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Peptide and Protein Synthesis by Segment Synthesis-Condensation

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The chemical synthesis of biologically active peptides and polypeptides can be achieved by using a convergent strategy of condensing protected peptide segments to form the desired molecule. An oxime support increases the ease with which intermediate protected peptides can be synthesized and makes this approach useful for the synthesis of peptides in which secondary structural elements have been redesigned. The extension of these methods to large peptides and proteins, for which folding of secondary structures into functional tertiary structures is critical, is discussed. Models of apolipoproteins, the homeo domain from the developmental protein encoded by the *Antennapedia* gene of *Drosophila*, a part of the Cro repressor, and the enzyme ribonuclease T_1 and a structural analog have been synthesized with this method.

IN THIS ARTICLE WE DESCRIBE EFFORTS IN OUR LABORATORY to prepare models for biologically active peptides and polypeptides in which we have focused our attention on secondary structural elements such as α helices and have, to a first approximation, been able to neglect tertiary structure (*1, 2*). We then proceed to the question of whether the principles that we have developed for the design of secondary structural units in such molecules can be extended to the replacement of naturally occurring secondary structural elements by redesigned units in proteins where folding to form tertiary structures is crucial.

How to Construct Model Proteins

One of the most powerful techniques for the construction of proteins has been the cloning of genes followed by the expression of the corresponding naturally occurring proteins in suitable host systems (*3*). To obtain modified protein structures, the techniques of site-directed or cassette mutagenesis have been used to modify the gene structures. Alternatively, with the considerable progress that has been made in DNA synthesis, both natural and modified proteins can be prepared through the expression of corresponding synthetic genes.

In view of the power of the molecular biological methods, it is reasonable to ask whether chemical synthesis of proteins remains a viable alternative. The Merrifield solid-phase procedure (*4*), in which the carboxyl terminus of the growing peptide chain is covalently anchored to a solid support, revolutionized peptide synthesis. When used in combination with purification techniques such as high-performance liquid chromatography (HPLC), the preparation of peptides 30 or 40 amino acids in length has become almost routine. However, when one proceeds to the stepwise synthesis of longer peptides and of small proteins in the range of 50 to perhaps 150 amino acids in length, the problems that arise in the purification and characterization of materials often become formidable. Although small proteins up to about 150 amino acids in length have been synthesized with the stepwise solid-phase method (*5*), the purification of such materials has been difficult and the homogeneity of the resultant peptides has been difficult to establish. Furthermore, if a family of mutants or structural analogs of such small proteins are to be prepared by the solid-phase method, most of the molecule must be resynthesized for each mutant, unless the mutation is near the amino terminus.

Nevertheless, the appeal of chemical methods for the preparation of small proteins remains high because such methods have flexibility that would be difficult or impossible to achieve by the molecular biological approaches. In particular, as we have already demonstrated for the opioid peptide β-endorphin, through chemical synthesis it is possible to introduce nonpeptidic structural regions, to replace a right-handed helix by a left-handed helix, and to use unnatural amino acids (*6*). Also, isotopically labeled amino acids useful for spectroscopic studies can be introduced at specific locations in the peptide or protein molecule by chemical methodology (*7*).

The classical approach to preparing peptides of 30 to 40 residues in length was to synthesize well-characterized protected peptide segments through solution-phase methods and then to couple the resultant segments in solution to make the desired product. Such a segment-condensation approach has been successfully applied to the synthesis of enzymes (*8*). Solution syntheses of the peptide segments allowed the purity of the growing peptides to be monitored at each stage of the synthesis, but the synthetic efforts were enormously tedious, limiting the number of peptides that could be constructed in a reasonable length of time. Furthermore, there was no assurance that the assembly of the protected peptide segments would proceed

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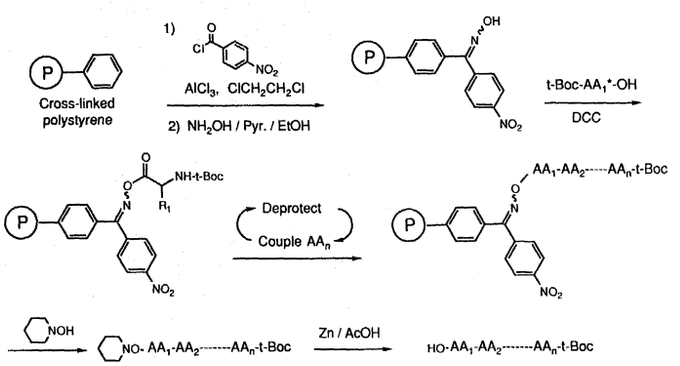
without complications, and often the yields for the coupling of the protected segments were low.

We have turned to the peptide-segment synthesis-condensation approach for synthesizing peptides in the range of 50 to approximately 150 amino acids, efficiently assembling the segments by a combination of solid-phase and solution-phase methodology. Several years ago we prepared an oxime polymer through *p*-nitrobenzoylation of the polystyrene–1% divinylbenzene copolymer typically used in Merrifield syntheses, followed by reaction of the polymer-bound ketone derivative with hydroxylamine to give a *p*-nitrobenzoyl oxime polymer (*9*) (Fig. 1). In our synthetic approach we couple a Nα-protected, side chain–protected amino acid through its COOH-terminal carboxyl function to the oxime by using dicyclohexylcarbodiimide (DCC). The resultant resin–amino acid linkage is sufficiently stable to allow the synthesis of peptide segments of four to ten residues in length. The peptide segments, with their side chains fully protected, can be removed from the polymer in a variety of ways. The most frequently used conditions involve treatment with *N*-hydroxypiperidine, followed by reaction of the resulting peptide *N*-hydroxypiperidyl ester with zinc and acetic acid to obtain the protected peptide COOH-terminal carboxylic acid. The protected peptides are then purified by reversed-phase HPLC or by crystallization, and characterized by amino acid analysis, nuclear magnetic resonance (NMR) spectroscopy, and mass spectroscopy.

A purified peptide segment can then be covalently reintroduced onto the solid support and condensed with additional soluble segments to give larger protected peptides. Typically, we can assemble protected peptide intermediates of ~20 residues in length by this sequential coupling procedure. Further condensation, between intermediates of greater than 20 amino acids, has usually been performed in solution. At this stage the method is less systematic and problems can arise. Couplings of the 20- to 40-residue intermediates do not always proceed in high yield, and the protected intermediate segments may not be readily soluble in solvents compatible with peptide synthesis.

We have not encountered solubility problems very frequently in our recent work. However, when such problems do arise, they can often be ameliorated by altering the length and composition of the intermediates or by changing the pattern in which the smaller segments are assembled. Thus alteration of a large segment does not necessitate the resynthesis of all of the segments over again.



* Side-chain protected amino acid.

Fig. 1. Synthesis of protected peptide segments on the oxime resin. Abbreviations: Pyr, pyridine; EtOH, ethanol; tBoc, *tert*-butyloxycarbonyl; Ac, acetyl.

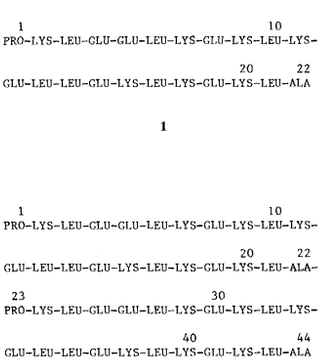


Fig. 2. Amino acid sequences of "idealized" peptide models for apolipoprotein A-I.

Preparation of Apolipoprotein Models by Segment Synthesis-Coupling

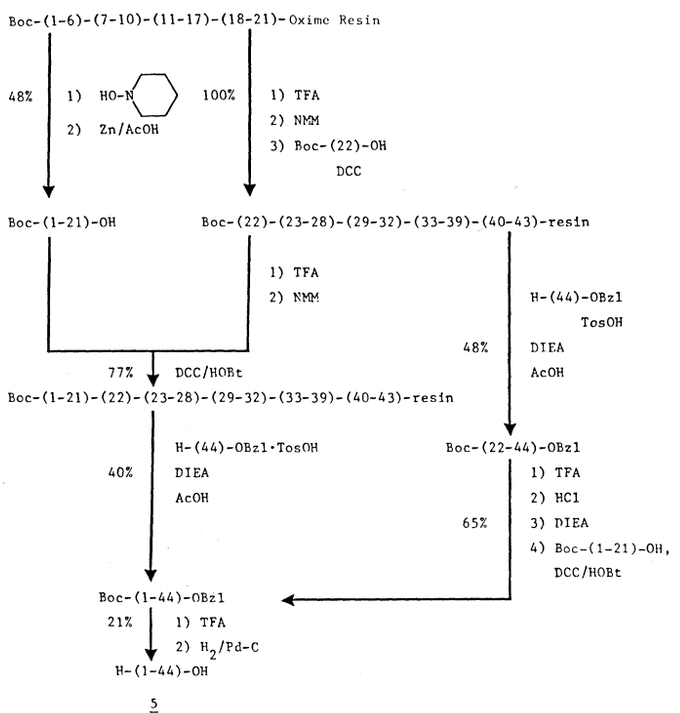
We chose apolipoprotein A-I (apo A-I) for our initial studies because of our previous experience in designing secondary structural models of surface-active peptides and proteins (*1*). The first major target (*10*) was the docosapeptide **1**, which we had previously prepared by the Merrifield stepwise procedure (*11*), and which had been shown to mimic the properties of human plasma apo A-I (Fig. 2). Apo A-I, the principal protein constituent of high-density lipoprotein (HDL) (*12*), was believed to consist of six to seven amphiphilic α helices, each 22 amino acids in length (*13*). We designed **1** so that it too could form an amphiphilic α helix, but would have no significant homology with the amino acid sequence of native apo A-I. The model peptide showed the essential surface properties of intact apo A-I, showed similar binding to phospholipid vesicles, and activated phospholipid for the catalytic action of the important enzyme lecithin–cholesterol acyltransferase (E.C. 2.3.1.43) in a closely related way (*11, 14*). Thus, it appeared that the secondary structure of apo A-I, rather than any specific amino acid, was responsible for biological function.

Scheme 1. Synthesis of peptide **1** using the oxime resin. Abbreviations: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl.

In the preparation of **1**, three protected peptide segments, the tetrapeptide **2** (positions 7 to 10 and 18 to 21 in **1** sequence), heptapeptide **3** (positions 11 to 17), and the hexapeptide **4** (positions 1 to 6) were used. The COOH-terminal amino acid anchoring the growing peptide chain to the oxime resin was Leu in each case.

The three purified and characterized protected peptide segments were assembled sequentially on the oxime support proceeding from the COOH-terminus to the NH_2-terminus (Scheme 1). Although we used either dicyclohexylcarbodiimide (DCC)/*N*-hydroxybenzotriazole (HOBt) or DCC/ethyl-2-(hydroxyimino)-2-cyanoacetate (EACNOx) for the coupling of peptide **2** with the oxime resin, the additive EACNOx was more effective than HOBt in suppressing racemization in the condensation of this COOH-terminal segment on the oxime resin (only 3% D-isomer formed under the conditions used). Aminolysis with Ala-OBzl (benzyl-protected COOH) in the presence of acetic acid yielded the fully protected docosapeptide. After purification on Sephadex LH-60, the docosapeptide was deprotected by treatment with 50% trifluoroacetic acid (TFA)–50% CH_2Cl_2 and catalytic hydrogenation (Pd on charcoal). By a combination of ion exchange chromatography on CM-cellulose, partition chromatography on Sephadex G-50, and desalting on Sephadex G-25 or G-15, the pure product was obtained in overall yield of 24% and 16% through the use of DCC-HOBt and DCC-EACNOx, respectively. Both preparations had properties identical with those of **1** synthesized in a stepwise fashion (*10, 11*).

We addressed ourselves next to the problem of whether the segment synthesis-condensation approach could be applied to the preparation of larger peptides that were unlikely to be obtained with sufficient purity through stepwise solid-phase synthesis. We attempted to synthesize a 44–amino acid peptide (44-mer) **5** that contained two repeating 22-mer segments that corresponded to the docosapeptide **1** (Fig. 2). One objective was to test the usefulness and convenience of our segment approach, and the other was to obtain a peptide with which we could assess the consequences of the



Scheme 2. Synthesis of peptide **5** by coupling on the oxime resin and in solution. Abbreviations: Ac, acetyl; NMM, *N*-methylmorpholine; Tos, toluenesulfonyl; DIEA, diisopropylethylamine.

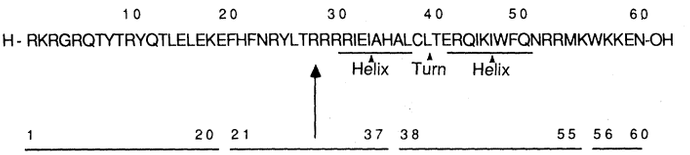


Fig. 3. Sequence of homeo box region of *Antp* protein and general synthetic strategy (*31*).

covalent attachment of two amphiphilic secondary structural domains. In Scheme 2, the routes by which we synthesized the 44-mer, either by coupling large segments on the oxime resin or by coupling large segments in solution, are shown (*15*). In the coupling of the large segments on the oxime resin, we used as a starting material the resin-bound 21-mer that was an intermediate in the synthesis of peptide **1**. This 21-mer, which eventually became the NH_2-terminal domain of peptide **5**, was cleaved from the resin and converted into a free carboxylic acid. Another sample of the resin-bound 21-mer, which was intended to yield the COOH-terminal domain, was extended toward the NH_2-terminus through the introduction of the Ala residue corresponding to Ala^{22} in the final peptide. We condensed the resin-bound 22-mer with the 21-mer containing the free carboxyl group. The resin-bound 43-mer was removed from the support by treatment with Ala^{44} benzyl ester to give the protected 44-residue peptide. In an alternate route, we condensed two major segments in solution to give the 44-mer. In this instance, we cleaved the resin-bound 22-mer containing Ala in the NH_2-terminal position from the resin through the use of Ala benzyl ester, which gave us the COOH-terminal 23-mer in a protected form in solution. Subsequently, the Nα-protecting group was removed and the product was condensed with the 21-mer containing the free COOH-terminal carboxyl group. The protected 44-mer samples obtained through condensation of the two major segments either on the polymeric support or in solution were then treated with 50% TFA–50% CH_2Cl_2 (v/v). Catalytic hydrogenation with 10% Pd on activated charcoal removed the remaining protecting groups. Addition of ammonium formate increased the reaction rate for the latter reaction. The free 44-mer **5** was purified by CM-cellulose ion exchange chromatography followed by partition chromatography on Sephadex G-25. Amino acid analysis and HPLC confirmed the purity of the final peptide. The overall yield in the final deprotection and purification step was 21%. Although peptides 44 residues in length are accessible by stepwise solid-phase synthesis, the heterogeneities that are present in the products of these synthetic procedures can be difficult to remove.

We have shown that the model 22-mer **1** mimicked many of the important properties of apo A-I (*11*). However, the covalent linkage of the two segments in the 44-mer gave a peptide that exhibited a marked increase in amphiphilicity relative to the 22-mer (*15*). The 44-mer showed a substantially greater α helicity in 50% trifluoroethanol than did the 22-mer and a considerably increased tendency to form peptide micelles in aqueous solution. We measured the limiting molecular area of the 44-mer adsorbed at amphiphilic surfaces such as phospholipid-coated polystyrene and at an air-water interface and demonstrated that the properties of the 44-mer were considerably closer to those of apo A-I than those of the 22-mer. The 22-mer occupied an area of ~22 $Å^2$ per amino acid at the air-water interface, suggesting that the peptide was not fully helical under these conditions. In contrast, we found that the 44-mer occupied 14 to 16 $Å^2$ per amino acid at both the air-water interface and on the phospholipid-coated polystyrene beads (*15*). The latter values are near the value obtained for apo A-I itself, 15 to 16 $Å^2$ per amino acid (*16*). These results suggest that the fundamental structural unit of the apolipoprotein is not the 22-residue peptide segment

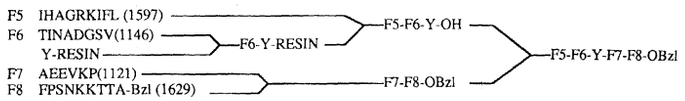


Fig. 4. Synthesis of major protected COOH-terminal segment of Cro repressor.

alone but rather is the 44-residue segment that consists of two helical regions separated by a linker region containing the helix breaker Pro. Note that in apo A-I, the putative amphiphilic α-helical segments are punctuated at regular intervals by either Gly or Pro residues, which presumably act as helix breakers. The punctuation of the helical segments by Pro and Gly residues may play a major role in allowing the apo A-I molecule to match the curvature of the surface of HDL.

Synthesis of a Small Protein Corresponding to the Homeo Domain of *Antennapedia*

The proposed homeo domain from the *Drosophila* developmental regulatory protein *Antennapedia* (*Antp*) (*17, 18*) has also been a target for segment synthesis-condensation. The homeo domain is a highly conserved 60–amino acid region found in many proteins that govern differential gene expression during early *Drosophila* development (Fig. 3) (*17, 19*). Because of their strongly basic character as well as the possibility emerging from sequence analysis that they could adopt the helix-turn-helix motif of bacterial and yeast repressors, homeo domains may mediate binding to DNA (*17, 20*). However, unlike the case of prokaryotic DNA binding proteins, the structural and functional characteristics of homeo domains and the proteins that contain them have not been elucidated in detail.

The 60–amino-acid region corresponding to the homeo domain of the homeotic protein *Antennapedia* is readily accessible through our segment synthesis-condensation method (*21*). The homeo domain was found to be a sequence-specific DNA binding region, and circular dichroism (CD) spectroscopic measurements on solutions of the peptide revealed the presence of α-helical structure both in the aggregated form of the peptide in aqueous medium and in 50% trifluoroethanol, supporting the likelihood that the domain binds to DNA through the postulated helix-turn-helix motif (*21*).

We synthesized the domain as follows: 11 small protected peptide segments (4 to 7 residues) prepared on the oxime resin were highly purified by HPLC and characterized by amino acid analysis and ^{252}Cf fission-fragment mass spectroscopy. The larger protected segments (17 to 20 amino acids in length) were synthesized by three or four consecutive couplings of the small peptides with the DCC-HOBt method. The large segments were removed from the resin and purified with a Sephadex LH-60 column (dimethylformamide, DMF). Purified material gave good amino acid analyses and showed the appropriate parent molecular ions by fission-fragment mass spectroscopy. Starting from the COOH-terminal pentapeptide benzyl ester, the large segments were coupled to each other by treatment with (*N*-ethyl-*N*′-dimethylaminopropyl)carbodiimide (EDC)–HOBt in solution to give the protected homeo domain (Fig. 3). After purification with Sephadex LH-60 (DMF), the resulting protected 60-mer gave satisfactory results on amino acid analysis and mass spectroscopy. Deprotection of the protected 60-mer was carried out by the low-high HF procedure (*22*), and the deprotected peptide was purified by gel filtration and HPLC to give the active DNA binding small protein. The synthetic *Antp* homeo domain was characterized by HPLC, SDS–polyacrylamide gel electrophoresis, amino acid analysis, peptide sequencing by the automated Edman procedure, and fission-fragment mass spectroscopy. Through footprinting experiments with deoxyribonuclease I, we demonstrated that the *Antp* homeo domain binds to specific sites (AAT tandem repeat) in the 5′ upstream region of the *Antp* gene (*21*).

Synthesis of Part of the Cro Repressor

Another DNA binding system that we are studying is the Cro repressor (Cro). This 66–amino acid protein is composed of three α helices and three β strands, as determined by x-ray diffraction and NMR analysis (*23*). The Cro repressor binds to double-helical DNA through a helix-turn-helix motif common to several DNA binding proteins (*24*). We have focused upon the preparation of the native Cro sequence, but our intention is to explore the specifics of the peptide-DNA interaction through the synthesis of appropriately mutated Cro analogs. Unlike our work on the homeo box domain, the synthesis of the Cro system is not yet complete. Our strategy for the total synthesis involves the preparation of two major intermediates which will then be coupled together to give the final product. The synthesis of one of these major intermediates, corresponding to the COOH-terminal half of the Cro repressor, has been completed; the synthetic scheme is illustrated in Fig. 4. In brief, four segments designated F5, F6, F7, and F8 were prepared, purified, and characterized. In Fig. 4, the molecular weight measured by fission-fragment mass spectroscopy for each of these segments is shown. After purification, segment F6 was coupled to resin-bound Tyr and then segment F5 was added. The product of these condensations was removed from the resin and was then combined with the protected peptide that resulted from the condensation of the F7 and F8 segments. The completion of the synthesis of Cro repressor awaits the synthesis of the NH_2-terminal half of the molecule.

Synthesis of Ribonuclease T_1 and a Structural Analog

An important question in protein engineering is whether a substantial redesign of the secondary structural unit in a molecule with tertiary structure can still allow a protein to fold properly. The molecule that we have been investigating with respect to this question is the small enzyme ribonuclease T_1 (RNase T_1). The enzyme is well characterized (*25*) with a firmly established amino

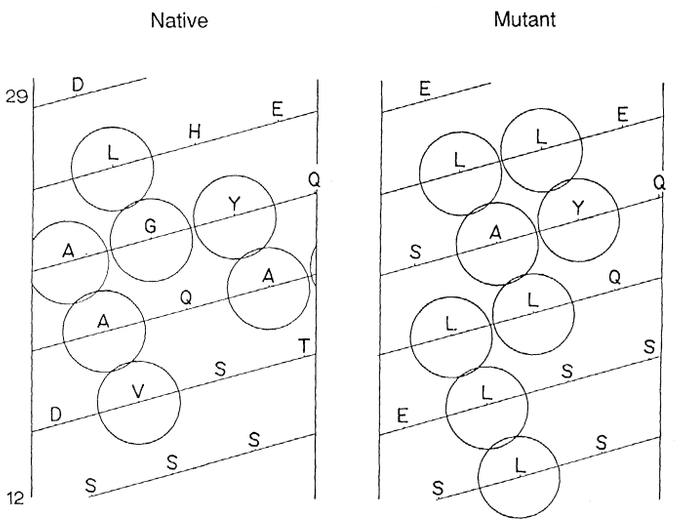


Fig. 5. Helical net diagrams of region from residues 12 to 29 of native and mutant RNase T_1.

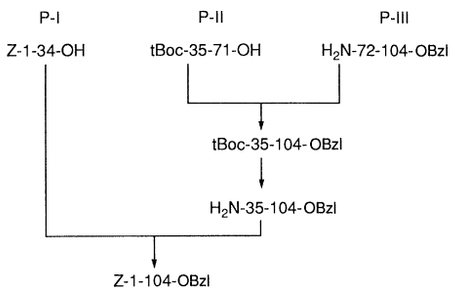


Fig. 6. Assembly of major peptide segments of RNase T_1 in protected form; Z, benzyloxycarbonyl.

acid sequence (*26*) and with a high-resolution x-ray structure (*27*). Folding studies have shown that the two disulfides in RNase T_1 can be reduced, and the denatured enzyme can be subsequently refolded and reoxidized to regain essentially full activity (*28*). The enzyme is very stable, has a high specific activity, and can be assayed by several methods. The three-dimensional structure reveals an 18-residue, 4.5-turn α helix on one side of the enzyme underlying a four-strand β sheet. This latter region, together with the adjacent loops, forms the active site. The helix is clearly amphiphilic and represents an excellent target for structural modeling (Fig. 5). To replace the naturally occurring α helix, a modified sequence has been designed that should fold into an α helix and be sterically compatible with the rest of the RNase T_1 structure (*29*). In the modified sequence, 11 of 18 residues of the helix have been changed from the native sequence to accentuate the amphiphilicity, and the net hydrophobicity of the helix has been increased by two residues. If we wanted simply to obtain this particular 11-site mutant enzyme, the most rapid strategy may have been to use synthetic oligonucleotides to construct a gene for the mutant enzyme (assuming that expression of the radically altered sequence would be feasible). This approach has been used successfully to generate several site-specific mutants of RNase T_1 at or near the active site of the enzyme (*30*). However, since we wanted to preserve the flexibility inherent in chemical synthesis, we chose to assemble the enzyme as a protected peptide using a convergent synthetic strategy.

The retrosynthetic strategy on which we have based our synthesis of native RNase T_1 began by breaking the amino acid sequence of the enzyme into three major segments consisting of 34, 37, and 33 residues and designated P-I, P-II, and P-III, respectively (*29*) (Fig. 6). These breaks in the amino acid sequence were chosen to allow the carboxyl termini of P-I and P-II to be Gly residues. Because the rate of coupling of two major segments typically slows with increasing segment size, it is desirable to have Gly residues as the carboxyl components in the coupling reactions of the major segments in order to avoid racemization. The amphiphilic α helix is located in the NH_2-terminal P-I segment. Each of the three major segments was assembled from five protected peptide segments of four to nine residues in length. To obtain the protected peptide sequence corresponding to native RNase T_1, we coupled the major segment P-II to P-III in solution and then added segment P-I to the combined P-II–P-III segment (Fig. 6). In the final step of our synthesis, we deprotected the protected 104–amino acid peptide by treatment with the low-high HF method (*22*).

The mutant intermediate peptide corresponding to P-I proved to be less soluble than native sequence P-I. By using a modified strategy the two immediate precursor segments of P-I were sequentially added to the 70-residue peptide composed of P-II and P-III. The intermediate, a protected 86-residue peptide, and the resulting mutant sequence (which was a 104-residue peptide) were both adequately soluble for further manipulations. Thus we could prepare the mutant 104-mer in a protected form and, as with the native system, this was deprotected by the low-high HF method.

Our preliminary results indicate that the replacement of the native amphiphilic α helix by the designed mutant helix results in the generation of active enzyme. This result shows that it is possible to apply the modeling approach that we have developed for secondary structural units in surface active peptides and proteins such as the apolipoproteins, peptide hormones, and peptide toxins (*1, 2*) to the redesign of a secondary structural region in a folded protein like RNase T_1.

Conclusion

The segment synthesis-condensation approach has been successfully applied to the preparation of apolipoprotein A-I models as well as to the homeo box peptide from *Antp*, a DNA binding protein. Our work on the Cro repressor is still incomplete and the studies on the small enzyme ribonuclease T_1 and its structural analog remain to be finished. However, the work that we have done to date demonstrates the versatility and power of the methodology we have used. The redesign of elements of secondary structure in folded proteins appears to be a valid probe of structure-function relations and extends the work that we began in less complex peptide hormones (*1, 2*). Although we cannot predict tertiary structure with confidence from primary amino acid sequence, we can replace native regions with novel amino acid sequences in such a way that secondary and tertiary structure is not significantly disrupted. Thus it appears that an empirical first step has been made toward the design of tertiary structure starting from primary amino acid sequences.

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31. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E. Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Research Articles

Molecular Sorting in the Secretory Pathway

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Proteins can be secreted from animal cells by either a constitutive or a regulated pathway; those destined for regulated secretion are actively sorted into dense-core secretory granules. Although sorting is generally assumed to be accomplished by specific carriers, the nature of these carriers remains elusive. In this study, peptide hormones were used as affinity ligands to purify a set of 25-kilodalton proteins from canine pancreatic tissue. Their ligand specificities and patterns of expression have the characteristics of sorting carriers.

IN MULTICELLULAR ORGANISMS, PROPER COMMUNICATION between cells requires precise temporal and spatial control of secretion. Many animal cells segregate their secretory products into distinct types of secretory vesicles. These vesicles differ in their proteolytic processing activities, in the sites at which they fuse with the plasma membrane, and in their dependence on external stimuli for release (*1–4*). Most epithelial cells manufacture two types of vesicles so that proteins can be selectively shuttled to either the apical or the basolateral surface (*5, 6*). Specialized secretory cells also segregate their secretory products into two types of vesicles, for constitutive and regulated release. For example, the mouse pituitary cell line AtT-20 packages adrenocorticotropic hormone (ACTH) in storage granules and releases it when the cell receives an external stimulus (*7, 8*). By contrast, the cell secretes several viral proteins and laminin by a constitutive pathway in which Golgi-derived vesicles fuse continuously with the plasma membrane (*9–11*). Segregation of proteins into hormone-regulated and nonregulated pathways has also been reported in the pituitary cell line GH_3 (*12*), PC-12 cells (*13*), and pancreatic beta cells (*14*). The observation that two secretory pathways can coexist in the same cell suggests that there is a selective mechanism to sort secretory proteins into either the

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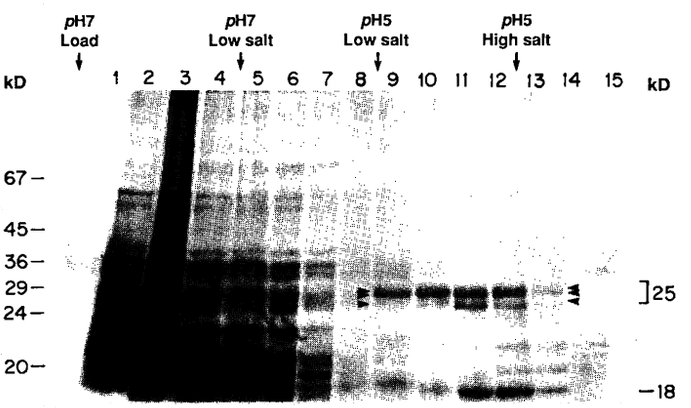

Fig. 1. Identification of proteins that bind to prolactin-Sepharose. Detergent-solubilized Golgi lysates were chromatographed on a prolactin-Sepharose column, and fractions were analyzed by 10 to 18 percent SDS-PAGE. (Lanes 1 to 4) Flow-through fractions from column during loading of Golgi lysates at neutral *p*H. (Lanes 5 to 8) Fractions collected during subsequent washings with neutral *p*H-loading buffer. (Lanes 9 to 12) Fractions collected on eluting the column with 50 m*M* potassium acetate (KOAc), *p*H 5.0, buffer. (Lanes 13 to 15) Fractions collected upon eluting column with 1*M* KOAc, *p*H 5.0, buffer. The positions of molecular standards (kilodaltons) are indicated on the left. A set of 25-kD proteins, which bound to the prolactin column, is indicated by the bracket on the right. Crude dog pancreas Golgi preparations, recovered from the interphase of a 1.3*M* sucrose cushion (*23*), were centrifuged for 1 hour at 100,000*g* and resuspended in 50 m*M* Hepes, *p*H 7.4, 400 m*M* KOAc, 0.5 m*M* EDTA (10 ml final volume for 250 g of wet tissue). Nikkol (Nikkol BL-8SY, Nikko Chemicals Co., Ltd., Tokyo, Japan) and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 2 percent and 0.5 m*M*, respectively, and the mixture was incubated on ice for 30 minutes. Insoluble materials were cleared by centrifugation at 100,000*g* for 30 minutes, and the composition of the supernatant was adjusted to 50 m*M* KOAc, 0.5 percent Nikkol, 15 mM Hepes, *p*H 7.4. Four 200-μl samples of the Golgi lysates were applied to a 200-μl affinity resin, prepared by coupling sheep prolactin (Sigma) to CNBr-activated Sepharose (Pharmacia) at 10 mg/ml. The column was washed with four column volumes of each of the following buffers: loading buffer (12.5 m*M* Hepes, *p*H 7.4, 50 m*M* KOAc, 0.5 m*M* EDTA, 0.5 m*M* PMSF, 0.5 percent Nikkol); elution buffer I (50 m*M* KOAc, *p*H 5.0, 0.5 m*M* EDTA, 0.5 m*M* PMSF, 0.5 percent Nikkol); and elution buffer II (1*M* KOAc, *p*H 5.0, 0.5 m*M* EDTA, 0.5 m*M* PMSF, 0.5 percent Nikkol). All procedures were carried out at 4°C. Proteins from each fraction were precipitated with 80 percent acetone at −20°C for 60 minutes before SDS gel electrophoresis.