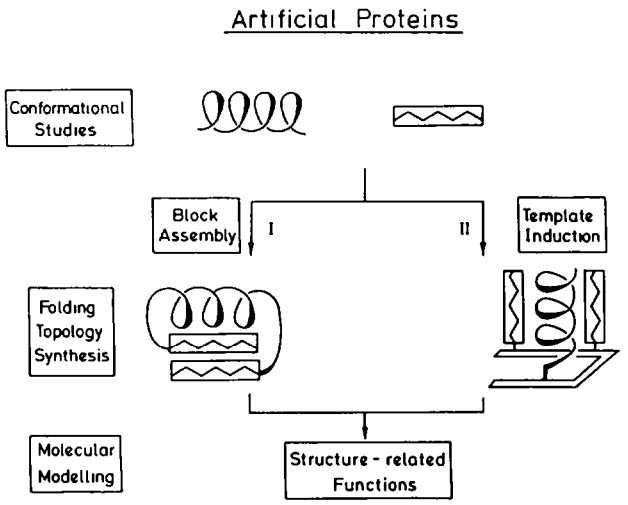

Figure 2 Schematic comparison of the two approaches towards the construction of artificial proteins described.

Building Blocks

The experimental basis for the construction of artificial proteins stems from detailed investigations on the relationship between primary sequence and secondary structure, aiming at the design of stable amphiphilic secondary structure blocks.

The elucidation of β-structure forming potential[12] was achieved by synthesizing

peptides of chain lengths between 6 and 10 residues containing alternating hydrophilic and hydrophobic amino acids by the liquid phase method[13] (Table 1). The strong solubilizing effect of the polymeric C-terminal protecting group polyethylene glycol (PEG) allowed to circumvent the difficulties arising from the notorious insolubility of β-forming peptides. Conformational studies were performed either with the free peptides or with PEG bound peptides, as it is known that PEG does not exert a detectable influence on the conformation of the attached peptide chain in solution[14].

TABLE 1

Critical number of dipeptide units n_c necessary for β–structure formation in various amphiphilic oligopeptides

Peptide	Solvent	n_c
$(Ile\text{-}Gln)_n$-Ile-Gly-PEG	H_2O	2
$(Ile\text{-}Thr)_n$-Gly-OH	CF_3CH_2OH	3
$(Thr\text{-}Val)_n$-Gly-OH	H_2O	4
$(Ser\text{-}Leu)_n$-Gly-PEG	H_2O	4
$(Ser\text{-}Ala)_n$-Ser-PEG	H_2O	>4

The development of β-structures at chain-lengths of 5-8 residues is comparable to the behaviour of hydrophobic homooligopeptides[15] and is also in the range observed for β–structure blocks in naturally occurring folding units[16]. This feature points to the high β–structure potential of this type of amphiphilic peptides in aqueous solution, best explained by the formation of a peptide bilayer–sheet[17]. However, sequence-specific features can be detected. For example, judging by circular dichroism, $(Thr\text{-}Val)_n$ shows high proportions of β-structure at the hexapeptide level already and the β-sheet structure is fully developed at the octapeptide level, while the sequence $(Ser\text{-}Leu)_n$ shows a strikingly sharp conformational transition from random coil to β-structure at the octapeptide level (see Fig. 3a). Moreover, sufficient overall hydrophobicity seems necessary besides amphiphilic character, as indicated by the fact that $(Ser\text{-}Ala)_n$ is unable to adopt a β-sheet conformation even at the nonapeptide level, whereas the side chain protected sequence $(Ala\text{–}Ser(OtBu))_4$-PEG shows fully developed β-structure in water.

The critical chain-length for helix formation proved to be in the range of 10-15 residues in helix supporting media like trifluoroethanol[18], depending on the helix forming potential of the individual residues. A drastic reduction of the critical

chain-length can be achieved by the incorporation of α–amino isobutyric acid (Aib), which has a strong helix-inducing effect due to its restricted conformational space[19]. The large number of helical Aib-containing peptides described[20] allows to define the following rules for the design of suitable helical building blocks:

(i) The sequence must contain a number of Aib residues sufficient to allow helix formation at the considered chain-length

(ii) The distribution of charged, hydrophilic and hydrophobic residues along the chain must result in an amphiphilic helix when the sequence is displayed in the helical–wheel representation (see Fig. 3b). Our results with model helices[21] establish that amphiphilic Aib-containing peptides of lengths of 15 residues already adopt well-developed helical conformations in helix supporting media and even in water. As an example, the CD spectrum (Figure 3c) in phosphate buffer of HCl·H–Pro–Ala–Aib–(Glu–Ala–Ala–Aib–)$_2$–Ala–Aib–Gly–PEG is shown.

Linearly Assembled Folding Units

Several findings show that our approach of assembling secondary structure blocks to structures of higher order is realistic. First, as mentioned above, current hypotheses about protein folding propose secondary structure elements as nucleation sites. Moreover, taking βαβ-folding units as an example, molecular modelling confirms that the parallel β-strands and the overlying α-helix are positioned in a very similar way in proteins of different size, function and sequence[22], pointing to the generality and importance of structure stabilization by hydrophobic interactions between β-strand and helix residues in the core of the folding unit.

So far, attempts for the construction of folded polypeptides have been based on conformational probability parameters for single amino acid residues derived from the statistical analysis of globular proteins[23]. However, it has been shown that the predictive power of these statistical parameters is not sufficient in the case of peptides[24]. In contrast, the use of experimentally characterized stable secondary structure blocks of the kind described above reduces the chain entropy of the unfolded linear polypeptide considerably by achieving the first nucleation step, and makes the folding process energetically more favourable. Furthermore, the formation of a hydrophobic core (increasing the entropy of the solvent) acts as an additional driving force for folding.

A folding unit designed on this basis[8] is shown in Fig. 3d. The (Leu-Ser)$_n$ β-sheet block was chosen because Ser is thought to increase water solubility and Leu was shown in molecular modelling to be more advantageous with respect to

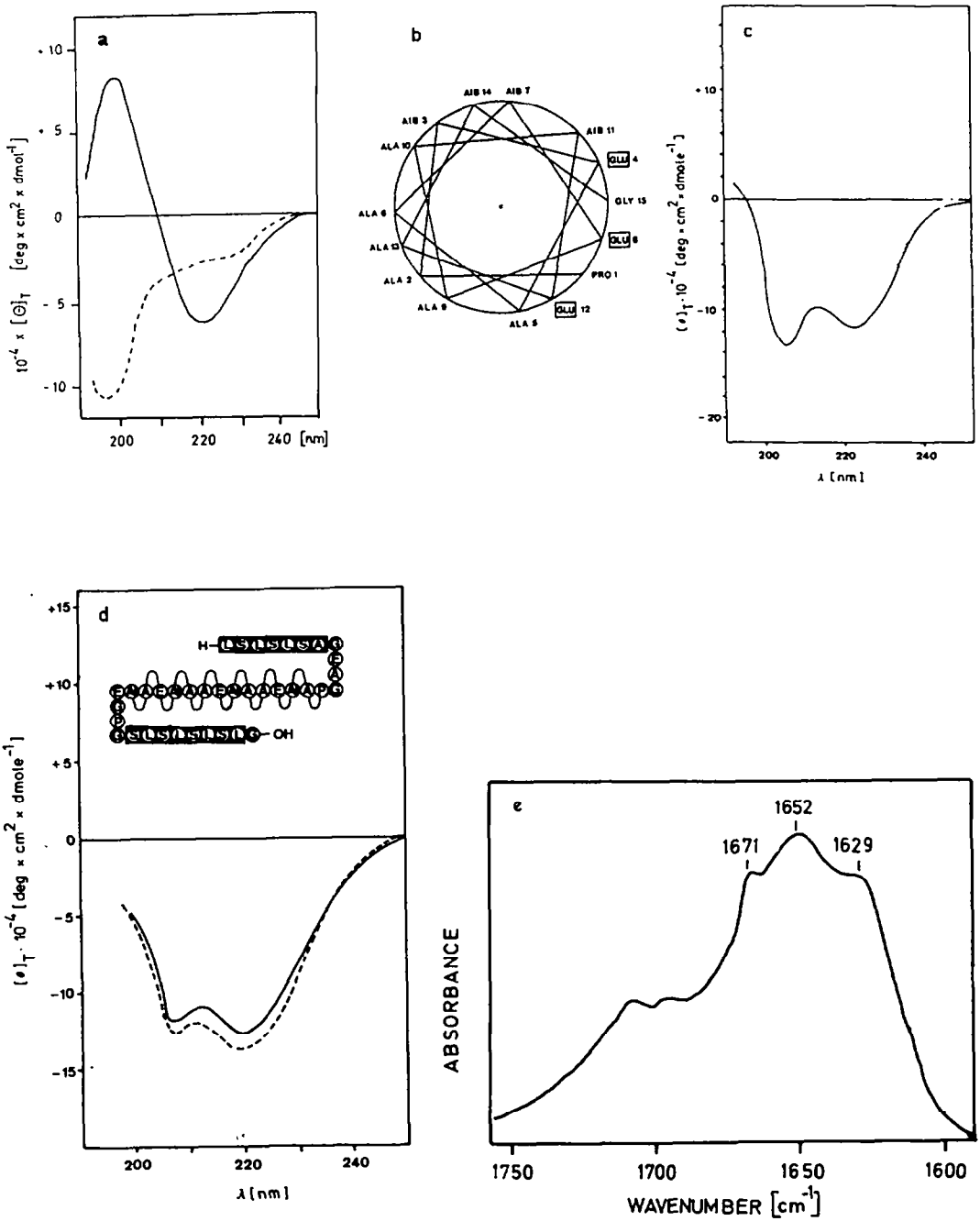

Figure 3 (a) CD spectra of PEG-bound CF_3SO_3H·H-Leu(Ser-Leu)$_3$, c = $7.7 \cdot 10^{-4}$M (· ··) and CF_3SO_3H·H–(Ser-Leu)$_4$, c = $8.5 \cdot 10^{-4}$ M (—) in water, showing the conformational transition from unordered to β-secondary structure at this chain-length. (b) The helix sequence Pro–Ala–Aib(–Glu–Ala–Ala-Aib)$_2$-Glu-Ala-Aib-Gly in the helical wheelrepresentation. (c) CD spectrum of HCl·H-Pro-Ala-Aib(-Glu–Ala-Ala-Aib)$_2$-Glu–Ala–Aib–Gly–PEG in phosphate buffer at pH 7, c = $1.3 \cdot 10^{-3}$ M (d) CD spectrum of the βαβ-model M I in aqueous solution at neutral pH, c = $7 \cdot 10^{-5}$ M (···) and c = $7 \cdot 10^{-6}$ M (—). (e) ATR-IR[25] of the βαβ–model M I in water, pH 7.

intramolecular packing compared to Val or Ile due to its somewhat greater flexibility. The block lengths correspond to those found in natural proteins. Spectroscopic data are in agreement with the hypothetical folded structure. The circular dichroism spectrum in aequeous phosphate buffer at pH 7 (Fig. 3d) is characterized by a dominating negative Cotton effect at 219-220 nm and a second one at 206 nm indicative of a highly ordered conformation, and is very similar to those found for α/β proteins[25]. The ATR-IR spectra[26] (Fig. 3e) show the expected bands for α (1652 cm^{-1}), β (1629 cm^{-1}) and loop (1671 cm^{-1}) structure. It should be reminded that $(Ser\text{-}Leu)_3$ is unable to adopt an ordered conformation under the same conditions. Thus, the spectrum points to an intramolecular stabilization of the N-terminal block. Although the ultimate proof for the presence of a folded structure is still missing, these studies provide valuable experimental information on the physicochemical and conformational properties of linearly assembled amphiphilic secondary structures. In our experience, the most critical obstacle on the way to artificial proteins is their high tendency for intermolecular aggregation. The optimal balance between hydrophobic (promoting intramolecular folding) and hydrophilic (providing solubility) character of the sequence of the polypeptide seems to be hard to attain by de novo design; finding the "Θ–conditions" for intramolecular folding of a polypeptide chain[27] for a given set of experimental conditions will be a most challenging task for future protein design. Recognition of these difficulties prompted us to look for alternative strategies for the construction of tertiary structures; our first results are presented below.

Template Assembled Synthetic Proteins (TASP)

As mentioned in the previous section, the most critical hurdle in the construction of artificial proteins is to bring about the intramolecular folding of the designed polypeptide chain. Our new concept aims to avoid this problem by the assembly of peptide blocks with high potential for taking up a secondary structure on a multifunctional carrier molecule (template). As shown schematically in Figure 4, amphiphilic α-helix and β-sheet forming oligopeptides are attached to the template which directs the arrangement of the peptide units to a predetermined folding topology, e.g. βαβ-, 4–helix–bundle- or β-meander-like folding patterns. In principle, a broad variety of multifunctional molecules exhibiting limited conformational flexibility can be used as templates. However, it must be noted that a definite propensity of the template molecule to adopt a conformation with appropriate orientations and relative distances is an essential condition for the construction of

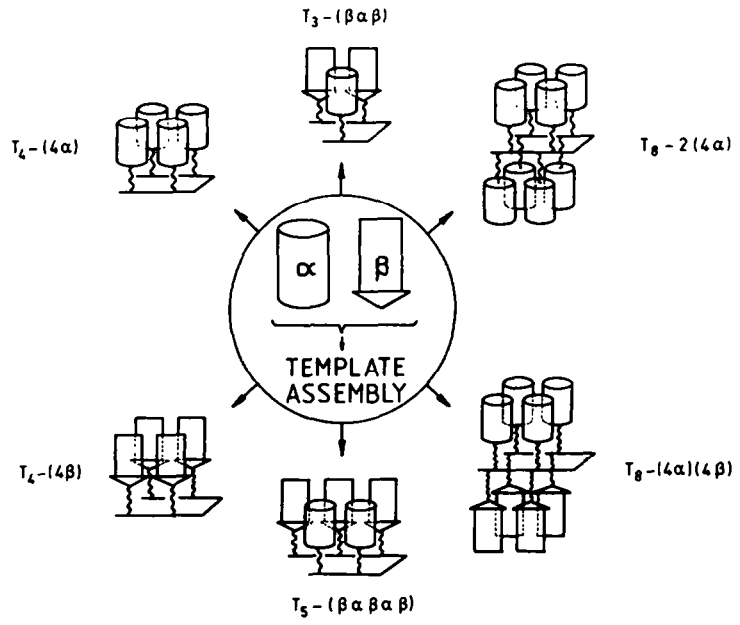

Figure 4 Schematic representation of template assembled proteins (T_n, T = template molecule, n = number of attachment sites on T; α, β, amphiphilic peptide blocks with tendency for α-helix (α) and β-sheet (β) secondary structure formation. Different examples of synthetically accessible folding topologies are shown.

complex globular structures that depend on a directing effect of the template ("template induced folding"). The use of highly flexible multifunctional carriers appears to be possible only if the attached peptide chains exhibit a high tendency for self-aggregation in a particular overall structure, as, for example, in the triple-helix formation of collagen models[28] or, indeed, of crosslinked procollagen itself[29]. Therefore, either open-chain molecules with particularly favoured solution conformations (e.g. specially designed linear oligopeptides) or conformationally constrained (e.g. cyclic) molecules are the most suitable candidates. In a first approach, we have focused our efforts on multifunctional oligopeptides as template molecules for the following reasons : (i) they are readily accessible to chemical synthesis, (ii) orthogonal main and side chain protection of the template molecule provides synthetic flexibility; thus, peptide blocks can be attached by segment condensation or stepwise synthesis, and peptide vectors can be assembled with parallel or antiparallel orientations, (iii) templates with tailor-made spatial geometry and flexibility can be generated by incorporation of nonnatural amino acids and peptide mimetics. Two of the TASP prototypes designed so far (TASP I and TASP II, Table 2) are discussed in more detail below.

TABLE 2

Sequences and designed topologies of TASP I and TASP II

TASP I		
T_3-($\beta_I\alpha_I\beta_I$)	T_3 :	Ac-Lys(α_I)-Pro-Lys(β_I)-Lys(β_I)-Polymer
	β_I :	Ac-(Thr-Val-)$_3$-
	α_I :	Ac-Glu-Ala-Leu-Aib-Ala-Glu-Leu-Aib-Glu-Leu-Glu-Ala-
TASP II		
T_4-($4\alpha_{II}$)	T_4 :	Ac-Lys(α_{II})-Lys-Lys(α_{II})-Pro-Gly-Lys(α_{II})-Glu-Lys(α_{II})-OH
	α_{II} :	H-Asp-Ala-Ala-Thr-Ala-Leu-Ala-Asn-Ala-Leu-Lys-Lys-Leu-

T_n: T = Template molecule; n = number of attachment sites on T; α, β, amphiphilic peptide blocks with tendency for helix (α) and β-sheet (β) secondary structure formation.

As indicated by molecular modelling studies, the template Lys-Pro-Lys-Lys in TASP I can adopt a low energy conformation with a β-turn of type I′ for Pro-Lys[30], in agreement with the high probability for this pair to occur at positions i+1 and i+2 in turns[31]. The template used for TASP II may exist in a low energy conformation with a β-turn of type II for Pro–Gly[30,32] connecting the antiparallel β–sheet segments. Due to the conformational characteristics of the tetramethylene amino side chain of lysine, a considerable directional persistence perpendicular to the plane of the template is predicted[27], i.e. the template is expected to enhance and stabilize tertiary structure formation. Sufficient torsional flexibility of the tetramethylene amino unit of lysine allows the attached peptide blocks to orient themselves for optimal packing and solvation. One of the most attractive features of TASP molecules is their synthetic accessibility. The template corresponds to a multifunctional carrier with specifications of capacity and average distance between attachment sites similar to those of soluble or insoluble supports in conventional peptide synthesis[13,33]. Furthermore, the synthesis of these branched peptides is much more efficient and less subject to the limitations (e.g. solubility and coupling problems) encountered in the synthesis of linear peptides of comparable size[34]. Simultaneous or successive assembly of the secondary structure blocks on the template lead to globular-like structures. TASP I & II were synthesized by standard protocols, applying both soluble[13] (TASP I) and insoluble[33] (TASP II) polymeric supports. The scheme for the synthesis of TASP II is shown in Fig.5. TASP I & II proved to be readily soluble in water over a wide range of experimental conditions (pH, temperature and concentration variation, addition of salts and organic solvents).

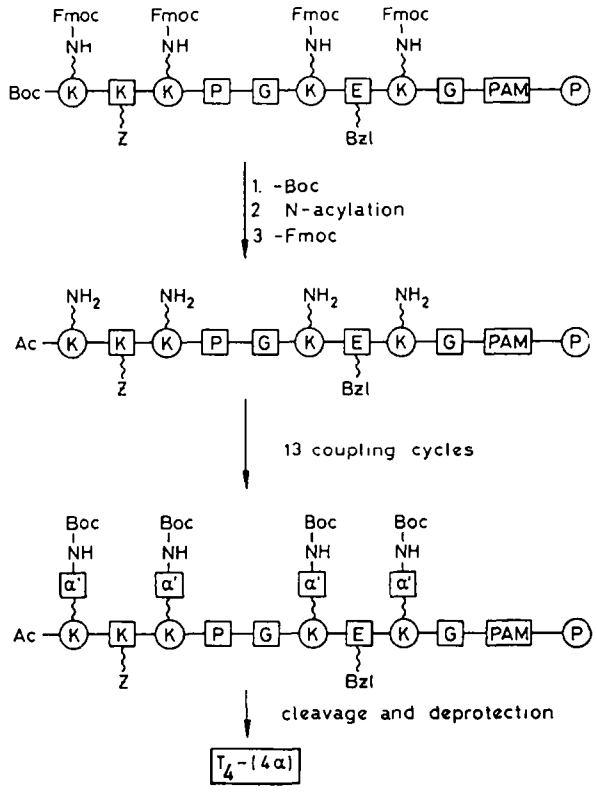

Figure 5 Scheme of the synthesis of TASP II showing the chosen orthogonal protection strategy. P : polystyrene resin, PAM : phenylacetamidomethyl, Boc : tert-butyloxycarbonyl, Fmoc : 9-fluorenylmethoxycarbonyl, Bzl : benzyl, Z : benzyloxycarbonyl . The template amino acids are given by their one-letter codes.

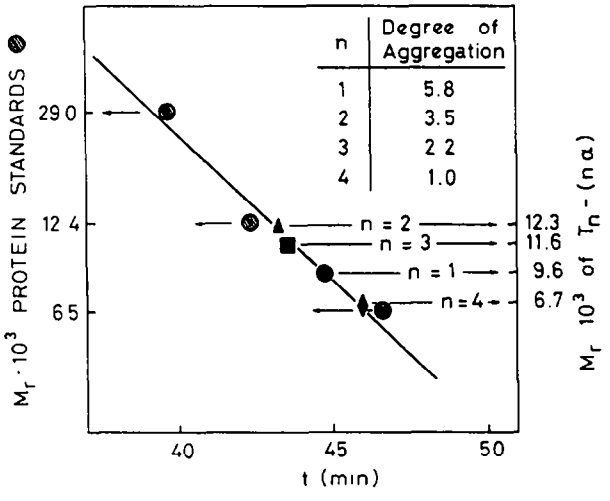

Figure 6 Molecular-weight determination by size exclusion HPLC of TASP II (n=4) and the partial template sequences with 1 (n=1), 2 (n=2), 3 (n=3) helix blocks in 50 mM Tris-buffer, 0.1 M KCl, pH 7. The molecular weight standards used are carbonic anhydrase (29000), cytochrome c (12400), aprotinin (6500). Insert : degree of aggregation (MW_{exp}/MW_{calc}) for n=1-4.

Very interesting findings were obtained with TASP II as to its tendency for aggregation. No significant aggregation could be detected in size-exclusion chromatography studies for the TASP 4–helix-bundle, while the partial template carrying 1, 2, and 3 helix block sequences did show association to aggregates of different sizes (Figure 6). The pronounced solubility and the greatly reduced tendency for aggregation of TASP molecules may be regarded as strong experimental evidence that the template enhances "intramolecular aggregation" of the amphiphilic peptides to a globular structure, in which the hydrophilic residues are exposed to the polar solvent.

TASP I-II have been further characterized by CD (Figures 7-8) and IR spectroscopy and experimental data are in full agreement with the postulated structures. TASP I (Fig. 7c) exhibits a negative Cotton effect at pH 7 (λ =218-222 nm) consistent with the presence of both an α-helical and a β-sheet structure. The spectra of the helix (Fig. 7a) and the two β–sheets (Fig. 7b) on the template are shown for comparison. In addition, the solid state IR absorption bands are indicative of the presence of both helical (ν = 1655 cm^{-1}) and β–sheet (ν = 1630 cm^{-1}) blocks. Remarkably, the critical chain length for the onset of secondary structure formation in the β-sheet block is significantly lower (6 residues) when it is synthesized on the template compared to the individual peptide block[12], an observation pointing to the secondary structure stabilizing effect of the template. However, it should be mentioned that the template exerts a β-structure inducing effect on the adjacent $(Val–Thr)_3$ sequences even in the absence of the helical block.

The template effect is even more dramatic for the template assembled prototype TASP II. As shown in Figure 8, TASP II exhibits a CD spectrum in aqueous solution typical for a helix conformation (negative Cotton effects at λ = 222 nm ($n–\pi^*$) and λ = 207 nm (π-π^*), crossover at λ = 198 nm, positive effect at λ = 190 nm, whereas the corresponding template–unattached 13-mer peptide adopts a predominantly random-coil conformation under identical experimental conditions (negative effect at λ = 197 nm, crossover at λ = 190 nm, Figure 8). Moreover, SDS addition to the 13–mer peptide induces a transition from disordered to helical conformation, whereas no significant change in the spectra is observed for the template–assembled 4-helix-bundle (not shown). We consider this as a clear indication that the helix blocks in TASP II are stabilized by hydrophobic contacts between interacting blocks in the core of the bundle similar to the long range interactions found in folded proteins. The contrast with our previous linear folding models must be emphasized. In the case of our helix-bundle protein, the single helix blocks in the "isolated" state have merely the potential to fold in a helix (as indicated by the results obtained with SDS) and it is the template that causes the formation of a helical strucure in TASP II. It is interesting to compare our findings with experimental evidence indicating the

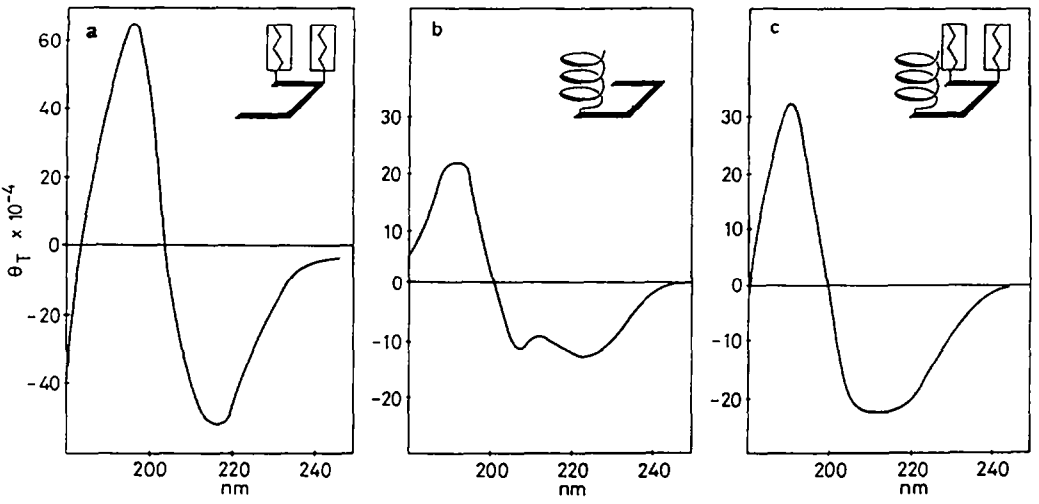

Figure 7 CD spectrum of TASP I (c) in H_2O : TFE 8:2, c = $1.18 \cdot 10^{-4}$ M; the spectra of the helix (Fig. 8a) and β-sheet (Fig 8b) blocks on the template are shown for comparison.

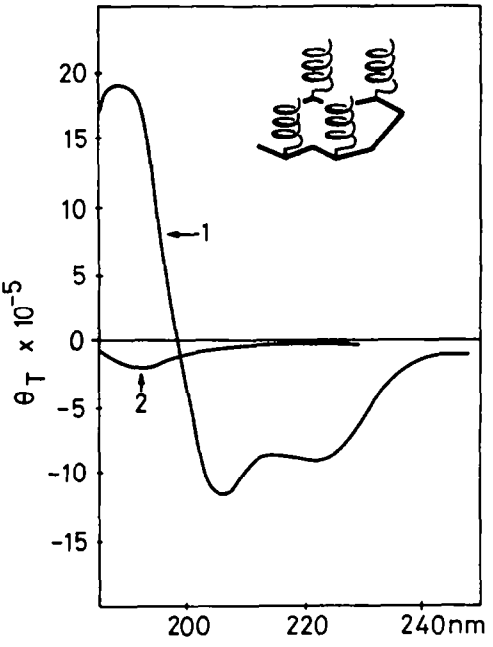

Figure 8 CD spectra of TASP II (1) and of the 13-mer helix α_{II} of TASP II (2) in H_2O, pH 7, c = 10^{-4} M.

concentration-dependent self–aggregation of "de novo" designed amphiphilic helices to 4-helix-bundle-like proteins in aequous solutions[9,35].

The present data on the conformational and solution properties of TASP II can only be rationalized if a 4-helix-bundle-like conformation, resulting from a template–induced intramolecular "collapse" of the amphiphilic helices is assumed, perhaps similar to the proposed "molten globule" folding mechanism described in the literature[36].

In summary, there is strong evidence that the template assembly of amphiphilic helices and ß–sheets represents a valuable concept for the construction of tertiary structures. By comparison to other current strategies for the de novo design of proteins, the novel "construction plan" presented here shows some distinct advantages :

- The strategy is applicable to the design of a wide variety of chain topologies;
- template-assembled proteins are readily prepared by the techniques of peptide synthesis and they show favourable solution properties;
- peptide building blocks can be designed independently of templates and can be assembled in a modular fashion.
- due to the structure directing effect of the template, the formation of a tertiary structure is not bound to the complex folding pathway of a linear polypeptide chain; the incorporation of functional properties into a predetermined tertiary structure can become a realistic goal in protein design.

It remains to be seen which of the two basic strategies for the construction of artificial proteins described here shall prove most successful; meanwhile, both approaches are helping us to a greater understanding of the rules that govern protein folding and topology.

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