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Review

Secondary structures of proteins and peptides in amphiphilic environments (A Review)

(amphiphilic surfaces/apolipoproteins/peptide hormones/peptide secondary structure/peptide toxins)

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ABSTRACT **Many peptides and proteins that act at lipid–water interfaces assume a unique amphiphilic secondary structure which is induced by the anisotropy of the interface. By using synthetic peptides in which these inducible amphiphilic structures have been optimized, one can show that the amphiphilic α helix is a functional determinant of representative apolipoproteins, peptide toxins, and peptide hormones. By increasing the amphiphilicity of the structurally important regions of the molecule, one can enhance the biological activity of the peptide even beyond that of the naturally occurring polypeptide. It is proposed that rigid amphiphilic secondary structures such as α helix, β sheet, or π helix will be found in most medium-sized peptides acting at membranes and lipid–water interfaces.**

A major objective of our research is to achieve an understanding of the structural determinants of biologically active peptides and polypeptides sufficient to permit us to design, in a rational fashion, new peptides with comparable or enhanced activities. Much progress has been made in the ability to predict tertiary structure from primary sequence information, but the point at which a macromolecule, such as an enzyme, possessing a great deal of tertiary structure can be designed from first principles has not yet been reached. Nevertheless, we have already succeeded in designing an enzymatic catalytic site by taking advantage of the preexisting tertiary structure present in an enzyme (1–3). In other words, we have carried out the "chemical mutation" of the catalytic site of an enzyme into a new reactive group capable of carrying out a different type of catalysis. For example, we have taken the hydrolytic enzyme papain and, by appropriate covalent modification with a flavin group, we have been able to obtain a semi-synthetic enzyme that oxidizes dihydronicotinamides. Ultimately, we would want to design not only the catalytic site of an enzyme but also a substrate binding site. Before such a project can be undertaken, one has to establish viable mechanisms by which tertiary structures can be generated.

The goals of our present work are more modest, and in the present review we shall discuss the progress we have made in designing biologically active peptides and polypeptides in cases in which the secondary structure rather than the tertiary structure is the dominant factor determining the binding characteristics of the peptides. It is the thesis of this work that, in those instances in which secondary structural characteristics dominate the biological activity and physical properties of a peptide or polypeptide, it is now possible to achieve the rational design of new sequences that have little homology to the naturally occurring ones but have similar or enhanced biological activity.

The present article focuses primarily on surface-active peptides—i.e., peptides that bind to amphiphilic surfaces such as phospholipid surfaces, membranes, receptors, etc. We are testing faces will have important regions comprising amphiphilic secondary structures complementary to those of the surfaces (4). To illustrate our approach to the design of surface-active peptides, we shall discuss the progress that has been made in preparing model peptides that mimic successfully the naturally occurring systems in the cases of apolipoproteins, peptide toxins, and peptide hormones.

The biological activity of a protein—be it an enzyme, a structural protein, or a receptor—strictly depends on its conformation because the functions of all proteins rely on the precise spatial positioning of several functional groups with respect to each other. Proteins achieve a well-defined three-dimensional structure by the juxtaposition of blocks of structural units which are stabilized by secondary structural forces (e.g., see refs. 5 and 6). Because secondary structures can be assumed only by peptides larger than a certain minimal size, proteins with the usual structural organization have to be at least 50–60 amino acids long. Below this limit, additional structural restraints are required to ensure the maintenance of a unique tertiary structure. Thus, the smaller proteins achieve rigidity by the presence of numerous intrachain disulfide bonds, and cyclic oligopeptides owe their unique conformation to the small peptide ring. Without these intrachain covalent bonds, medium-size peptides in general will not assume a unique, rigid, stereospecific conformation.

Yet, many oligopeptides serve as biological agents of exquisite specificity, even though they exist in solution in a multitude of ill-defined conformer states. In order to be able to express their specific function, these peptides must be induced to assume a special conformation. For these peptides, the inducer is usually a protein—an enzyme, a receptor, an antibody, etc. The induction is usually a result of stereospecific interactions between the ligand and the protein. Such interactions are limited by the number of groups that can be accommodated at a given "active site," usually not more than four or five groups. Thus, protein–ligand interactions are ideally suited to induce a specific conformation for small peptides acting as ligands.

It is most likely that most peptide hormones containing less than 10 amino acids achieve their active conformation this way. However, a large number of biologically active peptides have 10–50 amino acids and no intrachain linkages. How do these peptides maintain their active conformation? Do they undergo protein-induced conformational transitions? If not, is only a small fraction of the peptide active at any time? Because many of these peptides act on cell surfaces, they are in a very amphiphilic environment at the locus of their activity. We thought that in the case of many of these peptides this amphiphilic environment might induce the active conformation.

Abbreviations: apo A-I, apolipoprotein A-I; HDL, high density lipo-

APOLIPOPROTEIN

The first macromolecule to draw our attention in the course of our development of models for surface-active peptides was apolipoprotein A-I (apo A-I), the principal polypeptide constituent of high density lipoproteins (HDL) (7–9). From circular dichroism spectra of apo A-I solutions that suggested that the polypeptide was quite α-helical and from the amino acid sequence, it was proposed by Segrest *et al.* (10) a number of years ago that there are "amphipathic"—or, to conform to modern usage, amphiphilic—helical regions in the peptide chain. Subsequently, Fitch suggested (11) that through approximately two-thirds of the apo A-I molecule not only were there such amphiphilic α-helical regions but that these might be repeating units approximately 22 amino acids in length punctuated by helix breakers such as glycine or proline and connected by short peptide segments (hinges). The repeating pattern that Fitch found was not perfect; indeed, there were several cases in which hydrophobic residues appeared in otherwise hydrophilic regions of the helix or in which the converse was true. Nevertheless, Fitch's analysis of the repeating units appeared to us (4, 7, 11) to provide a very attractive hypothesis that could account for the ready ability of apo A-I to bind to the surface of the HDL particle.

In particular, if one considers the structural organization of the HDL particle with the phospholipids in a surface monolayer—the phospholipid head groups protruding into the aqueous solution and the fatty acyl chains oriented toward the interior of the particle—it could be readily visualized that the amphiphilic α-helical regions of apo A-I might lie on the surface of the HDL particle with the axes of the helices approximately tangential to the particle surface. This would allow the hydrophobic sides of the helices to penetrate into the space between the phospholipid head groups, thereby making contact with the hydrophobic chains of the phospholipid. The hydrophilic sides of the helices would be oriented toward the aqueous solution in the same direction as phospholipid head groups.

Although this picture appeared attractive, the question arose as to how such a proposal might be tested. As the application of physical chemical methodology to the study of protein–lipid interactions progresses, it should be possible eventually to determine directly the structural characteristics of apo A-I bound at the surface of the HDL particle. However, at the present time, solution of this structural problem by physical methods is a most formidable undertaking. Our approach to testing the hypothesis that it is the induction of amphiphilic α-helical segments in the apo A-I molecule that allows it to bind effectively to the phospholipid surface present in the HDL particle has been an organic chemical one. Specifically, we have undertaken to test the structural basis of peptide binding to the phospholipid surface by designing and preparing peptides that are predicted to have the desired secondary structural characteristics (4). In taking this approach, one route would be to prepare segments corresponding to regions of the naturally occurring apo A-I molecule. Although for a polypeptide for which tertiary structure is crucial to biological and physical properties it would be unsatisfactory to prepare relatively short regions of the molecule and hope that a reasonable simulation of the properties of the intact molecule might be achieved, such an approach does not appear comparably unattractive for peptide systems in which tertiary structure is of little importance and the secondary structural characteristics dominate the basic properties. Nevertheless, we have not considered that the preparation of peptides corresponding to the native sequences of the naturally occurring system is an attractive route to testing the importance of the secondary structural characteristics that we have been examining (4). There are two reasons for this which can be appreciated from a consideration of our studies on peptide models for apo A-I.

First, we had prepared a number of peptide segments corresponding to the naturally occurring sequences, but we found that the shorter segments were not effective models for apo A-I. For larger segments, such as a 44-amino acid peptide chosen from a region that appears to have a considerable potential to form amphiphilic α helices, we have observed a moderately effective simulation of some of the properties of the apo A-I molecule, but a peptide corresponding to a prototypic 22-amino acid amphiphilic α-helical region of apo A-I was a poor model for the intact polypeptide (12). Thus, the preparation of the natural segments of apo A-I does not give a particularly attractive entry into models for the polypeptide. However, beyond the lack of success of such peptides in simulating the properties of apo A-I, it must be pointed out that, even if the smaller peptides had been found to possess properties similar to those of the intact polypeptide, one could not be sure that the reason the small peptides were active was that their amphiphilic α-helical structure was important. The alternative proposal could be made that a specific amino acid sequence corresponding to a kind of "active site" might have been prepared fortuitously by choosing the right peptide segment of the intact molecule for synthesis.

For the reasons just outlined, in our design of models we have chosen to take a different route. If a secondary structural characteristic such as an amphiphilic α helix is indeed crucial to the biological and physical properties of a peptide, then it should be possible to construct a new sequence with minimal homology to the natural sequence but having a similar secondary structure (4). For example, we have designed, synthesized, and characterized a docosapeptide, peptide 1, which, in its structural characteristics and fundamental properties, epitomizes the apo A-I molecule (7). Peptide 1,

5 10
Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Leu-Lys-
15 20
Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala,

consists principally of three types of residues: glutamate, as the negatively charged hydrophilic residues; lysine, as the positively charged residues; and leucine, as the neutral hydrophobic residues. These amino acid residues were chosen because in each of their categories they have very high helix-forming potential (13). Additionally, because we have a great deal of experience in preparing peptides by the solid-phase method (14, 15) using alanine as the COOH-terminal residue, this residue was chosen as the COOH terminus of the model peptide. Because proline and glycine residues function as helix breakers in the naturally occurring sequence, we decided to place a proline residue at the NH_2 terminus of the model peptide, although this was probably not essential to its design. As can be seen from the helical projection (16) of peptide 1 (Fig. 1), approximately one-third of the amphiphilic α helix surface is hydrophilic, and the rest is hydrophobic.

First we asked whether peptide 1 forms an α helix in solution. The ultraviolet circular dichroism spectra over a range of peptide concentrations showed that there was considerable α-helical character for it. The measured $[\theta]_{222\ nm}$ was dependent on the peptide concentration. Quantitative analysis of the concentration dependence of the mean residue ellipticity gave results that could be interpreted in terms of a monomer–tetramer equilibrium for the peptide. At low peptide concentration we estimated from the $[\theta]_{222\ nm}$ that the peptide was approximately 30% α-helical, whereas at high concentration it was calculated to be about 50% helical (7), a value in reasonable accord with

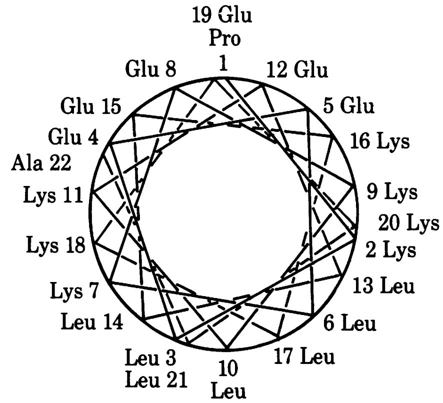

FIG. 1. **Peptide 1 represented to show amphiphilic α-helical segment on an Edmundson helical wheel.**

that estimated for apo A-I. The interpretation that the docosapeptide is involved in a monomer–tetramer equilibrium was confirmed by a molecular weight measurement: at high peptide concentration the molecular weight corresponded to that of the tetramer. For apo A-I there is a similar aggregation of the peptide chains, resulting in the formation of polymeric forms up to an octamer (17). Thus, it is only the helical conformation of peptide **1** that is conducive to self-association by forming tetrameric peptide micelles.

Another method of physical characterization of the apo A-I molecule has been through monolayer studies at the air–water interface. When the docosapeptide model was compared to apo A-I in behavior at the air–water interface, considerable similarity between these peptide systems was seen. In particular, the collapse pressure measured for the monolayer formed by either was approximately 21 dynes/cm (17).

A very important characteristic of apo A-I is its binding to phospholipid. It was the consideration of the way in which this molecule might bind to a phospholipid surface that initiated our modeling studies. Because we wished to make as quantitative a comparison as possible between the binding of our docosapeptide model and of apo A-I to phospholipids, we had to develop an appropriate experimental approach. We prepared unilamellar vesicles from egg lecithin according to the Korn and Batzri injection procedure (18), and we measured the binding of the peptides to the vesicles by using either a rapid ultrafiltration method or rapid gel filtration through a crosslinked Sepharose column (7). The peptide concentrations generally were measured with reagents such as fluorescamine or *o*-phthalaldehyde which detect free amino groups. When the binding of either apo A-I or peptide **1** to the egg lecithin vesicles was measured, we observed saturation behavior, and the dissociation constants measured for the peptides were nearly identical. Specifically, K_d was approximately 10^{-6} M for the binding of apo A-I to egg lecithin vesicles and 2×10^{-6} M for the binding of peptide **1**. Next, we measured the relative binding to cholesterol-containing vesicles in which the phospholipid/cholesterol ratio was 4:1. In this case, the binding of the apo A-I molecule to the vesicles was improved somewhat from that seen with pure egg lecithin, giving a K_d approximately one-third as large. In contrast, peptide **1** bound about 50% less tightly to the mixed vesicles than it did to pure egg lecithin (8).

Thus, although peptide **1** is still a reasonable model for apo A-I in its binding to the mixed vesicles, it does not behave as well compared to apo A-I as it does in the binding to pure egg lecithin. In examining the proposed helical regions (17) of apo A-I, we noted that, in some instances, polar hydrophilic residues were located in predominantly hydrophobic regions of the helices. In particular, there were two arginine residues (Arg-116 and Arg-123) located in what otherwise would be hydrophobic regions of apo A-I (7, 8). We wondered if the 3-OH group of cholesterol might have a deleterious interaction with the hydrophobic portion of the amphiphilic α helix of **1** that is inserted into the vesicles. In that event, the rather favorable interaction of apo A-I with the mixed lecithin/cholesterol vesicles might be due to the presence of polar arginine residues in the predominantly hydrophobic regions of the respective amphiphilic α helices. Therefore, we undertook to incorporate an arginine residue into the hydrophobic region of an amphiphilic α helix like **1** in order to assess the role of the 3-OH function of cholesterol in peptide–cholesterol interaction.

The newly designed docosapeptide, peptide **2**,

```
               5                   10
Pro-Lys-Leu-Glu-Glu-Leu-Lys-Glu-Lys-Arg-Lys-
           15                  20
Glu-Leu-Leu-Glu-Lys-Leu-Lys-Glu-Lys-Leu-Ala
```

contained an arginine residue at position 10 in what would be otherwise a completely hydrophobic region on the amphiphilic α helix (see helical projection of peptide **1** in Fig. 1). Binding of this peptide to cholesterol-containing vesicles was appreciably tighter than to the pure egg lecithin vesicles. This behavior was strongly reminiscent of that of apo A-I itself (19). Although the differences in the binding constants for the interaction of peptide **2** with the cholesterol-containing vesicles and with the pure egg lecithin vesicles are small, they nevertheless are comparable to the difference seen for apo A-I. This clearly demonstrates that, through the approach that we have undertaken, it is possible to fine-tune the binding of amphiphilic peptides to lipid or phospholipid surfaces. Because we use an experimental system that allows us to determine directly thermodynamic quantities such as dissociation constants, we are able to assess the interactions of peptides with lipids or phospholipids quantitatively.

PEPTIDE TOXIN

In considering the modeling of increasingly complicated amphiphilic secondary structures, we thought that it would be worthwhile at the next stage to try to design a peptide containing not only an amphiphilic secondary structural feature but also an active center. In surveying possible candidate peptides for this type of modeling our attention was drawn to the bee venom toxin, melittin. This 26-amino acid peptide is an activator of phospholipase A_2 and is able to lyse erythrocytes. Consideration of the amino acid sequence of the peptide and of various data in the literature concerning binding to phospholipids and other physical characteristics (20), led us to the conclusion that the NH_2-terminal 20 amino acids of the peptide chain might be forming a rather hydrophobic amphiphilic α helix (21). According to this picture, the proline residue present in this region might cause a kink in the helix. Alternatively, melittin might have a structure in which the proline would be flanked on either side by two shorter helical segments. In addition to the amphiphilic α helix, according to our analysis, there appears to be a hexapeptide active site region at the COOH terminus containing a cluster of positive charges. Indeed, melittin-(1–20), which lacks this hexapeptide portion, does not lyse erythrocytes but apparently is quite capable of binding to them (22).

To test our structural hypothesis for melittin we designed

peptide 3,

5
H_2N-Leu-Leu-Gln-Ser-Leu-Leu-Ser-Leu-Leu-
10 15
Gln-Ser-Leu-Leu-Ser-Leu-Leu-Leu-Gln-Trp-
20 25
Leu-Lys-Arg-Lys-Arg-Gln-Gln-$CONH_2$

which was made as nonhomologous as possible to the native peptide in the NH_2-terminal 20 amino acids (21). Because our picture of melittin suggested that the proline residue probably was not an important factor in the lytic activity of the peptide, in our structural modeling we decided to replace this residue with serine. The hydrophobic side of the helix in peptide 3 (Fig. 2) was made up of leucine residues which we chose for their high helix-forming potential, hydrophobicity, and electrical neutrality. Although glutamine seemed to be the optimal choice for the neutral hydrophilic residues, some serine residues were included to increase the hydrophilicity of the model, permitting us to match the amphiphilicity of the native peptide. In our model peptide 3, a tryptophan was retained at position 19 for studies of intrinsic fluorescence, and the COOH-terminal hexapeptide portion of melittin was also preserved.

Circular dichroism measurements on solutions of the model peptide at pH 7.0 suggested (concentration dependency of the mean residue ellipticity at 222 nm) that peptide 3 might be aggregating. By sedimentation equilibrium centrifugation peptide 3 appeared to be tetrameric at a concentration of 2.5×10^{-5} M (21). Melittin similarly forms tetramers (20, 23). From the circular dichroism data, the helix content of peptide 3 was calculated to be 69% for the tetramer and 35% for the monomer (21). In the case of melittin the corresponding values are 48% and 18%, respectively.

Both peptide 3 and melittin form stable monolayers at the air–water interface. Their surface pressure–area curves show discontinuities at 45 and 22 dynes/cm, respectively, indicating collapse of the monolayers. The higher collapse pressure and also the larger limiting area per molecule of peptide 3 compared to melittin indicated that peptide 3 is able to form a longer amphiphilic segment than melittin.

The hemolytic activity of the model peptide as measured by a 30-min-incubation assay was found to be appreciably greater than that of melittin (21). Peptide 3 has a higher surface affinity than native melittin, consistent with a more extended helical structure for the model peptide, and this is important for cell lysis. We have examined the kinetics of the lysis of human erythrocytes by melittin and model peptide 3 (24). A complete description of the results is beyond the scope of this article, but briefly our findings show that melittin or the model peptide binds rapidly to the outer surface of the erythrocyte membrane, and the surface-bound peptides produce transient openings through which hemoglobin molecules can escape. At the same time, melittin loses its ability to cause rapid lysis, presumably by translocation through the bilayer. In a substantially slower process, internalized melittin produces transient membrane openings in a steady state. Comparison of the results with peptide 3 to those with melittin show that, on a molar basis, the synthetic analogue produces a fast process that is similar to that caused by melittin but is more efficient than the latter in the slow phase. The results we have obtained indicate that the functional units sufficient for the activity of melittin-like cytotoxic peptides are a 20-amino acid amphiphilic α helix with a hydrophobic/hydrophilic ratio that is >1 and a short segment with a high concentration of positive charges.

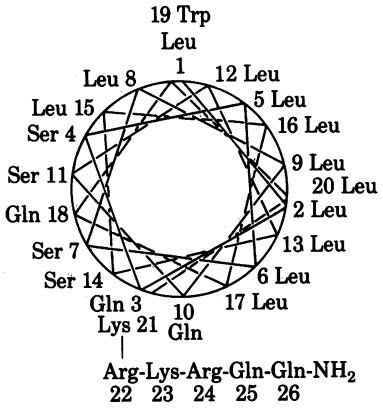

FIG. 2. Peptide 3, represented to show amphiphilic α-helical segment on an Edmundson helical wheel.

HORMONE

In view of our findings with the model peptides which simulate the activity and properties of apo A-I and melittin and the limited possibilities of well-defined secondary structures for peptides, we explored the possibility that amphiphilic secondary structures could be of importance in hormones as well (21, 25). We examined first the question of which hormones might contain amphiphilic helical regions and focused our attention on peptides that are at least 20 amino acids in length and do not contain multiple disulfide bridges. We concluded that human β-endorphin (Fig. 3), a 31-amino acid peptide with potent opiate activities, is an excellent candidate for the structural approach used for the design of models for apo A-I and melittin (25). Our analysis of the amino acid sequence of β-endorphin revealed a potential amphiphilic α or π helix in the COOH-terminal region, residues 13–29. In the α-helical arrangement, residues 13–29 would form an amphiphilic structure in which the hydrophobic domain would cover half of the surface of the helix and would twist around the length of the helix with the hydrophilic residues being either neutral or basic. In the π-helical form, these residues would form a similar amphiphilic structure except that the hydrophobic domain would run straight along the length of the helix. The importance of lipid interactions in the stabilization of helical structure on the opiate receptor has been suggested for β-endorphin. Also, the possibility exists that an amphiphilic helical structure in β-endorphin could stabilize the molecule against proteolytic degradation, either through intramolecular hydrophobic interaction with the enkephalin region or by intermolecular interactions.

Unlike residues 13–29, residues 6–12 of human β-endorphin have little propensity for formation of secondary structure and are not hydrophobic. We have suggested that residues 6–12 serve as a spacing link between the specific enkephalin sequence (residues 1–5) and the amphiphilic helical region (25). We have now designed, synthesized, and tested three β-endorphin models, peptides **4**, **5**, and **6** (ref. 26; unpublished data).

Peptide 4 is homologous to β-endorphin in residues 1–19 and nonhomologous in residues 20–31 (Fig. 3). Residues 13–31 of peptide 4 could form an amphiphilic α helix which would be similar to that postulated for β-endorphin in this region, except that the hydrophobic domain would extend to the COOH terminus and lay straight along the length of the helical structure (Fig. 4 *Left*). Peptide 4 exhibited behavior similar in many respects to that of β-endorphin, including strong binding to both δ- and μ-opiate receptors, high activities in opiate assays on guinea pig ileum and rat vas deferens preparations, and considerable resistance to proteolytic degradation. The most notable differ-

	5 10
β-Endorphin	H_2N-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-
Peptide 4	H_2N-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-
Peptide 5	H_2N-Tyr-Gly-Gly-Phe-Met-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Pro-
Peptide 6	H_2N-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-

15 20 25 30
Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
Leu-Val-Thr-Leu-Phe-Lys-Gln-Leu-Leu-Lys-Gln-Leu-Gln-Lys-Leu-Leu-Gln-Lys-OH
Leu-Leu-Gln-Leu-Trp-Gln-Lys-Leu-Leu-Lys-Gln-Leu-Gln-Lys-Leu-Leu-Gln-Lys-OH
Leu-Leu-Lys-Leu-Leu-Gln-Lys-Leu-Leu-Leu-Gln-Lys-Leu-Phe-Lys-Gln-Lys-Gln-OH

FIG. 3. Amino acid sequences of β-endorphin and peptides **4**, **5**, and **6**.

ences between peptide **4** and β-endorphin included the ability of peptide **4** to self-associate at low concentration, the slowness of its action in the two assays, and its considerable nonspecific binding to rat brain homogenates.

Peptide **5** was designed as a complete structural model of β-endorphin. Only the [Met^5]enkephalin region at the NH_2 terminus, the specific opiate receptor recognition site, and Pro-13 as a helix-breaking residue were retained from the natural sequence of β-endorphin. [There were three additional sequence homologies (Ser-10, Leu-14, and Leu-17) which are coincidental to the design of peptide **5**.] In the region residues 6–12, an alternating Ser-Gly sequence was used to mimic the hydrophilic spacer region proposed for the corresponding β-endorphin residues. This sequence is likely to form a flexible hydrophilic peptide chain with little tendency for secondary structure formation. For peptide **5** (Fig. 4 *Center*), residues 14–31 were selected

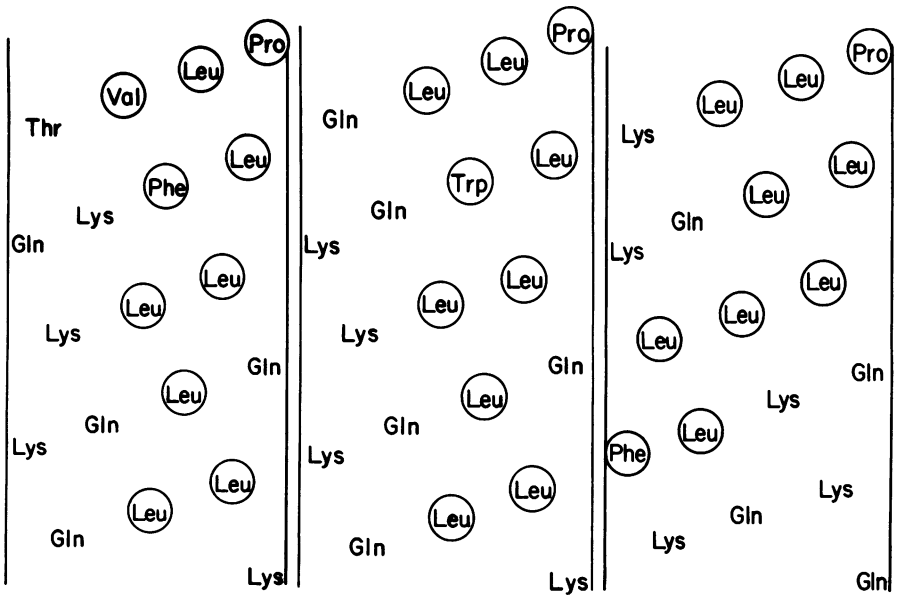

FIG. 4. Residues 13 through 31 represented on an α-helical net (26). (*Left*) Peptide **4**. (*Center*) Peptide **5**. (*Right*) Peptide **6**.

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