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COMMENTARY

STUDIES OF PEPTIDE CONFORMATION IN THE DESIGN OF PEPTIDE AGONISTS

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In the proper biological milieu most peptides take well-defined conformations which are responsible for their unique properties. The study of peptide conformations is fundamental not only to the understanding of the action of chemotactic agents, enzyme inhibitors, peptide hormones, and toxins, but also to the elucidation of the factors that stabilize the three-dimensional structures of proteins. The physical techniques that have been employed in probing peptide conformations are mainly spectroscopic, including nuclear magnetic resonance, infrared, Raman, X-ray and ultraviolet circular dichroism spectroscopy. For some enzyme inhibitors, it has been possible to study directly the structures of their complexes with the respective enzymes by the use of techniques such as X-ray crystallographic analysis. However, in many instances such as the peptide hormones it has not been feasible to study the complexes of the biologically active peptides with their receptors, frequently because of major technical problems such as the lack of availability of sufficient quantities of receptors in their biological environments.

An alternative approach to the elucidation of the biologically relevant conformations of peptides has involved the synthesis of peptide analogs in which the effects of amino acid composition and sequence on peptide structure and function were probed. In the past, however, this approach has been limited to the replacement of a single or, at most, a few amino acids in each analog in order to determine the role of the specific side chains in the interaction of the peptides with their ligands or receptors. In many of these studies very little attention was given to the overall topology of the peptide.

In the approach that we have used for the construction of models for peptides and proteins ranging from apolipoproteins through chemotactic agents, hormones and toxins, we have prepared synthetic peptides designed to form secondary structures similar to those of the naturally-occurring systems in the appropriate regions, but employing non-homologous primary sequences of amino acid residues [1, 2]. To test our hypotheses concerning the importance of particular secondary structural conformations for the biological activity of the peptides under study, we have examined the abilities of the model peptides to reproduce the properties of the natural peptides despite the significant differences between the models and the natural systems in their primary amino acid sequences in the regions of interest.

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Models of apolipoprotein A-I

We have proposed that peptides and proteins which bind to biological interfaces where the environments are amphiphilic form complementary amphiphilic secondary structures [3]. In our initial test of this hypothesis we employed our peptide modelling approach in the investigation of the importance of secondary structure in the activity of apolipoprotein A-I (apo A-I), the major protein constituent of plasma high density lipoproteins [4]. The examination of the 243 residue sequence of apo A-I suggested the presence of 6 or 7 rather homologous 22 residue units capable of forming α-helical structures, each having a hydrophobic domain lying along one side of the helix [5, 6]. The importance of the formation of such amphiphilic helical structures in the apo A-I molecule when this molecule binds at the hydrophobic–hydrophilic interface on the lipoprotein surface had already been proposed [7].

In our peptide modelling approach, we designed a 22 residue model peptide which reproduced the most prominent characteristics of the repeating amphiphilic α-helical segments proposed for apo A-I and examined the physical and biological properties of this model [4]. The distribution of the amino acid residues of the model on the surface of an α-helix is illustrated in Fig. 1 using a helical net diagram. In the α-helical form, the hydrophobic residues are segregated in a single domain that lies along the length of the helix, parallel to the helix axis, and covers approximately one-third of the helix surface. The hydrophilic face of the α-helix consists of approximately equal numbers of basic and acidic residues, with the basic residues positioned nearest the hydrophobic–hydrophilic boundary. In the design of our model peptide, leucines were employed as hydrophobic residues, lysines as basic hydrophilic residues, and glutamates as acid hydrophilic residues. All of these residues are believed to have a high α-helix-forming potential. Additionally, since helix-disrupting proline residues occur at regular intervals in the apo A-I sequence, this residue was used at the N-terminus of our model peptide. An

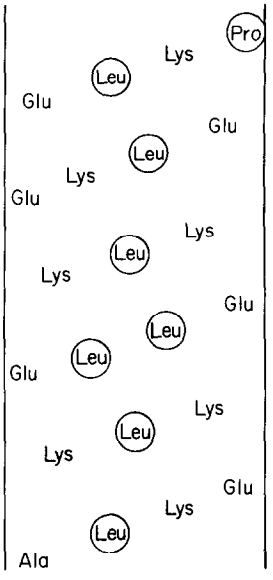

Fig. 1. Distribution of residues in the amphiphilic helical model peptide mimicking apolipoprotein A-I, as illustrated in a helical net diagram. The circled residues are hydrophobic and form a continuous surface lying along the helix axis.

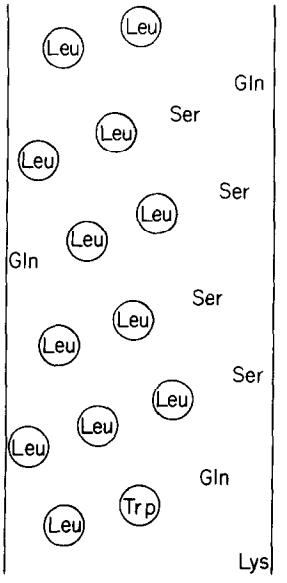

Fig. 2. Distribution of residues in the amphiphilic helical region of the model melittin peptide, as illustrated in a helical net diagram. The circled residues are hydrophobic and form a continuous surface lying along the helix axis.

alanine residue was present at the C-terminus for synthetic reasons. The design we employed resulted in a model apo A-I peptide which has a high potential to form a secondary structure that epitomizes the amphiphilic α-helical structures proposed for a large part of the apo A-I molecule, but which has minimal sequence homology to any regions of the native polypeptide. We found that the model apo A-I peptide reproduced all of the fundamental properties of apo A-I itself. Not only did the model peptide show binding to phospholipid similar to that exhibited by the apo A-I protein, comparable surface activity in monolayers formed at the air–water interface, but also binding of the model peptide to lecithin vesicles resulted in the substrate activation necessary for the catalytic action of the enzyme lecithin:cholesterol acyltransferase [8]. The similarity of the properties exhibited by the model peptide to those of apo A-I, combined with the principles employed in its design, provided compelling evidence that the fundamental properties of apo A-I result from the amphiphilic α-helical structures that it can form and do not depend on any unique sequence of amino acid residues in its primary structure. Subsequent to investigation of the properties of the 22 amino acid model peptide, we prepared the 44 amino acid peptide which is the corresponding dimer [9]. We found that the 44 peptide simulates even better the properties of apo A-I, and on this basis have suggested that the basic structural unit responsible for the activities of apo A-I consists of two amphiphilic α-helices flanking a β-turn region.

A model toxin

In the next stage of our modelling approach to the elucidation of the biologically active conformations of important peptides, we investigated the design of a model for melittin, a 26 residue hemolytic peptide that forms the main toxin component of bee venom [10, 11]. We proposed that the lytic activity of melittin on erythrocytes depended on the presence of a 20 residue amphiphilic α-helix which was rather hydrophobic and which was connected at its C-terminal end to a highly basic hexapeptide that functions as a kind of "active site". The synthetic peptide that we prepared to test this hypothesis modelled the amphiphilic α-helix postulated for melittin with the same considerations for hydrophobic–hydrophilic balance, charge distribution, maximal α-helix-forming potential and minimal homology to the natural sequence as were employed in developing models for apo A-I. In the case of the melittin model, the hydrophobic surface of the helix was composed of leucines and covered two-thirds of the surface area, while the hydrophilic surface was comprised of neutral Ser and Gln residues. The helix in the model was connected to a highly basic hexapeptide at its C-terminus in which the amino acid sequence of the corresponding region of melittin itself was retained. The distribution of the residues of the model amphiphilic α-helical region on the helix surface is illustrated in the helical net diagram in Fig. 2.

The model melittin peptide self-associated in a tetrameric form with high α-helical content and formed stable monolayers at the air–water interface with a high collapse pressure, a low compressibility and a small limiting area, as expected for an amphiphilic α-helical structure and which corresponded to the properties of melittin [10]. Most importantly, the model peptide lysed egg lecithin vesicles and lysed human erythrocytes with kinetics similar to those observed for naturally-occurring melittin [11].

Design of peptide agonists with hormonal activity

In the next stage of our modelling studies, we turned to the examination of peptide hormones. We

proposed that there are three categories of biologically active structures formed by peptide hormones [2, 10, 12]. In the first category one encounters relatively short peptides such as [Met^5]-enkephalin and [Leu^5]-enkephalin where the entire structure constitutes a specific recognition site that determines the interaction of the hormone with its receptors. To probe the structural requirements in such recognition sites, generally it is important to investigate a large number of synthetic analogs as is usually done in classical pharmacological studies of small organic molecules. In the second category of peptide hormone structures, including compounds like insulin, the molecules encountered are complex, frequently involving the presence of multiple disulfide linkages, and tertiary structural considerations often play a major role. In the third category of peptide hormones which will form the main focus of the remainder of this article, we have suggested that the sequences which are composed of 10 to 50 amino acids and which have, at most, one disulfide bridge the binding to the amphiphilic environment of a receptor might require the presence of a complementary amphiphilic secondary structure in the peptide. To a first approximation, in designing models for such peptide hormones we have been able to neglect tertiary structural considerations. There are several functions that the amphiphilic secondary structures in the third category of peptide hormones may have. These regions may function to present the specific recognition sites (active sites) of the hormones in the proper geometry to interact in a productive fashion with the corresponding receptor. A second possible role of the amphiphilic secondary structures in peptide hormones may be to assist the diffusion of the hormones to the receptors. In particular, induction of the amphiphilic secondary structures when the peptide hormones encounter surfaces near the receptors may allow the problem of diffusion of the hormone to the receptor to be reduced to a two-dimensional one rather than a three-dimensional problem. In principle, the hormone can utilize the amphiphilic secondary structures in moving along a surface searching for its receptor. A third point to be considered is that, although in a first approximation of our modelling of peptide hormones and other surface active peptides we have neglected folding, there may well be intramolecular interactions of the amphiphilic secondary structural region with other regions of the hormone molecule which could lead to stabilization of the hormone to enzymatic degradation.

The approaches we have used for the design of analogs of peptide hormones which act as agonists are very similar to the methodology we have employed for the apolipoproteins and peptide toxins [1, 2]. First, we have searched the peptide sequences for regions that might form amphiphilic secondary structures. This search can be carried out initially with the use of simple paper and pencil diagrams like the helical net diagrams [13] of Figs. 1 and 2 or the Schiffer–Edmundson helical wheel diagrams [14] we have presented in other articles [1, 2]. Additionally, we have used literature information on the identification of the "active site" or specific recognition sites where such information is available. In many of the peptide hormones for which we have designed analogs, at least part of the specific recognition site lies at the N-terminus. Once we have a rough picture of the possible structural regions in a hormone molecule which can include the specific recognition site, amphiphilic secondary structure regions and often a spacer region, we build models using either space-filling models or a computer graphics system. Inspection of the models not only shows us in greater detail the steric interactions of the various structural regions but also can provide us with a feeling for the prominence of particular functional groups whether they be found in a specific recognition site, in a linker, or in a secondary structural region. After we are in a position to propose a structural model for the biologically active conformation of the peptide, we proceed to the design of one or more analogs to test our hypothesis. What we have tried to do initially has been to maintain the amino acid sequence of the specific recognition site and to construct the amphiphilic secondary structural regions from amino acids where the sequence is as different as possible from the natural one but has the potential to form a comparable secondary structure. In the cases of some hormones such as β-endorphin, we have also explored the replacement of the linker region by non-homologous segments. In designing the secondary structural regions of our model agonists, we have tried to choose the proper type of amino acid for a particular type of secondary structure. A problem which we encounter here is that information about the probability that certain residues tend to go into specific secondary structures comes generally from structural information obtained by X-ray crystallography on globular proteins [15]. However, it is by no means clear that the amino acids which would tend to go into certain secondary structures in a globular protein necessarily will form similar structures when they are present in a peptide or protein which has a structure which is induced by a biological interface such as a phospholipid surface. Nevertheless, at this point the best we can do is to proceed with the information we have from globular proteins on the frequencies with which particular residues are found in certain secondary structures. Other aspects of the models which must be considered are the maintenance of the overall charge balance, the distribution of charged groups in the amphiphilic secondary structural regions, and the maintenance of hydrophobic–hydrophilic balance. Finally, and perhaps most importantly in terms of the modelling approach, we try as much as possible to reduce the sequence homology between the secondary structural regions of the analogs and those of the naturally-occurring peptides which we are trying to simulate. This is done, of course, in order to strengthen the argument that in these regions it is really the secondary structure which matters rather than the specific amino acid structure. Clearly, if one were to focus entirely upon obtaining the most active analog, it would not necessarily be the best course of action to eliminate specificity in the secondary structural regions to the extent which we usually do in developing our models. There is a considerable likelihood even in the amphiphilic secondary structural regions where the surface characteristics of the

secondary structure dominate the effect of the region on the biological and physical properties of the hormone that there still may be appreciable specificity due to the recognition of certain residues [16, 17]. However, in making a test of a structural model for a peptide hormone we believe that it is very important to examine the more extreme cases that we can generate by reducing the sequence homology between the model and the natural hormone in the putative secondary structural regions.

Having discussed the background of our modelling approach, its application to apolipoproteins and toxins, and the way in which we have extrapolated the approach to the design of peptide analogs, we will turn briefly to some specific examples of the design of agonists of important peptide hormones.

First, we will consider the case of calcitonin. We have proposed a structural model for this 32 amino acid, calcium-regulating hormone in which we suggested that there are three structural regions important to the biological activity of calcitonin [18, 19]. These included: a cyclic segment containing 7 residues at the N-terminus which is tied together through a disulfide bond between Cys-1 and Cys-7; an amphiphilic helix spanning the region from residue 8 to position 23, a Pro residue; and a hydrophilic "spacer" unit covering the sequence from residue 23 to residue 32, the carboxyl-terminal proline amide. In our first design of a peptide agonist analog for calcitonin, which we designated MCT-I, we tried to optimize the amphiphilic α-helical structure in the region from residues 8–22 (Fig. 3), while maintaining as minimal a sequence homology to natural calcitonin as is possible [18]. While the surface characteristics and other physical properties of this analog such as its α-helicity were accentuated relative to those of salmon calcitonin I (SCT-I), the most active naturally-occurring calcitonin, MCT-I was between 10- and 20-fold less potent than SCT-I, showing biological activity comparable to that of the most active mammalian calcitonin, the porcine species. Examination of the biological activity of several of the naturally-occurring calcitonins, together with their putative amphiphilic helical structures, led us to the proposal that the significant differences between the structures of SCT-I and MCT-I in the amphiphilic secondary structural region are likely to involve the Glu residue at position 15 of SCT-I which is a Leu residue in the corresponding position of MCT-I and the Trp residue at position 12 of MCT-I which is a Leu residue in the corresponding position of SCT-I, both of these positions being located on the hydrophobic face of the α-helical segment. All of the natural variants of calcitonin have hydrophilic amino acids, usually Asp or Glu, in position 15. Further, the mammalian hormones which are less active than some of the hormones from lower species have aromatic residues in positions 12, 16 or 19, while the ultimobranchial peptides do not [20]. In view of these observations we designed our second model, MCT-II, to be similar to MCT-I with the exception that on the hydrophobic face of the amphiphilic helical region of MCT-II a Leu residue was placed in position 12 and a Tyr residue was put in position 22, as in the case of SCT-I itself. In Fig. 3 we show the amphiphilic helical regions of MCT-I, MCT-II and SCT-I.

We found that the biological activity of the second model, MCT-II, was similar to that of SCT-I despite the low sequence homology, particularly for the hydrophilic residues, between the analog and the natural species in the region from residues 8 to 22 [19]. We found that the receptor binding ability of MCT-II in brain and kidney membranes, the activation of adenylate cyclase, and the hypocalcemic potency of the MCT-II analog were comparable to those of SCT-I. These results demonstrate that the amphiphilic α-helical structure of MCT-II is important for binding to calcitonin receptors. Additionally, our findings for MCT-II show that the presence of a hydrophilic residue, either an Asp or Glu, which usually occurs on the hydrophobic face of the α-helical region in the natural calcitonins is not required for high biological activity. Also, our results suggest that the presence of Trp at position 12 in MCT-I has a substantial effect in terms of the relatively lower biological activity of MCT-I.

Recently, we obtained evidence that calcitonin gene-related peptide (CGRP), a 37 residue peptide hormone which has been postulated to be a neurotransmitter [21], contains a significant amphiphilic secondary structural region [22]. We found that, in a structure-forming solvent such as 50% trifluoroethanol, the ellipticity measured in the circular dichroism spectrum at 222 nM suggests that approximately 46% α-helix is formed at a CGRP concentration of 4×10^{-6} M, a result similar to that seen under similar conditions for salmon calcitonin. Also, for rat CGRP the formation of stable monolayers at the air–water interface has been demonstrated, and the surface pressure at which these monolayers collapse was found to be similar to the monolayer collapse pressure of SCT-I. In an analogy to our analysis of the calcitonin structure, we have proposed that CGRP consists of three structural regions. The first corresponds to the region from the N-terminus through residue 7 of calcitonin and

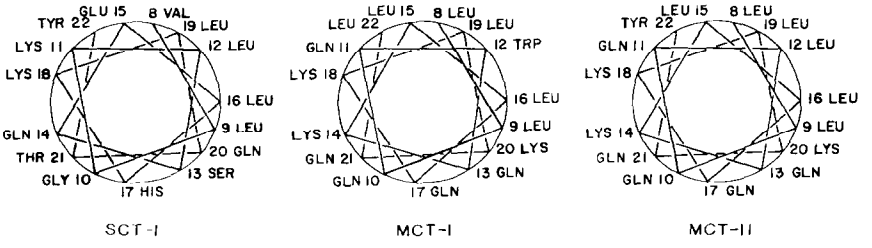

Fig. 3. Schiffer–Edmundson helical wheel projections of the amphiphilic helical regions from residues 8–22 in SCT-I, MCT-I and MCT-II.

includes the disulfide linkage between residues 2 and 7 of CGRP. Additionally, there is a potential amphiphilic helix which runs from residue 8 to a point which is uncertain but could be anywhere from residue 25 to residue 29. Finally, there is a spacer region from the C-terminal end of the helical region to the C-terminal residue of the entire peptide. It is possible that the structure could involve a β-turn from residues 28 through 31, including the Pro residue at position 29. We have considered the possibility that if such a β-turn were formed it could allow the spacer region of CGRP to fold over the proposed amphiphilic helix in such a way that the Phe amide present at the C-terminus of both the human and rat forms is brought near to the disulfide loop connecting positions 2 and 7. We are presently in the course of testing models that have been constructed and designed to contain potentially amphiphilic α-helical structures which should aid us in determining the physical, chemical and pharmacological properties of CGRP in relation to the secondary structure of the hormone.

Another hormone which we have modelled extensively is β-endorphin [22–26]. In this instance we have suggested the presence again of three basic structural units [23, 24]. These include: a specific opiate recognition site at the N-terminus (residues 1–5); a hydrophilic connecting link (residues 6–12); and the potential amphiphilic helix which comprises the region from residues 13–31. There is an ambiguity as to whether we are dealing with an amphiphilic α-helix or an amphiphilic π-helix in the C-terminal region. The hydrophobic domain would twist along the length of the helix in the α-helical form. On the other hand, in the π-helical form the hydrophobic domain would lie straight along the length of the helix. At the present time one cannot state with confidence which of these two hypotheses is the right one. However, in view of our substantial experience in the construction of α-helices we have built our β-endorphin models using this type of structure as a starting point for our design of the C-terminal region. Several peptide models for β-endorphin containing amphiphilic helical regions in their C-termini have been built by us. One of the most unusual agonists that we have constructed is a peptide in which, in addition to the design of an amphiphilic α-helical region from residues 13–31 with a sequence possessing minimal homology to that of β-endorphin, we included a hydrophilic spacer region from residues 6 to 12 having minimal homology to the equivalent region of the natural hormone. Besides this we have designed a peptide which is a negative model in which many of the important features of β-endorphin are retained with the important difference being, however, that in the model system in an α- or π-helical conformation of the C-terminal region the amphiphilic character present in our model peptides is absent. Our studies with the negative model, as well as with the various positive models for the amphiphilic helical region, have provided considerable evidence for the importance of an amphiphilic helical structure in the region from residues 13 to at least 29 and possibly 31. This structure affects the resistance to proteolysis of the natural hormone and makes some contribution to the interaction of the hormone with the opiate δ and μ receptors. We found that to observe high opiate activity on the rat vas deferens the amphiphilicity of the C-terminal helical structural region is essential. In contrast, however, interaction with the opiate receptors on the guinea pig ileum did not seem to have a similar requirement. We have also constructed a peptide model in which the whole C-terminal segment from residues 13–31 was constructed employing only D-amino acids [26]. Circular dichroism spectroscopy has provided evidence that under structure-forming conditions the C-terminal region of the model peptide assumes a left-handed helical arrangement. Furthermore, the observed receptor binding properties, stability to enzymatic attack and analgesic activity of this model peptide showed that it was very β-endorphin-like. In view of the major difference between the chirality of the amphiphilic helical region of β-endorphin and that of the model peptide we have concluded that the receptor binding and biological activity are dependent on the surface characteristics of the C-terminal helical region of β-endorphin rather than on the peptide backbone structure. Recently, we initiated studies on the preparation of β-endorphin models in which whole regions will be replaced systematically by non-peptide structure units. Initially, we focused on the hydrophilic link (residues 6–12) connecting the specific opiate recognition site at the N-terminus to the amphiphilic helix in the C-terminal region [22]. A model peptide containing a hydrophilic linkage consisting entirely of a repeating sequence involving a single amino acid, the *S*-isomer of γ-amino-γ-hydroxymethylbutyric acid, was prepared (Fig. 4). Although the new hydrophilic link does contain amide bonds, the distribution of these bonds is different from that which one sees in peptide structures. We observed that the β-endorphin model containing the new hydrophilic linkage and also having a right-handed helical region non-homologous to β-endorphin from residues 13 to 31 and having a twisted hydrophobic face exhibited binding to μ and δ receptors in guinea pig whole membrane preparations which was slightly enhanced relative to the binding observed with β-endorphin itself. Moreover, this model inhibited the twitching of electrically stimulated rat vas deferens with a potency about 7-fold less than that of human β-endorphin. Finally, the antinociceptive behavior of the model was comparable to that of a peptide agonist we had prepared

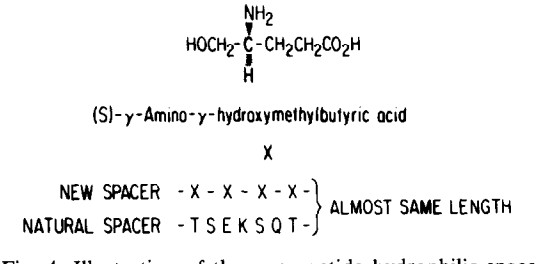

Fig. 4. Illustration of the non-peptide hydrophilic spacer region of a β-endorphin model consisting of four units of (*S*)-γ-amino-γ-hydroxymethylbutyric acid. For comparison, the sequence of the spacer region of human β-endorphin from residues 6–12 is also shown.

earlier that had the same amphiphilic helical region but had the human β-endorphin sequence in the hydrophilic linker region. These results encourage us to replace other regions of β-endorphin by non-peptide analogs and to pursue similar model-building in the cases of other hormones.

Several additional hormones have been shown by us to fit the hypothesis that amphiphilic secondary structural regions can be induced in either structure-forming solvents or at appropriate surfaces. The peptides examined by us include glucagon [16], growth hormone-releasing factor (GHRF), corticotropin-releasing factor [27], and parathyroid hormone. In the case of GHRF we were able to prepare a model 29 amino acid peptide which differed in 13 residues in the region 7–29 from the amino acid sequence of the naturally-occurring hormone, yet had a comparable potency.

Conclusion

The proposal that many peptides ranging from 10 to 50 residues in length contain important amphiphilic secondary structural regions has been tested successfully through the design of model peptides. This hypothesis provides strong guidelines for the preparation of new peptide agonists which can have higher stability and comparable, if not greater, potency than the corresponding natural hormones. While the most extensive applications of our modelling approach have been in the design of peptide agonists having hormonal activity, there are many applications to the preparation of models for other surface active peptides and proteins such as apolipoproteins [3, 28, 29], chemotactic agents [30], and toxins [10, 11].

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