105. Approaches to Synthetic Vaccines

Design of Epitope-Containing Amphiphilic Peptides Matching the Antigenic Structure in the Native Protein

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A general procedure for the design of synthetic vaccines with the retained conformational features of protein antigenic determinants is described. This new concept emerges from detailed studies on the relationship between primary sequence and secondary structure formation of synthetic peptides and takes advantage of the amphiphilic nature of epitope-containing peptide segments in the native protein to accomplish structural modifications. These segments, for example amphiphilic helices or β-sheets, are stabilized by the insertion of secondary structure-inducing amino-acid residues on the hydrophobic part of the peptide without affecting the spatial arrangement of functional residues on the hydrophilic side. The availability of amphiphilic peptides with tailor-made conformational properties, *e.g.* helices, β-sheets, and, moreover, assemblies of these blocks to structures of higher order ('folding units'), allows the presentation and stabilization of continuous as well as discontinuous epitopes by this approach. This strategy is exemplified for the case of two discontinuous epitopes taken from lysozyme, which are matched to host molecules with adequate conformational features by the help of computer-assisted molecular modelling. The implications of this new concept for the design of synthetic vaccines are discussed with special emphasis to the important role of peptide synthesis and chemical structure modification.

Introduction. – Synthetic vaccines have been the subject of much interest recently [1] [2]. Major advances in the fields of gene and hybridoma technology, together with progresses in the use of synthetic peptides in immunological research, have paved the way for the development of totally synthetic vaccines, lacking the disadvantages of classical vaccines, *e.g.* difficulties in culture or storage, residual contamination of the virus or parasite, or its reversion to virulence.

The demonstration that immunization with short synthetic peptide sequences of a given protein can induce specific antibodies against the native protein – even to those parts of the protein which are not themselves immunogenic [3] – has prompted intensive research in the field of protein chemistry. Many attempts have been made to predict the location of antigenic sites in proteins from specific features of their primary, secondary, or tertiary structure [4–6]. However, the question to which extent the balance between sequential, structural, and topological information must be maintained in an antigenic determinant to achieve high antibody specificity is open so far. Although short synthetic peptides have been commonly used for the elucidation of antigenic sites in native proteins, this approach suffers from limitations arising from the high conformational flexi-

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bility of short amino-acid sequences. As was recently pointed out by *van Regenmortel* [7], the method chosen for antigenicity studies largely determines the results obtained. For example, antibodies raised against synthetic peptides seem to recognize regions of high mobility in the native protein, resulting in an overestimation of the role of segmental flexibility for antigenicity; on the contrary, monoclonal antibodies to a given protein are also able to locate discontinuous, conformationally well defined sites.

Interestingly, there is increasing experimental evidence pointing to the importance of the three-dimensional conformation of antigenic determinants for their specific recognition by antibodies [8]. Due to their high chain-flexibility, short peptides are not suitable for the preservation of these conformational and topological features contributing to the induction of specific antibodies; they represent *a priori* a tool of only limited value for mimicking the complex conformational and dynamic properties of antigenic epitopes on the protein surface.

From these considerations, it becomes obvious that synthetic-vaccine optimization requires the development of model systems with an adequate balance in sequential and conformational information. We have recently developed a strategy for the construction of peptides with tailor-made structural features [9]. As we shall demonstrate in this article, it can be applied in the approach of one of the most delicate problems in present synthetic vaccine research, *i.e.* the production of epitope-containing peptides that match more closely the antigenic structure in the native protein [7].

Foundations of the Concept. – 1. *The 'Flexibility' of Peptide Segments.* The fact that 'flexible' peptide segments are able to elicit the synthesis of antibodies, which recognize well-defined protein topologies, has caused some confusion. A closer look at the problem shows that the term 'flexibility' is used in a very ambiguous way. The 'flexibility' of a short peptide-chain adopting a 'random-coil' conformation (and amenable to analysis by statistical mechanics procedures [10]) has a different quality than the 'flexibility' (better: 'mobility') of peptide segments in proteins as determined by NMR or crystallographic refinement data of protein structures: if the conformational restrictions present in the native state were retained in 'isolated' peptide segments excised from the dynamic protein surface, these fragments had to be considered as conformationally constrained rather than 'flexible' molecules. However, due to the lack of structure-stabilizing long-range interactions, these conformational restrictions are no longer operative in 'isolated' peptides, resulting in an additional degree of conformational freedom, for example a different distribution of the end-to-end distance r [11]. In other words, a 'flexible' segment on a protein surface can still contain substantial conformational information.

In spite of the substantial loss of topological information, short peptides are able to mimic some relevant features of the native protein as shown by recognition experiments. This surprising finding may be rationalized by an extension of the above conformational considerations: as short-range interactions play a dominant role in the folding process of a polypeptide chain [12], it is possible in some favorable cases that even small peptide segments, *e.g.* excised from a 'turn region' of a protein, can adopt a native-like preferred conformation in solution. However, this short-range stabilization of structural features is restricted to a very small entity of conformational probes, mainly because the critical chain length for the onset of ordered structures like helices or β-sheets is well beyond the size of sequential peptide segments usually applied in synthetic vaccine research so far [13].

We may conclude here that the conservation – or at least stabilization – of the native conformation in a synthetic vaccine is of fundamental concern even for mobile antigenic segments of the protein, as conformational features of the antigenic determinant seem to be essential for obtaining high-affinity antibodies.

2. *Conformational Properties of Amphiphilic Peptides.* The detailed analysis of the topology of peptide segments located on the surface of proteins offers a major key for the construction of artificial epitopes with preserved conformational features. Most important, the architecture of such segments is characterized by their amphiphilic nature, consisting of a functional (hydrophilic) and a structural, *i.e.* structure-stabilizing (hydrophobic), part *(Fig. 1)*.

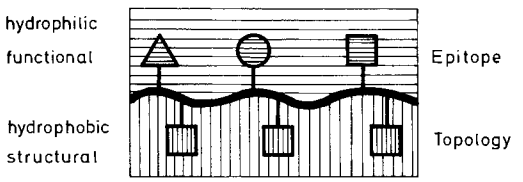

Fig. 1. *The amphiphilic nature of epitope segments.* Adequate modifications of the peptide on its hydrophobic side result in the stabilization of the conformational features of the epitope (hydrophilic side) and compensate for the loss of structure-supporting long-range interactions in the free peptide segment.

In the native state of the protein, the spatial orientation of the functional groups is determined by specific long-range interactions of side chains on the hydrophobic side with other internal parts of the folded polypeptide, this topological information being lost in short 'isolated' protein fragments. However, the chemical nature of the structural side of the amphiphilic segment seems of no concern with respect to epitope recognition. For this reason, the overall conformation of the peptide can be stabilized by adequate modifications (*e.g.* amino-acid replacement, insertion of peptide mimetics) on its hydrophobic side, thus compensating for the loss of long-range interactions. In this way, the conformation of amphiphilic peptide segments – in the first place stretches with helical or β-pleated-sheet conformations – can be highly conserved without the spatial orientation of functional epitope-forming residues themselves being affected. The hydrophobic side of an epitope segment can be considered as the 'workshop' of the peptide chemist, where the whole spectrum of chemical tools – particularly the insertion of unusual amino acids and mimics – can be used to sustain the 'roof', *i.e.* backbone folding, of the epitope. Consequently, 'peptide engineering' will become a major element in synthetic vaccine chemistry.

3. *Amphiphilic Peptides with Tailor-Made Topology: Helices, β-Sheets, Folding Units.* Conformational investigations on model peptides have provided considerable insight into the basic principles of secondary structure formation [14–16]. From these studies, some general rules for the construction of amphiphilic helices and β-sheets have been derived. It could be shown that peptide sequences of the general formula $(A\text{–}P)_n$ (P = polar, hydrophilic residue, *e.g.* epitope-containing sequence; A = hydrophobic residue with voluminous side chain) are potent β-sheet formers in aqueous solution for $n = 3$–4 [17]. For the problem at hand, amphiphilic β-sheets taken from the surface of a

protein are stabilized by substitution of the residues directed to the interior of the protein by strongly β-sheet-inducing amino acids, *e.g.* Ile, Val, or Leu (*cf.* **3** in *Fig. 3*).

As to the construction of amphiphilic helices, the insertion of a strongly helix-inducing residue, *e.g.* 2-aminoisobutyric acid (Aib), in the hydrophobic side of the helix cylinder provides a useful tool for the stabilization of the helical conformation (*cf.* **1** in *Fig. 3*) [18]. Peptides with the general sequence $(P\text{-}P'\text{-}A\text{-Aib})_n$ (A = apolar residue, *e.g.* Met, Ala; *P*,*P'* = hydrophilic residues, *e.g.* amino acids from a helical epitope) adopt a stable helical structure for $n \geq 3$ even in aqueous solution [19]. Depending on the content of Aib per helical turn, amphiphilic helices with tailor-made topologies have been constructed [19].

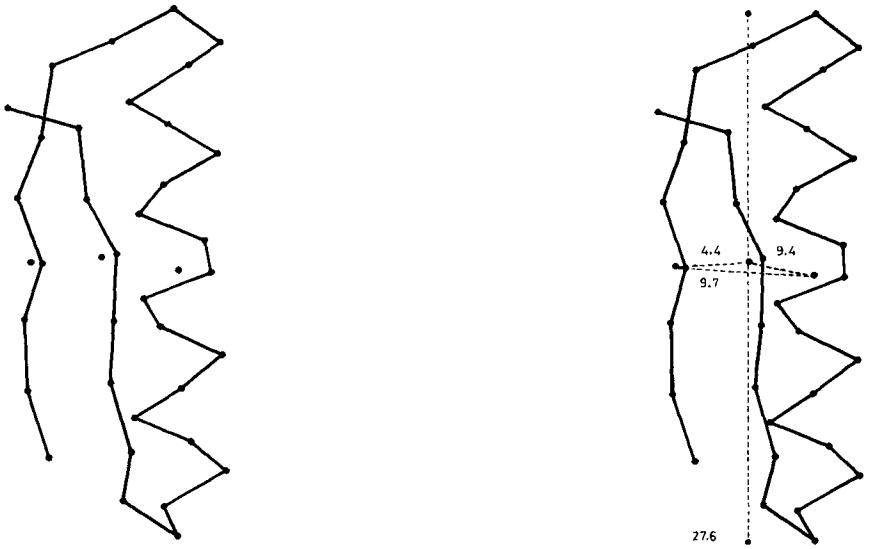

Fig. 2. *C(α)-Backbone of a $\beta\alpha\beta$-folding unit from flavodoxin (Met-1 to Val-35)* [24]. Atomic coordinates are from the *Brookhaven Data Bank* ([34], File No. 4FXN). The indicated distances (dotted lines) are expressed in Å.

The knowledge of the secondary-structure-forming potential of amphiphilic oligopeptides constitutes the basis for the construction of polypeptides exhibiting a tertiary structure in solution [9]. According to this concept, amphiphilic peptide blocks with high potential for secondary-structure formation are assembled so as to fold in a globular structure such as a $\beta\alpha\beta$-folding unit (*Fig. 2*). The amphiphilic nature of the peptide blocks acts as major driving force for the adoption of a folded conformation, resulting in a hydrophobic core (*cf.* yellow zone in *Fig. 5*) and a hydrophilic surface. Again, intramolecular hydrophobic packing interactions are responsible for the overall stability of the structure, whereas the hydrophilic surface of such artificial proteins is available for functional information, *e.g.* presentation of epitopes.

The design and synthesis of folding units of the $\beta\alpha\beta$-type (comprising 30–40 amino-acid residues) suitable for the construction of synthetic vaccines (compare *Figs. 2* and *5*) have recently been reported [20]. Consequently, the basic experimental foundations for the design of synthetic vaccines are already established.

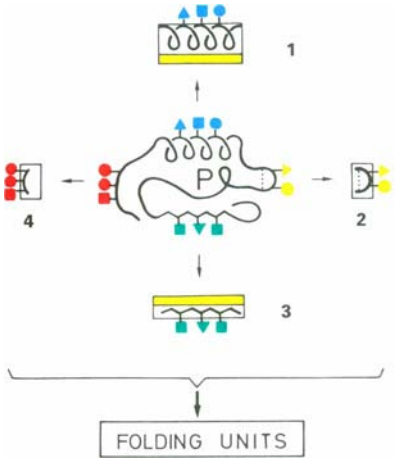

Fig. 3. *Conformationally dependent antigenic sites* (colored symbols) *in the native protein* (P) *and their presentation as stabilized short peptide segments with retained topological features.* **1** = α-helix; **2** = β-turn; **3** = β-sheet; **4** = unordered segment or loop. Hydrophobic surfaces are displayed in yellow.

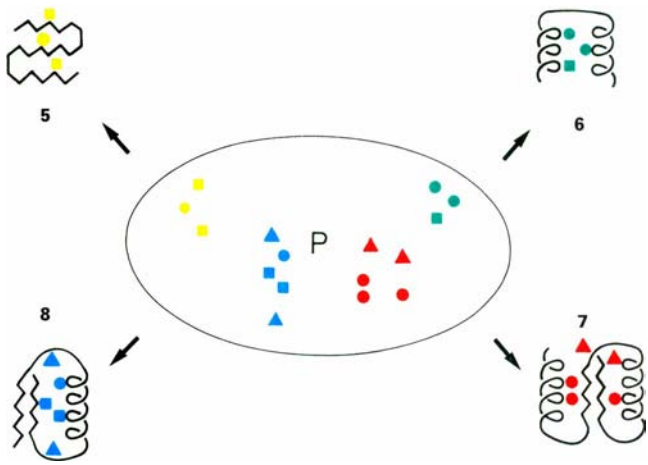

Fig. 4. *Folding units as hosts for 'discontinuous' antigenic sites* (colored symbols). **5** = β-meander; **6** = α*l*α-fold (*l* = connecting loop); **7** = βαβα-fold; **8** = βαβ-fold; P = native protein.

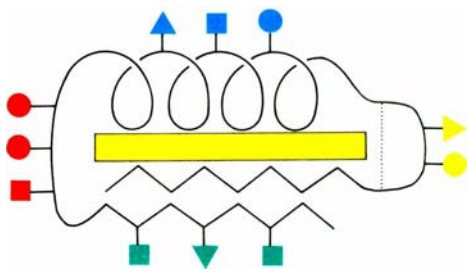

Fig. 5. *The βαβ-folding unit as tentative model for a synthetic vaccine representing the condensed protein surface* (compare to *Fig. 3*)

The Design of Epitopes. – According to recent data [7], the entire accessible surface of a protein is thought to be a continuum of overlapping epitopes potentially capable of binding to appropriate paratopes [8]. Thus, in the light of this model, a discrimination between continuous and discontinuous epitopes is of little use. Linear stretches of residues (considered as continuous or sequential epitopes) are only exceptionally found on the surface of a protein. Consequently, the majority of epitopes are of the discontinuous type, *i.e.* they are made up of distant residues brought together by folding of the protein backbone.

As shown in *Fig. 3*, one method to preserve the topology of different epitopes consists in their incorporation in stable secondary-structure blocks (hosts for antigenic determinants) exhibiting conformational properties similar to the corresponding segment in the native structure. For the presentation of epitopes, the complete hydrophilic surface of amphiphilic helices (**1** in *Fig. 3*) or β-sheets (**3** in *Fig. 3*) is disposable. Most interestingly, this approach can be applied even if the exact three-dimensional structure of a protein is not known. For example, amphiphilic helices may be predicted by empirical rules based on the primary sequence ('helical-wheel' concept) [21]; as outlined before, they can then be stabilized by modifications in the hydrophobic part (symbolized by yellow zones, *Fig. 3*) in order to maintain the conformational features of the epitope.

Determinants consisting of β-turn-like structures (**2** in *Fig. 3*) feature the exposure of only a limited number of residues. Stabilization of antigenic β-turns has been accomplished by cyclization reactions [22].

Peptide segments without structural order or loops (**4** in *Fig. 3*) cannot be stabilized in their native conformation by the same procedure as helices, β-sheets, or turns. These stretches are preferentially transferred to folding units which offer adequate topologies for the incorporation of epitopes not being part of defined secondary structures. Accomodation of loops or nonregular segments in tertiary folds (**4** in *Fig. 3*) mimics their environment in the native protein and might, therefore, result in a similar conformational arrangement of these epitopes, *e.g.* an exposure of the essential residues *(cf. Fig. 5)*.

The present concept can likewise be applied in a similar manner for the construction of epitopes, assembled from residues far apart from each other in the primary sequence. A variety of folding units with distinct topological character, *e.g.* β-meanders (**5** in *Fig. 4*), $\alpha l\alpha$- (**6**), $\beta\alpha\beta\alpha$- (**7**), or $\beta\alpha\beta$- (**8**) units, is accessible as host molecules for the incorporation of this type of discontinuous epitopes [23]. Computer-assisted molecular modelling serves as a powerful tool for an optimal matching of antigenic determinants to the selected hosts.

A further development of this approach consists in the assembly of different epitopes within a single folding unit. This method exhibits the highest potential for the construction of synthetic vaccines *(Fig. 5)*. As folding units may be considered to be artificial proteins of minimum size (*ca.* 30–40 residues), important topological features of the native protein are gathered on a 'condensed' surface, a 'maximum of antigenicity' being concentrated on a minimum of surface ('antigenic hedgehog').

Examples. – To demonstrate the feasibility of the proposed strategy for the construction of synthetic vaccines, the concept outlined here was applied to the design of epitope-containing synthetic peptides corresponding to antigens with well-defined three-dimensional conformation. Matching of antigenic determinants to selected host molecules was performed by means of computer-assisted molecular modelling, some principle aspects concerning the geometry of the various structures being examined in a first step.

Recent data suggest that folding units, especially of the $\beta\alpha\beta$-type, show nearly identical topological patterns for a variety of primary sequences [24]. The geometrical parameters, *e.g.* sheet-sheet distance (4.4 Å), helix-sheet distance (9.55 Å), and length of the helix-block (27.6 Å), are exemplified in *Fig. 2* for a $\beta\alpha\beta$-unit taken from flavodoxin (Met-1 to Val-35) [25]. They should be of general validity for all $\beta\alpha\beta$-folds comprising 30–45 residues, including artificial (non-protein) folding units exhibiting $\beta\alpha\beta$-topology. For this reason, these values can be directly applied to any $\beta\alpha\beta$-model selected as host for the transfer of an antigenic determinant from a native protein.

These figures compare rather well with the dimensions of antigenic sites (usually expressed in terms of volume, surface or C(α)-C(α) distances of residues far distant in sequence. C(α)-C(α) distances range from 21 to 36 Å for lysozyme epitopes [26]. In a specific example, a monoclonal antibody raised against hen-egg-white lysozyme (HEWL) revealed an epitope consisting of 10 residues within a surface of 13 × 6 Å [27]. As the $\beta\alpha\beta$-folding unit affords a minimal volume of 6 × 18 × 30 Å, the available surface of such folding units is clearly in the range needed for matching extended 'discontinuous' epitopes.

We demonstrate here the practicability of our strategy for two well-defined epitopes of HEWL [28] *(Fig. 6)*.

The first epitope (*Epitope 1*, displayed in red) includes the residues Arg-5, Glu-7, Ala-10, Lys-13, and Arg-14, which are all located on the hydrophilic side of an amphiphilic helical segment in the protein. In *Epitope 2* (green), comprising Trp-62, Asp-87, Thr-89, Asn-93, Lys-96, Lys-97, the Trp residue belongs to a more unordered region being relatively far apart from the other residues in the primary sequence. Consequently, this epitope cannot be modelled by a simple amphiphilic secondary structure, but must be incorporated in a folding unit with adequate backbone folding. *Fig. 7a* shows the natural sequence of *Epitope 1* with the exposed antigenic residues displayed in red. To demonstrate the above approach, the epitope residues were transferred to the helix of a $\beta\alpha\beta$-folding unit *(Fig. 7b)*. As can be seen from *Figs. 7a* and *b*, the match of *Epitope 1* onto the α-helix of the folding unit results in an optimal fit for this antigenic site.

A similar result is obtained by structure stabilizing modifications on the hydrophobic side of the natural helix as outlined above. Hydrophobic amino acids in the natural sequence of lysozyme, *i.e.* Lys[1]-Val[2]-Phe[3]-Gly[4]-Arg[5]-Cys[6]-Glu[7]-Leu[8]-Ala[9]-Ala[10]-Ala[11]-Met[12]-Lys[13]-Arg[14]-His[15] were substituted by helix-inducing Aib residues, resulting in the following stabilized sequence: Val-Phe-Aib-Arg-Cys-Glu-Aib-Ala-Ala-Aib-Aib-Lys-Arg-Aib. Amino-acid substitutions were only performed on the hydrophobic side of the helix, the functional side of the model peptide matching exactly the topology of the epitope in the protein.

It should be noticed that Val-1, Phe-2, Cys-5, and Ala-9 of the model helix are located on the hydrophilic side of the helical cylinder, and their substitution by more polar or even charged residues would probably bring along a further stabilization of the secondary structure due to an enhanced amphiphilicity of the peptide. However, modifications on the hydrophilic (functional) side were strictly avoided to exclude any disturbing effect on the recognition of the polar residues comprising the antigenic site.

The same procedure was applied to the helical segment of *Epitope 2*, the Trp residue being neglected in the designed model peptide Asp-Ala-Aib-Thr-Ala-Ala-Aib-Asn-Ala-Aib-Lys-Lys-Ala-Aib *(Fig. 8)*. As can be seen from *Fig. 8*, the antigenic residues (Asp-1,

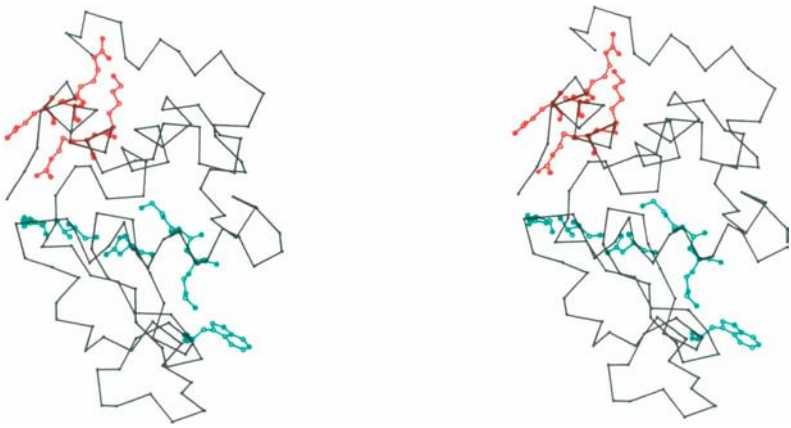

Fig. 6. *Stereoplot of the hen-egg-white lysozyme (HEWL) C(α)-backbone* [33] *with 2 antigenic determinants* (*Epitope 1*, red; *Epitope 2*, green) [28]. The side chains, C(α)-atoms, *N*-terminal N-atoms and C-terminal COOH groups are shown for the relevant amino acids. Heteroatoms (N,O) are represented as full circles. The atomic coordinates of HEWL are taken from the *Brookhaven Data Bank* ([34], File No. 6LYZ).

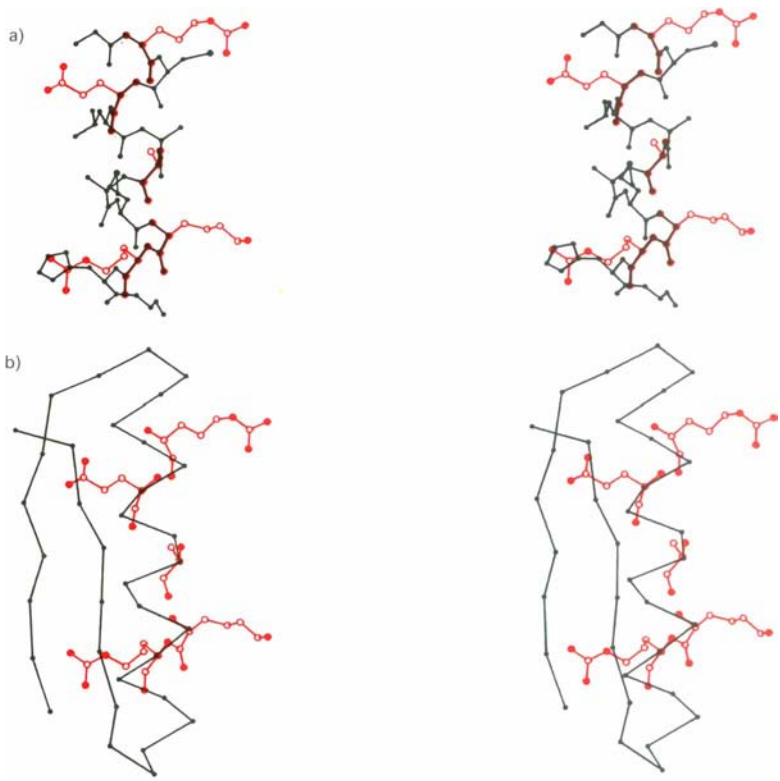

Fig. 7. a) *Stereoplot of the HEWL-(4–16) sequence in its native conformation.* All side chains are displayed, the *Epitope-1* side chains (Arg-5, Glu-7, Ala-10, Lys-13, Arg-14) are shown in red. Other conventions are the same as in *Fig. 6*. b) *Stereoplot of the fitting of* Epitope 1 *side chains* (red) *in their native conformation to the C(α)-backbone of a flavodoxin βαβ-fold* (Met-1 to Val-35, compare to *Fig. 2*). Other conventions are the same as in *Fig. 6*.

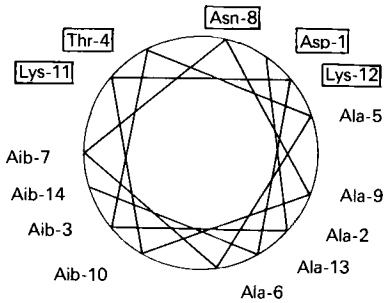

Fig. 8. *'Helical-wheel' representation* [21] *of a stabilized model helix with the conserved spatial arrangement of antigenic residues from* Epitope 2. Epitope residues are framed.

Thr-4, Asn-8, Lys-11, Lys-12) are concentrated on the hydrophilic side of the helix, exhibiting a spatial arrangement very similar to the native protein. Most notably, helices with the corresponding modified sequence have proved to be stable in aqueous solution, *i.e.* the native conformation of the epitope remains conserved [29].

Interestingly, the transfer of *Epitope 2* (green in *Fig. 9*) on a $\beta\alpha\beta$-folding unit did not result in a satisfactory match of the critical residues. Especially, residue Trp-62 requires a somewhat different geometry than provided by a β-sheet host. In this case, matching was successful when the C(α)-backbone of a 'helix-loop-helix'-type host (avian pancreatic polypeptide, APP [30]) was used (red in *Fig. 9*). The C(α)-backbone of HEWL Ser-60 to Asp-101 carrying *Epitope 2* is displayed in black. The small protein APP represents a modified '$\alpha l\alpha$'-folding unit (*cf.* **2** in *Fig. 4*) with a *N*-terminal Pro-helix. The backbone of the Pro-helix offers a somewhat different geometry for the fit of the remote Trp residue.

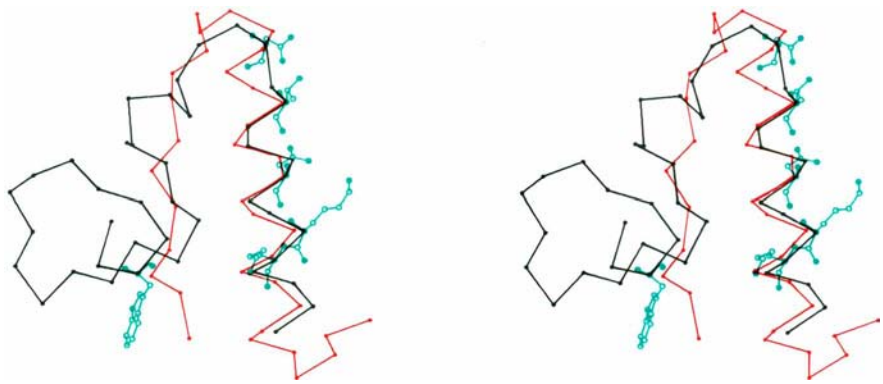

Fig. 9. *Stereoplot of the C(α)-backbone of the Ser-60 to Asp-101 HEWL sequence with the* Epitope 2 *side chains* (green), *showing the best fit of* Epitope 2 *to the selected host, avian pancreatic polypeptide* (APP, [30] C(α)-backbone displayed in red). The atomic coordinates of APP are from the *Brookhaven Data Bank* ([34], File No. 1PPT). Other conventions are the same as in *Fig. 6*.

Stabilization of the Pro-helix originates from hydrophobic contacts of conserved Pro-residues in position 2, 5, and 8. This example clearly demonstrates that the accessibility of various folding unit topologies *(Fig. 4)* is a prerequisite for matching more complex discontinuous epitopes.

Conclusions and Outlook. – A general procedure is presented which allows the conservation of the conformational properties of protein epitopes by synthetic peptides with chain lengths of 15–40 amino-acid residues. Due to intrinsic limitations of the approaches in use, only antigenic sites with high segment mobility in the protein have constituted a realistic target for the construction of synthetic vaccines so far. With the various procedures described here for the retention of epitope topologies, the whole protein surface – comprising continuous and discontinuous determinants – becomes accessible for the modelling of antigenic properties. Therefore, improvements in the empirical prediction of antigenic sites from the primary sequence of a protein – most notably the prediction of secondary structures and amphiphilic stretches – become of utmost concern.

In our laboratory we have recently suceeded in the synthesis of $\beta\alpha\beta$-type folding units [20] and presently focus our interest on molecules of the type shown in *Fig. 5*. Immunological studies are performed on free peptides as well as on polyethylene-glycol-bound sequences [31]; as indicated by preliminary results, these PEG-peptides may provide some interesting features with respect to their immunological behavior [32].

In conclusion, we may note that the present approach takes advantage from well-established experimental foundations concerning the relationship between amino-acid sequence and secondary-structure formation of peptides. Most notably, the stabilization of a specific backbone conformation by chemical manipulations remains a privileged domain of the peptide chemist. Consequently, the developed concept should also strongly stimulate the field of peptide synthesis. Much intuition will be needed to realize the depicted goals; the new strategy for the construction of synthetic vaccines might become a valuable piece in the complex puzzle of immunological research.

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