Synthesis of Secretin. IV.[1] Secondary Structure in a Miniature Protein

Agnes Bodanszky, Miguel A. Ondetti, Viktor Mutt, and Miklos Bodanszky

Contribution from the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio, The Squibb Institute for Medical Research, New Brunswick, New Jersey, and the Karolinska Institutet, Stockholm, Sweden.
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Abstract: The optical rotatory dispersion and circular dichroism spectra of natural and synthetic (porcine) secretin preparations are indistinguishable from each other. These spectra suggest a polypeptide of low-helix content; in this respect secretin is similar to lysozyme. Peptides corresponding to partial sequences of secretin exhibit spectra which with increasing chain length gradually approach those of the hormone itself. The comparison of the rotational spectra of the fragments with those of secretin allows a general description of the conformation of the molecule.

The question whether peptide hormones possess a certain architecture, a preferred conformation, raises itself rather naturally. It follows from our present knowledge of enzymes and other proteins with highly specific functions that their architecture is a prerequisite for the specificity in their biological activity. In the case of hormones such a requirement is far from being obvious. They could, even without possessing a particular conformation, develop one during the process of attachment to the receptor site. Nevertheless in some hormones the existence of a more or less defined geometry follows directly from their covalent structure: the 20-membered ring lends considerable rigidity to the molecules of oxytocin, vasopressin, and vasotocin and the three disulfide bridges introduce a serious limitation to the freedom of conformation in insulin. No such limitations can be assumed in single-chain peptide hormones and unless the more subtle forces of hydrogen-bonding and side-chain interactions determine their geometry, corticotropin, bradykinin, angiotensin, gastrin, cholecystokinin-pankreozymin, caerulein, etc. could exist only as random coils. In fact measurements of the rates of diffusion through membranes[2] seem to indicate that some of these hormones have considerable conformational freedom.

It is rather difficult to visualize that any of the presently available techniques could give a definite answer to the question of conformation in oligopeptides. While the objection to the X-ray crystallographic description of protein architecture, namely, that it is valid only for the solid state and not in solution, is probably not serious, such an objection could easily be raised in the case of smaller, single-chain peptides. No drastic conformational change can be expected during crystallization of a highly complex, globular protein. A small, single chain, however, could undergo serious changes on crystallization, due to packing forces. Therefore if and when X-ray crystallographic evidence for the architecture of peptide hormones will be forthcoming, it will have to be treated with certain reservations. At this time most of the peptide hormones are available only as amorphous solids. Their lack of crystallizability is suggestive in itself: it could be the consequence of a conformational freedom absent in globular proteins, many of which were obtained in crystalline form.

Conformational analysis of proteins by means of rotational spectra[3-5] has obvious limitations. The information deduced from spectral evidence can at best give some general description of the molecule, such as helix content, some types of folding, or perhaps the presence of cyclic areas. Nevertheless the examination of ORD and CD spectra is still the principal method of investigation for peptides and proteins not available in the crystalline form necessary for X-ray studies.

Secretin, the gastrointestinal hormone for which the expression "hormone" was coined, was isolated[6] (from porcine intestines) in pure form only recently. The availability of the pure hormone led to the elucidation of its structure[7,8] and to its total synthesis.[9-12] The synthetic experimentation which necessarily will follow could very well be guided by some knowledge of the conformation of the molecule. If the secretin

(1) For previous papers of this series see ref 10–12.
(2) L. C. Craig, E. J. Harfenist, and A. C. Paladini, *Biochemistry*, **3**, 764 (1964).
(3) S. N. Timasheff and M. J. Gorbunoff, *Ann. Rev. Biochem.*, **36**, 13 (1967).
(4) J. A. Schellman and C. Schellman in "The Proteins," Vol. II, H. Neurath, Ed., 1964, p 1.
(5) P. J. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).
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(7) J. E. Jorpes, V. Mutt, S. Magnusson,and B. B. Steele, *Biochem. Biophys. Res. Commun.*, **9**, 275 (1962); V. Mutt, S. Magnusson, J. E. Jorpes, and E. Dahl, *Biochemistry*, **4**, 2358 (1965).
(8) V. Mutt and J. E. Jorpes, Presented at the Fourth International Symposium on the Chemistry of Natural Products, Stockholm, 1966; V. Mutt and J. E. Jorpes, "Pharmacology of Hormonal Polypeptides and Proteins," N. Back, L. Marini, and R. Paoletti, Ed., Plenum Press, New York, N. Y., 1968, p 569.
(9) M. Bodanszky, M. A. Ondetti, S. D. Levine, V. L. Narayanan, M. von Saltza, J. T. Sheehan, N. J. Williams, and E. F. Sabo, *Chem. Ind.* (London), 1757 (1966).
(10) M. Bodanszky and N. J. Williams, *J. Am. Chem. Soc.*, **89**, 685 (1967).
(11) M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *ibid.*, **89**, 6753 (1967).
(12) M. A. Ondetti, V. L. Narayanan, M. von Saltza, J. T. Sheehan, E. F. Sabo, and M. Bodanszky, *ibid.*, **90**, 4711 (1968).

chain exists in a preferred conformation, then it is likely that this architecture is a prerequisite for biological activity. Attempts at improvements, *e.g.*, synthesis of analogs with higher selectivity in their hormonal spectra, should aim at molecules with secondary structures similar to that of secretin. The work connected with secretin, isolation, degradation, and synthesis, provided compounds eminently suitable for a systematic study of the question outlined above. The identity of the rotational spectra of natural and synthetic secretin contributed to the already accumulated evidence—supporting the correctness of the structure proposed by Jorpes and Mutt.[7,8] Moreover these spectra suggest that the molecule of the hormone exists in a preferred conformation, and allow a description of this conformation in general terms.

Experimental Section

Natural secretin was prepared from porcine intestines as described earlier.[7] The synthetic samples were obtained both by stepwise synthesis[10,11] and by fragment condensation.[12] Peptides S_{22-27} and S_{15-27} were prepared stepwise, S_{9-27} and S_{5-27} are products of fragment condensation. Synthesis of S_{1-6} and S_{1-14} will be described, in a separate paper. The peptide S_{15-27} was used in the form of its dichloroacetate salt, the hexapeptide amide S_{22-27} as the hydrochloride, and all other peptides in the form of their acetate salts.

The solutions were prepared from weighed amounts of solid samples and distilled water. The initial concentration of the solutions was 1–2 mg/ml and appropriate dilutions were made with distilled water to keep optical density below 2. Cylindrical fused quartz cells with 1- and 10-mm path length were used. The pH of the solutions was measured with a Radiometer Model 4 pH meter, but the pH was not adjusted to a particular value, since in preliminary experiments no significant dependence of the spectra on pH could be observed, at least not in the pH range used. All spectra were taken at room temperature, with a Cary Model 60 spectropolarimeter and Model 6001 CD attachment.

For "denaturation" expriments[13] 2 ml of a 2.35% solution of secretin was dissolved in 1.8 ml of 8 *M* urea solution (prepared from urea freshly recrystallized from 50% ethanol) and incubated for 2 hr at 60° in a sealed tube. Rotation was measured after cooling to room temperature. As a blank, 0.2 ml of a 2.35% solution of secretin was dissolved in 1.8 ml of distilled water.

Results and Discussion

Early studies of oligopeptides indicated that a helical structure can exist in chains containing as few as five or six amino acids.[14] Therefore it seemed of interest to examine the spectra of two hexapeptides, S_{1-6} and S_{22-27}, the N-terminal and C-terminal sequences of secretin (I). Larger fragments like the peptides S_{1-14},

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2
15 16 17 18 19 20 21 22 23 24 25 26 27

secretin (I)

S_{15-27}, S_{14-27}, S_{9-27}, S_{5-27} were also studied. Comparison of ORD and CD spectra of natural and synthetic secretin and examination of the changes occurring on "denaturation"[13] of secretin concluded the series. From the point of view of these studies it is particularly advantageous that secretin contains no amino acids with strongly absorbing side-chain chromophores, like tyrosine or tryptophan, because their high absorption in the 200–300-mμ region makes ORD and CD measurements less accurate and would easily overshadow the electronic transitions of the peptide group which are influenced by the conformational changes of these molecules. The single phenylalanine residue has only moderate absorption around 260 mμ.

Helix Content. To evaluate our measurements on secretin and the secretin peptides, helix contents were calculated in several conventional ways.[15–17] This was done with full awareness of the fact that the calculations leading to a "helix content" are based on analogies and could be misleading. Also, even if helical portions of the molecule indeed exist, it would be difficult to differentiate between an α helix or some other kind of helical conformation.[18,19] The term helix can nevertheless conveniently be used to describe the existence of a regularly folded part of the peptide chain, especially where this regularity reveals itself in Cotton effects strikingly similar to those shown by molecules with a well-established helical conformation.

The ORD and CD spectra of both natural and synthetic secretin are very similar to those of egg white lysozyme[20] (Figures 1 and 2). This fact alone is worth mentioning because, while several small peptides exhibit anomalous dispersion,[21,22] to our knowledge none of these showed close similarity to the Cotton effects found in proteins. Calculations[23] based on closely resembling spectra of secretin and lysozyme indicate low helix contents (10–30%) for both compounds. In the case of secretin this would correspond to one or two turns. Potentially, even more than two turns are possible because in short helical segments the standard values of b_0 and $[m]_{233}$ lead to an underestimation of the helix content (*cf.* J. T. Yang, ref 23, p 270).

Partial Sequences. In search for data supporting the assumption of a helical region the detailed study of peptides corresponding to partial sequences of the

(13) N. S. Simmons, C. Cohen, A. G. Szent-Györgyi, D. B. Wetlaufer, and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 4766 (1961).
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(18) F. Quadrifoglio and D. W. Urry, *ibid.*, **90**, 2757 (1968).
(19) D. C. Phillips, *Sci. Am.*, **215**, 78 (1966); *cf.* also ref 20.
(20) C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature*, **206**, 757 (1965).
(21) A. F. Beecham, *Tetrahedron Letters*, 957 (1966).
(22) A. F. Beecham, *ibid.*, 4757 (1965).
(23) The b_0 value obtained from a Moffitt plot, −94, corresponds to about 15% helix. The change of rotation at 233 mμ on unfolding with urea showed about 10% helix. Calculations based on the modified two-term Drude equation gave A_{225} −646 and A_{193} +870. The helix content calculated from these values would be greater than 20%. On the other hand A_{225} and A_{193} are not in the linear relationship required by this method for estimation of helix content. In fact the lack of linearity between A_{225} and A_{193} suggests that some other conformation might also be involved besides the helical and random regions. Rotational spectra of lysozyme, treated in the same manner, show more than 30% helix and the same lack of linearity between A_{225} and A_{193}, while X-ray data have shown a helix content of about 30%, but also some β-folded sheets, and "disordered" regions in this enzyme.
The 223-mμ trough of the ORD curve can also be a measure of helix content. If the parameters suggested by J. T. Yang (in "Poly-α-Amino Acids," G. T. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1967) are used, $[m']_{223}$(helix) ∼ −15,000 and $[m']_{233}$(coil) ∼ −2000, the observed value for secretin, $[m']_{233}$ −4600, gives the helix content of 20%. Similarly, from CD curves using $[\theta]_{222}$(helix) ∼ −32,000, $[\theta]_{222}$-(coil) 0, the observed value for secretin, $[\theta]_{222}$ −6800, gives 21% helix content.

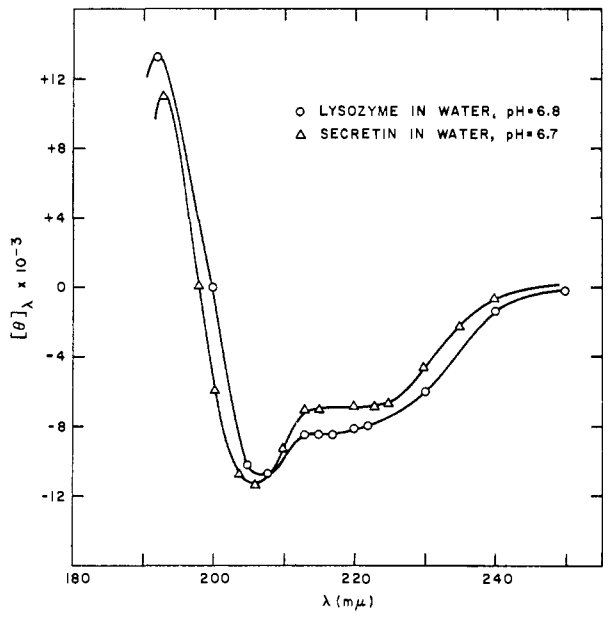

Figure 1. ORD spectra.

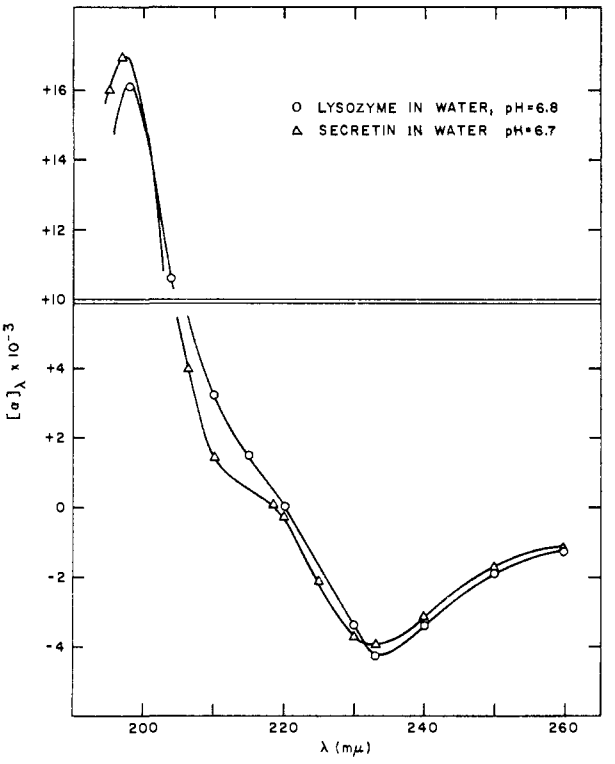

Figure 2. CD spectra.

hormone was undertaken. The ORD and CD spectra of these peptides are shown in Figures 3–6.

The C-terminal hexapeptide (S_{22-27}), Leu-Leu-Gln-Gly-Leu-ValNH$_2$,[24] with an unusually heavy proportion of hydrophobic side chains, was the first in the series of (synthetic) peptides to be studied. The ORD curve shows a very slight trough at 233 mμ; the CD

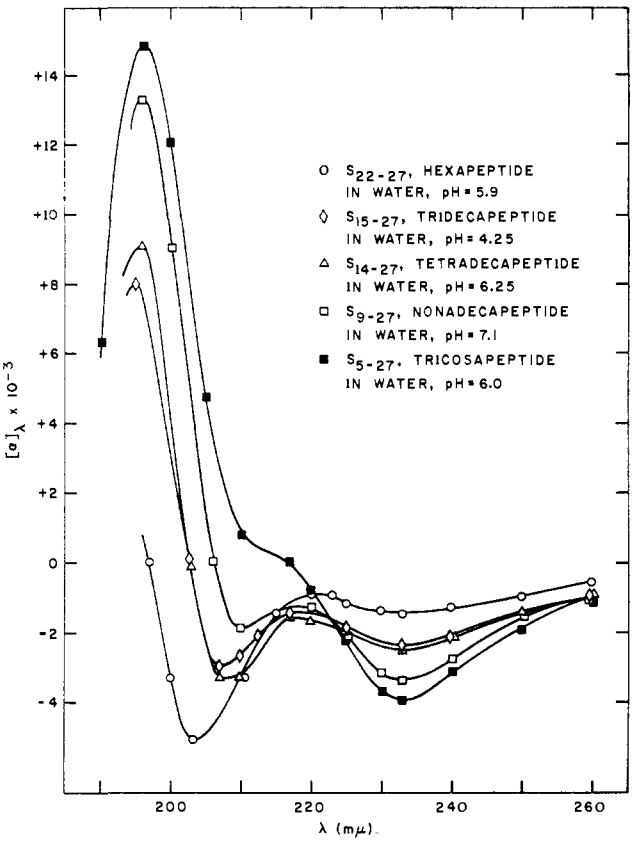

Figure 3. ORD spectra.

curve exhibits the strong negative trough near 200 mμ, found in random polypeptides.[25] The C-terminal tridecapeptide (S_{15-27}), identical with one of the thrombinic[26] fragments of secretin, exhibits a trough at 233 mμ, almost twice as deep as the one in the ORD curve of the hexapeptide; the CD curve of S_{15-27} shows a double trough at 200 and 223 mμ, a notch at 217, the lower wavelength trough being three times as intense as the higher wavelength one. The spectrum of the tetradecapeptide S_{14-27} is not significantly different from that of S_{15-27}.

Lengthening of the chain shows some more effects of folding in the rotational spectra. The 233-mμ trough in the ORD spectrum of the nonadecapeptide S_{9-27} is deeper, while the lower wavelength trough is more shallow and appears at slightly higher wavelength than in the shorter chains. In the CD spectrum the ratio of the short- and long-wavelength trough is changing with the length of the chain, the short-wavelength trough decreasing, the other trough increasing, as can be seen in synthetic polypeptides with increasing helix content.[18] Additional lengthening of the chain by four more amino acids leads to the tricosapeptide S_{5-27}, which reveals ORD and CD spectra almost identical with the 27-membered chain of secretin itself. The trough in the ORD with a specific rotation of −4000°, a shoulder at 210 mμ, and the high peak at

(24) This hexapeptide amide corresponds to the C-terminal tryptic fragment of porcine secretin; *cf.* ref 7.

(25) G. Holzwarth and P. Doty, *J. Am. Chem. Soc.*, 87, 218 (1965).
(26) V. Mutt and J. E. Jorpes, *Recent Progr. Hormone Res.*, 23, 483 (1967).

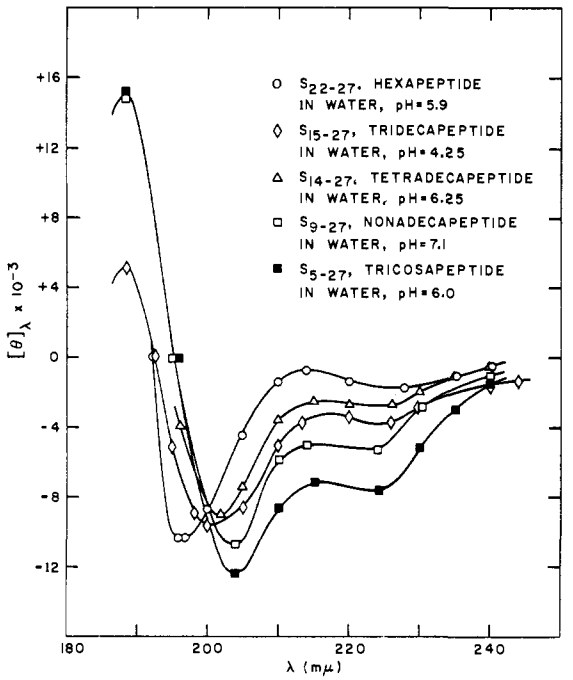

Figure 4. CD spectra.

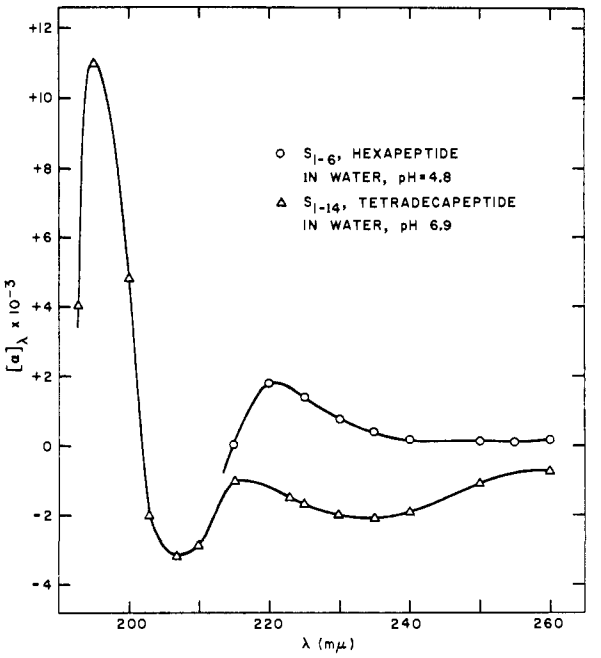

Figure 5. ORD spectra.

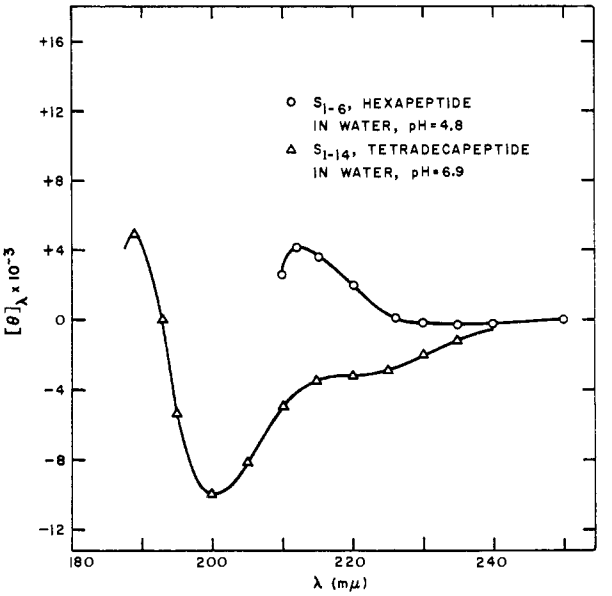

Figure 6. CD spectra.

195 mμ with a specific rotation of more than 10,000° are very similar to those of partly helical proteins like lysozyme,[19] and show many similarities to helical model polypeptides.[27] The circular dichroism spectra of the tricosapeptide and of secretin show strong resemblance to the CD of lysozyme[18] in that they all exhibit a trough at 222 mμ and a deeper one at 206–208 mμ. There is also a large positive peak near 190 mμ. The positions and signs of dichroic bands are very similar to the ones found in largely helical proteins, like metmyoglobin,[25] but the depth of the troughs, the $[\theta]_{222}:[\theta]_{208}$ ratio, is inverted.

The rotational spectra of the N-terminal hexapeptide, S_{1-6}, a fragment found in chymotryptic digests[26] of the hormone, seem to be strongly influenced by side-chain absorption (Figures 5 and 6). Histidine and phenylalanine are present in this peptide. The ORD spectrum has no trough at 233 mμ, but a peak at 220 mμ, similar to the ORD of phenylalanine itself. In the ORD of the N-terminal tetradecapeptide S_{1-14} (Figures 5 and 6) a slight 233-mμ trough appears, and the 220-mμ peak seems almost to be hidden by the 208-mμ trough present in all the ORD curves of peptides smaller than 23 residues in the series. A comparison of the spectra invites the speculation that the shoulder appearing in the secretin ORD spectrum could be traced back to the low-wavelength absorption band of the phenylalanine side chain, since it also appears in the spectrum of the tricosapeptide which likewise contains phenylalanine near the N-terminal end. On the other hand a similar shoulder present in the ORD curve of helical poly-L-glutamic acid[27] has been attributed to the 206-mμ $\pi^0 \rightarrow \pi^-$ transition[25] of the peptide bond.

The Helical Region of Secretin. With increasing chain length the partial sequences S_{22-27}, S_{15-27}, and S_{14-27} show an increase in the extreme values of their Cotton effects.[28] Nevertheless neither the wavelength of their minima and maxima nor the values of rotation at the extrema point to a helical structure. Then, in the nonadecapeptide S_{9-27} and especially in the tricosapeptide S_{5-27}, the characteristics of a helical molecule impressively emerge, and these characteristics remain unchanged when the chain length reaches 27, the complete sequence of the hormone. The impression is created that the amino acids between positions

(27) E. R. Blout, I. Schmier, and N. S. Simmons, *J. Am. Chem. Soc.*, 84, 3193 (1962).

(28) The gradual change might arise from an increase in the population of molecules present in a preferred conformation.

5 and 13 are an essential part of the helical region, but the spectra of S_{1-14} seem to contradict this assumption. Indeed it is puzzling that neither the N-terminal nor the C-terminal half of the molecule seems to form a helical stretch, and yet the whole molecule and even a 23-membered part of it appear to contain a helix. It occurred to us that this contradiction could be resolved by taking into consideration the distribution of the amino acids with nonpolar (hydrophobic) side chains. Such amino acids are present in positions 6 (phenylalanine), 10, 13 (leucine), 17 (alanine), 19, 22, 23, 26 (leucine), and 27 (valine).[29] (The threonine residues in positions 5 and 7 are potentially hydrophobic, but threonine can play the role of a polar amino acid as well.) The distribution of nonpolar residues in the N-terminal half of the chain (positions 6, 10, and 13) is strongly reminiscent of the distribution of hydrophobic residues in the helical regions of hemoglobin. The conclusions of Perutz, Kendrew, and Watson,[30] that such residues are located on one side of a helical stretch, which in turn is in contact with and stabilized by a second hydrophobic region, can be easily adopted for secretin if we assume that the short helix formed in the N-terminal part lacks sufficient stability until a water-poor region is created[31] by the C-terminal sequence with its conspicuous accumulation of hydrophobic side chains (in positions 22, 23, 26, and 27). It is tempting to speculate that these two regions are in close contact with each other, *i.e.*, that the phenylalanine (6) and leucine (10 and 13) side chains form hydrophobic bonds with the side chains of the leucine (22, 23, and 26) and valine (27) residues near the C-terminal. The postulate of a secondary structure envisaging such a general folding of the molecule brings (Figure 7) into harmony the dissonant spectra of the partial sequences. The stabilization of helical portions by hydrophobic bonds on one side of the helices was recognized also in lysozyme.[20] In this enzyme, however, also some β-sheet structure occurs which is not revealed in its rotational spectra. The presence or absence of β-sheet conformation in secretin remains an open question.

Of the 20 amino acids commonly occurring in proteins tyrosine, tryptophan, cystine, methionine, isoleucine, lysine, and proline are absent in secretin. The lack of proline, an "antihelical" residue,[30] is possibly of some significance: in a comparatively short chain the conformational restrictions created by proline could prevent the arrangement of the molecule in an architecture which is probably a prerequisite for its activity. Since the hydrophobic pocket created by the nonpolar side of the helical stretch and by the C-terminal sequence forms an "inside" of the molecule,

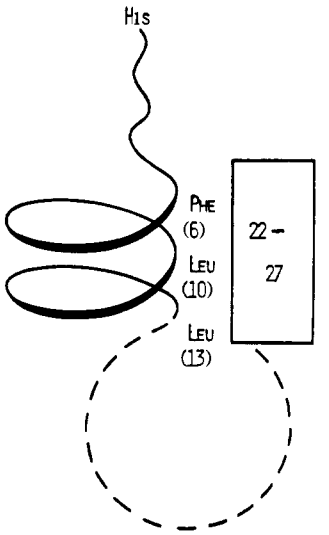

Figure 7. A schematic representation of the proposed conformation of secretin. This figure intends to show that there is a helical portion in the region between amino acids 6 and 13 but does not want to state the exact length of the helix. In the demonstration of the stabilization through folding of the chain and formation of a hydrophobic pocket the C-terminal sequence 22–27, rich in amino acids with hydrophobic side chain, is represented as a block, to indicate the uncertainties, *e.g.*, in the direction of the chain.

it follows that the hydrophilic amino acids of the helix and the N-terminal tetrapeptide sequence are on the "outside" and in contact with the receptors.[32] These regions are indeed critical for the biological properties of the hormone, *e.g.*, even peptide S_{2-27} is practically inactive and very little activity is shown by the 3-isoaspartic acid analog of secretin.[33] The existence of a preferred conformation in secretin and its evolution by contributions from both ends of the molecule are in harmony with the observations that in secretin, unlike corticotropin or gastrin, the whole chain seems to be necessary for hormonal activity. A solution containing both thrombic fragments (S_{1-14} and S_{15-27}) still shows no helicity and is also inactive[34] in biological tests.

Salt Bridges. Secretin contains no cystine and hence no disulfide bridge can contribute to its conformation. On the other hand, its four arginine, the N-terminal histidine, the glutamic acid, and two aspartic acid residues provide ample opportunity for the formation of bridges by ion pairing. Nevertheless the rotational spectra suggest that the salt bridges play at most a minor role in the determination of the preferred conformation. The similarity between the spectra of S_{22-27} and S_{15-27} indicate that the two arginine residues and the aspartic acid residue present in S_{15-27} and absent in S_{22-27} do not contribute significantly to the conformation. In S_{14-27} one more arginine residue appears, yet the spectra are almost identical with those of S_{15-27}. In S_{9-27} the one additional arginine and the new glutamic acid are of little consequence. Similarly no major effect can be seen in the peptide S_{1-14} even

(29) The pancreatic hormone glucagon is closely related to secretin, *e.g.*, in 14 positions they have identical amino acids. It is equally striking that hydrophobic side chains occur in glucagon in positions 6, 10, 13, 14, 19, 22, 23, 25, 26, and 27; while in seven of these the amino acid is different from the one in the same position in secretin, eight out of the ten places correspond to positions of nonpolar amino acids in secretin. *Cf.* M. H. Blanchard and M. V. King, *Biochem. Biophys. Res. Commun.*, **25**, 298 (1966); W. B. Gratzer, E. Bailey and G. H. Beaven, *Biochem. Biophys. Res. Commun.*, **28**, 914 (1967); W. B. Gratzer, G. H. Beaven, H. W. E. Rattle, and E. M. Bradbury, *European J. Biochem.*, **3**, 276 (1968); B. Weinstein, *Experientia*, **24**, 406 (1968).

(30) M. F. Perutz, J. C. Kendrew, and H. C. Watson, *J. Mol. Biol.*, **13**, 669 (1965).

(31) W. Kauzmann, *Biophys. J.*, **4**, 43 (1964).

(32) Since there is no significant difference between the spectra of S_{5-27} and S_{1-27}, the N-terminal 4 or 5 amino acids are seemingly not part of the helical stretch.

(33) M. A. Ondetti and J. Pluscec, unpublished.

(34) V. Mutt, unpublished.

though it contains an arginine, an aspartic acid, and a glutamic acid residue. It is quite possible that salt bridges are present in secretin but that they are not directly revealed in the ORD and CD spectra, unless the gradual enhancement with increasing chain length in the values of the extrema are an expression of their contribution to a stable conformation. That the aspartic acid in position 3 has no major effect on the conformation is shown by the similarity of the spectra of S_{5-27} and secretin.

Conclusion

Our results suggest that a preferred conformation could exist in molecules as small as a tricosa- or heptacosapeptide even in the absence of strong conformation-determining factors such as disulfides or salt bridges. Rather the sum of subtle side-chain interactions seems to play an important role in determining the architecture. On the other hand, a certain number of amino acids with appropriate side chains must be present in the molecule before the weak interactions can result in a stable conformation. The 27-membered chain of secretin seems to have more than sufficient length to produce a secondary structure.[35]

(35) An inspection of a model of secretin assembled from space-filling (CPK) atomic models suggests that the side chain of leucine-26 is sandwiched between the side chains of phenylalanine-6 and leucine-10 and similarly the side chain of leucine-23 lies between those of leucine 10 and leucine-13. Of course, these suggestions can be considered only as possibilities and not as evidence. The secondary structure of secretin should be established by X-ray crystallography. In preliminary experiments secretin was crystallized both as a flavianate and as a salt of 4-hydroxyazobenzene-4′-carboxylic acid.

An Application of Transient Nuclear Magnetic Resonance Methods to the Measurement of Biological Exchange Rates. The Interaction of Trifluoroacetyl-D-phenylalanine with the Chymotrypsins

Brian D. Sykes

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305. *Received May 21, 1968*

Abstract: Transient nmr methods have been applied to the measurement of the rate constants of biological exchange reactions. The experimental method is based upon the measurement of the spin–lattice relaxation time in the rotating frame and is applied to the binding of trifluoroacetyl-D-phenylalanine to α-chymotrypsin, DFP-chymotrypsin, and chymotrypsinogen A. Trifluoroacetyl-D-phenylalanine is shown to bind to all three enzymes and the forward and reverse rate constants are determined for the first two. The rates are [k_1, M^{-1} sec^{-1} (k_{-1} sec^{-1})]: α-chymotrypsin, 1.0×10^4 (4.9×10^2); DFP-chymotrypsin, 1.6×10^4 (16.6×10^2). These rates are appropriate to conformational changes occurring in the enzymes upon inhibitor binding.

Nuclear magnetic resonance (nmr) methods have been extensively applied to the investigation of biological systems.[1] High-frequency nmr,[2] relaxation time and line width measurements,[3] and halide ion probe techniques[4,5] have been used to determine the structure, conformation, and motion of enzymes and proteins in solution. While interpretation of the nmr spectrum of the macromolecule is limited by its inherent complexity, much information can be obtained from the nmr spectrum of a small molecule that is able to probe the environment of the macromolecule by rapid exchange between free solution and attachment to the macromolecule. Hence, for an inhibitor exchanging between free solution and the active site of an enzyme

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad (1)$$

an increase in the nmr line width of nuclei on the exchanging inhibitor molecule is often interpreted in terms of rotational restriction of the inhibitor within the active site, an increased rotational correlation time while bound to the enzyme, or an interaction with nuclei in the active site of the enzyme.

It has also been shown that a shift in the resonance frequency of nuclei on the inhibitor molecule can occur upon binding to the enzyme.[6,7] In principle, then,

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