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Studies on the Conformation of Secretin

The Position of the Helical Stretch

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An analog of the C-terminal tricosapeptide of secretin, with aspartic acid replacing glutamic acid in position 9 and lysine substituted for arginine in position 21, was prepared. The synthesis was carried out in solution by stepwise chain lengthening with the application of the *in situ* technique. The ord-cd spectra of this new analog closely resemble the spectra of the tricosapeptide with the unaltered secretin sequence and of the analog in which only arginine-21 was replaced by lysine and of secretin itself. The incorporation of aspartic acid instead of glutamic acid-9 resulted in an N-terminal sequence that has a considerably reduced probability of assuming a helical conformation. The observation that the helix content remained unchanged adds support to a model of secretin in which the helical stretch is near the C-terminus. The role of an acidic residue in position 9 is also discussed.

The gastrointestinal hormone secretin was discovered by Bayliss and Starling (*1*) and isolated in pure form, half a century later, by Mutt and Jorpes (*2*). The sequence of the 27 amino acid residues that constitute the single chain of the hormone (Fig. 1) was determined by Mutt, Jorpes, and Magnusson (*3*) and was proved by synthesis (*4–6*).

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

FIG. 1. The amino acid sequence of porcine secretin.

The ord-cd spectra of aqueous solutions of secretin (*7*, *8*), and also of its C-terminal tricosapeptide fragment, closely resemble the spectra of lysozyme. This similarity suggested that secretin has a partially helical conformation. A study of shorter peptides with partial sequences of secretin (*7*, *8*) led to the conclusion that the helix is stabilized by long-range cooperative interactions. More recent experiments (*9*, *10*) with analogs, in which the side-chain carboxyl groups of the residues in positions 9 and 15 are replaced by carboxamides, revealed that ion pairs play a significant role in the folding of

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the peptide chain and thereby in the stabilization of the helix. Aspartic acid in position 3 was not replaced because the N-terminal tetrapeptide, while essential for full activity, has little or no effect on the conformation of the hormone (*7*). The observation that the C-terminal tricosapeptide, a relatively small molecule, has a well-defined secondary-tertiary structure opened up opportunities for studying, through synthetic models, the forces that determine the architecture of proteins.

DISCUSSION

Considerations that were based on the distribution of nonpolar residues (*11*) and on solvent effects on the ord-cd spectra of fragments (*8*) resulted in placing the short helical stretch near the N-terminus (*7*, *8*). Yet, additional experimental work for a more definitive determination of the position of the helix seemed to be desirable. An analog of the C-terminal tricosapeptide of secretin, with arginine-21 replaced by lysine (21-Lys-$S_{5\text{-}27}$), has been synthesized in this laboratory (*12*). This substitution provides a site for labeling and for cross-linking experiments. The helical character recognized in the spectra of secretin appeared unchanged in the spectra of 21-Lys-$S_{5\text{-}27}$; thus, substituting lysine for arginine-21 did not alter the conformation of the chain. During the preparation of this tricosapeptide, the spectra of the deprotected nonadeca-, eicosa-, heneicosa-, and docosapeptide intermediates were also examined (*10*, *12*). Interestingly, the helical character did not appear suddenly at the introduction of the nonpolar residue, phenylalanine, in position 6, but emerged gradually. In the nonadecapeptide, the short-wavelength trough at 208 nm (ord), usually attributed (*13*) to contributions by "random"[2] chains, is quite noticeable, but this trough gradually disappears as the chain is lengthened. Concomitantly, the "helical" trough at 233 nm becomes more pronounced. The presence of an N-terminal *t*-butyloxycarbonyl group had at least as much effect in this respect as the incorporation of the next residue. Therefore, it appeared that not the addition of any particular residue but the lengthening of the chain, probably through folding and shielding, is responsible for the stabilization of a helix in these compounds. This observation also suggested the C-terminal half as the location of the helical stretch and prompted an analysis of the sequence with the aid of the empirical conformational parameters proposed by Chou and Fasman (*14*, *15*). Notwithstanding some possible reservations[3] in the application of these parameters, we deemed them quite valuable for designing analogs that were expected to provide additional experimental evidence for the question under study: the position of the helical stretch in secretin.

In secretin, the $\langle P_\alpha \rangle$ value (*14*) calculated for the sequence 6–13 is 1.07 and is 1.17 for the C-terminal part of the chain encompassing residues 17–24. Only the latter, higher value permits the *prediction* (*15*) of a helix. Nevertheless, this difference between the two $\langle P_\alpha \rangle$ values is not sufficient to allow a clear choice between the two halves of the molecule, and yet, because of the low helix content calculated from the ord-cd

[2] The expression "random" is used to describe such parts of a chain which do not take up a conformation that can be described in simple terms of geometry but does not mean that these parts may not have a preferred conformation.

[3] For a discussion on empirical conformational parameters, cf., e.g., (*27*).

spectra, only one of these segments can be helical. In order to clarify this issue, we decided to build an analog of S_{5-27} or of 21-Lys-S_{5-27}, in which the $\langle P_\alpha \rangle$ value of the sequence 6–13 is significantly decreased. To achieve this, we decided to replace glutamic acid-9, because of its high P_α value. To render the alteration of the molecule conservative, aspartic acid was selected as the replacement. Thus, as in the unaltered sequence, a carboxyl group is present in the side chain of the residue in position 9. The role of a carboxyl group in this position was examined in our recent studies (*10*) which involved the synthesis of 9-Gln-S_{5-27}. The measurable loss of "structure" in this analog could be attributed to the elimination of an ion pair with the resulting loss of stabilization of folding, although the replacement of Glu (P_α 1.53) by Gln (P_α 1.17) also reduced the $\langle P_\alpha \rangle$ value of the 6–13 segment from the original 1.07 to 1.02. Therefore, an examination of the 9-Asp analog was expected to shed light on two problems: the role of the carboxyl group in ion-pair formation and the participation of this particular residue (Glu) in a potentially helical region. Since the P_α value of Asp is 0.98, the $\langle P_\alpha \rangle$ calculated for the 6–13 stretch was reduced to 1.0. For reasons of practicality, i.e., the availability of intermediates in this laboratory, 9-Asp-21-Lys-S_{5-27} was synthesized.

EXPERIMENTAL[4]

Capillary melting points are reported uncorrected. The solvents were reagent grade: DMF was stored over a Linde 4XA molecular sieve, and TFA and DIEA were distilled. For tlc, precoated silica gel plates (Merck or a hard-surfaced plate from Quantum Industries) and, where noted, microcrystalline cellulose plates (Analabs) were used with the following solvent systems: A, *n*-BuOH–AcOH–H_2O (4:1:1); B, *n*-BuOH–Pyr–AcOH–H_2O (30:10:3:12); C, *n*-BuOH–AcOH–H_2O (4:1:5), upper phase; uv absorption, ninhydrin, fluorescamine spray (*16*), chlorination, and charring were used for detection.

For quantitative amino acid analysis, samples were hydrolyzed with constant-boiling HCl in evacuated, sealed ampoules at 110°C for 16 hr, and the ratio of amino acids determined on a Beckman–Spinco 120C instrument according to the method of Spackman *et al.* (*17*). Homonorleucine was added as an internal standard. For enzymatic hydrolysis, aminopeptidase M (Röhm) was applied (*18*).

For analysis by fluorescence of CCD, an Aminco–Bowman spectrophotofluorometer fitted with a photon counter was used. Aliquots from individual tubes were diluted to 3.0 ml with 0.1 *M* phosphate buffer (pH 8.4) and mixed with 1.0 ml of fluorescamine reagent (2 mg/10 ml of acetone). The excitation wavelength was set at 390 nm and counted for 10 sec at 480 nm.

All *in situ* operations (*9, 19*), including coupling, deblocking, and isolation of intermediates, were carried out either in 40-ml glass centrifuge tubes fitted with standard

[4] The abbreviations for amino acids and protecting groups are those of the IUPAC-IUB Commission on Biochemical Nomenclature (*28*). In addition, the following abbreviations are used: CCD, countercurrent distribution; DIEA, diisopropylethylamine; DCZ, 2,6-dichlorobenzyloxycarbonyl; DMF, dimethylformamide; DNP, 2,4-dinitrophenyloxy; ONO, *o*-nitrophenoxy; ONP, *p*-nitrophenoxy; TFA, trifluoroacetic acid.

24/40 ground-glass tapered joints or in 150-ml Teflon centrifuge tubes with standard machined 34/45 tapered joints.

t-Butyloxycarbonyl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminyl-glycyl-L-leucyl-L-valinamide (*I*). The protected pentadecapeptide,[5] Boc-Leu-Arg(NO_2)-Asp(Bzl)-Ser(Bzl)-Ala-Arg(NO_2)-Leu-Gln-Lys(DCZ)-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (1.5 g, 0.66 mmole), was placed into a 150-ml Teflon centrifuge tube and treated with TFA (6 ml) for 15 min at room temperature. The TFA was removed *in vacuo* on a rotary evaporator and the residue triturated with ether (100 ml) and centrifuged and the solid washed twice with ether (100 ml of ether each time). The trifluoroacetate salt was dried *in vacuo* over NaOH. Small samples were examined on silica gel tlc, single spot at R_fA 0.67. The partially deprotected pentadecapeptide was dissolved in DMF (15 ml) and soon turned into a gel; a Thermolyne-Max-Mix shaker was used to facilitate dissolution. Tertiary base (DIEA) was added dropwise until an alkaline reaction was detected above the surface of the solution with moist indicator paper. Boc-Arg(NO_2)-DNP (20) (5 mmoles in 5 ml of DMF) was added in portions with stirring, along with enough DIEA to maintain a slightly alkaline reaction mixture until the reaction was complete as determined by tlc. DMF was removed *in vacuo* and the residue treated with EtOAc (100 ml) and ether (25 ml) and centrifuged. The supernatant was decanted and the product was washed with EtOAc (5 × 100 ml) and dried, at first with a stream of nitrogen and finally in a desiccator over P_2O_5. The product (1.65 g, 0.66 mmole) was homogeneous on tlc, R_fA 0.88, mp 318°C dec.

t-Butyloxycarbonyl-O-benzyl-L-seryl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminyl-glycyl-L-leucyl-L-valinamide (*II*). The protected hexadecapeptide (I) was partially deprotected as described above with TFA and the trifluoroacetate salt was acylated in DMF with Boc-Ser(Bzl)-ONO (*19*) (0.624 g, 1.5 mmoles) and 1-hydroxybenzotriazole (*21*) (0.23 g, 1.5 mmoles) added as the catalyst. Completion of the reaction was ascertained by ninhydrin and fluorescamine spot tests. The product was precipitated with EtOAc (100 ml) and centrifuged after evaporating the DMF. The supernatant was decanted and the solid washed with EtOAc (4 × 50 ml) and dried. The product appeared homogeneous on tlc, R_fA 0.84; yield 1.69 g, 0.64 mmole; mp 326°C.

t-Butyloxycarbonyl-L-leucyl-O-benzyl-L-seryl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminyl-glycyl-L-leucyl-L-valinamide (*III*). A portion (0.6 g, 0.23 mmole) of II was placed into a 40-ml centrifuge tube and deprotected and acylated as described for II. Boc-Leu-ONO (*19, 22*) (0.2 g, 0.57 mmole) and 1-hydroxybenzotriazole (*21*) (0.08 g, 0.52 mmole) were used. After evaporating the DMF, EtOAc (30 ml) was used to triturate the product. The solid was then washed with EtOAc (3 × 30 ml) and dried. The product appeared homogeneous on tlc, R_fA 0.84; yield 0.62 g, 0.22 mmole; mp 324°C dec.

t-Butyloxycarbonyl-β-benzyl-L-aspartyl-L-leucyl-O-benzyl-L-seryl-nitro-L-arginyl-

[5] This protected pentadecapeptide amide was prepared by K. W. Funk (cf. also Refs. (*12*) and (*18*)).

L-leucyl–nitro-L-arginyl–β-benzyl-L-aspartyl–O-benzyl-L-seryl–L-alanyl–nitro-L-arginyl–L-leucyl–L-glutaminyl–N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl–L-leucyl–L-leucyl–L-glutaminyl–glycyl–L-leucyl–L-valinamide (*IV*). Compound III (0.58 g, 0.21 mmole) was dissolved in TFA, but an insoluble material remained. The solution was centrifuged and decanted. (The source of the insoluble material (45 mg) was found to be the molecular sieve, Linde 4XA, that had been used to dry EtOAc.) The TFA was evaporated *in vacuo*, and a solid was precipitated with ether. The trifluoroacetate salt was acylated with Boc-Asp(Bzl)-ONO (*19*) in the presence of 1-hydroxybenzotriazole (*21*), as described for compounds II and III. The product (0.53 g, 0.18 mmole), homogeneous on tlc, decomposed at 322°C.

t-Butyloxycarbonyl-O-benzyl-L-seryl–β-benzyl-L-aspartyl–L-leucyl–O-benzyl-L-seryl–nitro-L-arginyl–L-leucyl–nitro-L-arginyl–β-benzyl-L-aspartyl–O-benzyl-L-seryl–L-alanyl–nitro-L-arginyl–L-leucyl–L-glutaminyl–N^{ε}-2,6-dichlorobenzyloxycarbonyl–L-lysyl–L-leucyl–L-leucyl–L-glutaminyl–glycyl–L-leucyl–L-valinamide (*V*). Compound IV (0.52 g, 0.18 mmole) was deprotected and acylated with Boc-Ser(Bzl)-ONO (*19*) using 1-hydroxybenzotriazole (*21*) as a catalyst as described for compound III. Yield, 0.52 g, 0.17 mmole; mp 322°C dec.; tlc R_fA 0.76.

t-Butyloxycarbonyl-L-threonyl–O-benzyl-L-seryl–β-benzyl-L-aspartyl–L-leucyl–O-benzyl-L-seryl–nitro-L-arginyl–L-leucyl–nitro-L-arginyl–β-benzyl-L-aspartyl–O-benzyl-L-seryl–L-alanyl–nitro-L-arginyl–L-leucyl–L-glutaminyl–N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl–L-leucyl–L-leucyl–L-glutaminyl–glycyl–L-leucyl–L-valinamide (*VI*). Boc-Thr-DNP (*5*) was used to acylate the trifluoroacetate salt of V (0.5 g, 0.16 mmole), as described for compound I. The material (0.5 g, 0.15 mmole) appeared homogeneous on tlc, R_fA 0.9; mp 324°C dec.

t-Butyloxycarbonyl-L-phenylalanyl–L-threonyl–O-benzyl-L-seryl–β-benzyl-L-aspartyl–L-leucyl–O-benzyl-L-seryl–nitro-L-arginyl–L-leucyl–nitro-L-arginyl-β-benzyl–L-aspartyl-O-benzyl–L-seryl–L-alanyl–nitro-L-arginyl–L-leucyl–L-glutaminyl–N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl–L-leucyl–L-leucyl–L-glutaminyl–glycyl–L-leucyl–L-valinamide (*VII*). Compound VI (0.40 g, 0.12 mmole) was deprotected and acylated as described for II and III, using Boc-Phe-ONO (*19*) and 1-hydroxybenzotriazole (*21*). Yield, 0.41 g, 0.12 mmole; mp 322°C dec.; homogeneous on tlc, R_fA 0.84.

t-Butyloxycarbonyl-L-threonyl–L-phenylalanyl–L-threonyl–O-benzyl–L-seryl–β-benzyl-L-aspartyl–L-leucyl–O-benzyl-L-seryl–nitro-L-arginyl–L-leucyl–nitro-L-arginyl–β-benzyl-L-aspartyl–O-benzyl-L-seryl–L-alanyl–nitro-L-arginyl–L-leucyl–L-glutaminyl–N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl–L-leucyl–L-leucyl–L-glutaminyl–glycyl–L-leucyl–L-valinamide (*VIII*). Compound VIII (0.12 mmole) was prepared as described for VI. On tlc, the compound appeared as a single spot, R_fA 0.77; yield 0.37 g, 0.11 mmole; mp 327°C dec. *Anal.* Calcd for $C_{162}H_{237}N_{39}O_{44}Cl_2$: C, 55.5; H, 6.8; N, 15.6; Found: C, 55.1; H, 6.5; N, 15.7.

L-Threonyl–L-phenylalanyl–L-threonyl–L-seryl–L-aspartyl–L-leucyl–L-seryl–L-arginyl–L-leucyl–L-arginyl–L-aspartyl–L-seryl–L-alanyl–L-arginyl–L-leucyl–L-glutaminyl–L-lysyl–L-leucyl–L-leucyl–L-glutaminyl–glycyl–L-leucyl–L-valinamide (*IX*). Compound VIII (120 mg, 34 μmole) was treated in TFA (3 ml) for 15 min at room temperature. The solvent was evaporated *in vacuo* and the residue was triturated with ether, filtered, washed with ether on the filter, and dried *in vacuo* in a desiccator over NaOH. The solid was then dissolved in 80% AcOH (25 ml) and hydrogenated at atmospheric pressure

in the presence of Pd black catalyst for 40 hr (*23–25*). After filtering off the catalyst, the uv spectrum showed incomplete removal of the nitro protecting group. Hydrogenation was repeated with fresh catalyst. The catalyst was filtered and the residue evaporated *in vacuo*, redissolved in a small amount of water, and lyophilized.

The crude peptide was then dissolved in both layers (2 ml each) of the solvent system *n*-butanol–0.1 *M* phosphate buffer (pH 7) (*26*) and distributed through 100 transfers in a Craig instrument (3 ml of each phase per tube). The distribution of the peptide was

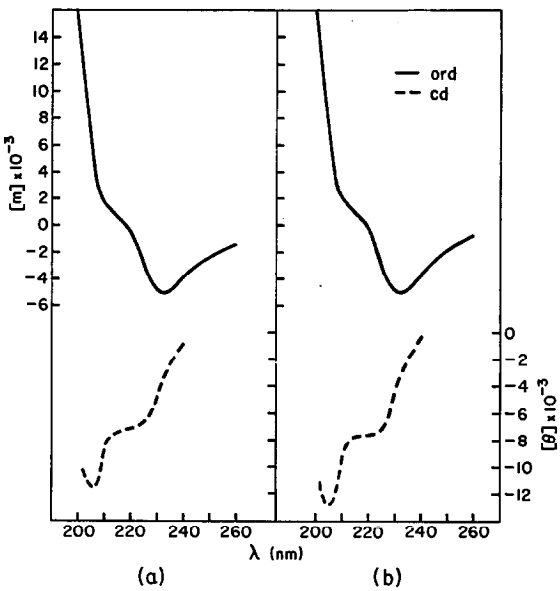

FIG. 2. Ord and cd spectra of (a) secretin and (b) 9-Asp-21-Lys-S_{5-27}, both in water.

first scanned by spot tests and then determined quantitatively by fluorescamine on 0.1-ml aliquots. There was one major peak with $k = 4.8$ (Fig. 3). The contents of tubes No. 75–90 were pooled and diluted with 95% EtOH. After storing the solution in the cold overnight, the precipitated phosphates were filtered and the filtrate taken up in 15 volumes of water (3 liters), acidified to pH 3, and treated with alginic acid (E. Mendell and Co., 2.5 g) (*26*). The alginic acid was collected on a filter, washed with ice-cold 0.001 *N* HCl, and eluted with 0.2 *N* HCl (20 ml). The eluant was passed (2–3 ml/min) through a column (1.25 × 19 cm) of DEAE-Sephadex (A-25) in the acetate cycle and lyophilized. A portion (25 mg) of this material was passed (ca. 1 ml/min) through another column (1.5 × 32 cm) of Sephadex G-25. The purified peptide (17 mg, 6 μmole) appeared homogeneous on thin layers of silica gel (R_fB 0.28) and cellulose (R_fA 0.48 and R_fC 0.63). On electrophoresis (Whatman 3MM paper) at pH 6.4, water–pyridine–acetic acid (1350:150:6), the peptide traveled toward the cathode with a mobility 0.4, the same as that of arginine; in 1 *M* AcOH, with 0.6 × Arg. Amino acid analysis: Lys, 1.2; NH_3, 4.4; Arg, 3.0; Asp, 2.0; Thr, 1.8; Ser, 2.9; Glu, 2.0; Gly, 1.1; Ala, 1.0; Val, 0.9; Leu, 6.0; Phe 0.8. Amino acid analysis after hydrolysis

with aminopeptidase M (18): Lys, 1.2; NH_3, 1,5; Arg, 3.0; Asp, 1.6; Thr, 1.5; Ser and Gln, undetermined; Gly, 1.1; Ala, 1.0; Val, 1.0; Leu, 6.0; Phe, 1.4.

A sample of the purified peptide was dissolved in water (pH 6.6 ± 0.1), centrifuged, and the ord-cd spectra (Fig. 2) were recorded at room temperature with a Cary Model 60 spectropolarimeter fitted with a Model 6001 cd attachment. Cylindrical fused

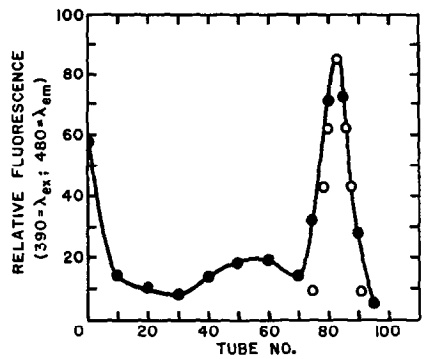

FIG. 3. Countercurrent distribution (100 transfers) of the tricosapeptide 9-Asp-21-Lys-S_{5-27}: (●) experimental values, (○) calculated values ($k = 4.8$).

quartz cells of 1-, 5- and 10-mm pathlength were used. The concentration of 9-Asp-21-Lys-S_{5-27} in the solution (0.4 mg/ml) was calculated from the recovery of quantitative amino acid analyses. The ord-cd spectra of secretin were recorded as reported in Ref. (7). The values of mean residue ellipticity (in degree cm^2/dmole) and of mean residue rotation were not corrected for the refractive indices of the solutions.

RESULTS AND CONCLUSION

The ord-cd spectra of the synthetic tricosapeptide analog, 9-Asp-21-Lys-S_{5-27} (Fig. 2), closely resemble those of 21-Lys-S_{5-27}, S_{5-27} and also of secretin itself. Thus, the helix content of the chain remained unchanged even though the probability of a helix in its N-terminal half was considerably reduced. This new evidence strongly supports a model in which the C-terminal region of the molecule is helical. The role of the N-terminal part in determining the conformation seems to be stabilization of the helix through folding, which in turn requires the participation of ion pairs. The aspartyl residue in position 9 can provide the negatively charged group for one of these ion pairs.

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