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On the biologically active structures of cholecystokinin, little gastrin, and enkephalin in the gastrointestinal system

(conformational energy/peripheral cholecystokinin receptor/gastrin receptor)

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ABSTRACT **The biologically active conformations of a series of four peptides [four cholecystokinin (CCK)-related peptides and enkephalin] in their interactions with gastrointestinal receptors have been deduced using conformational computational analysis. The two peptides that interact exclusively with peripheral-type CCK receptors are the heptapeptide COOH-terminal fragment from CCK (CCK-7) and the analogous sequence from cerulein (CER-7) in which threonine replaces the methionine proximal to the NH_2 terminus. The two peptides that interact exclusively with the gastrin receptor in the stomach are the active COOH-terminal fragment of little gastrin and the COOH-terminal tetrapeptide sequence common to all of these peptides, CCK-4. We find that preferred conformations for the peripherally active peptides CCK-7 and CER-7 are principally β-bends, whereas little gastrin and CCK-4 are fundamentally helical. In the class of lowest energy structures for both CCK-7 and CER-7, the aromatic rings of the tyrosine and phenylalanine lie close to one another whereas the tryptophan indole ring points in the opposite direction. This structure is superimposable on the structures of a set of rigid indolyl benzodiazepine derivatives that interact with complete specificity and high affinity with peripheral CCK receptors further suggesting that the computed β-bends are the biologically active conformation. The biologically active conformation for CCK-4 and the little gastrin hexapeptide has also been deduced. By excluding conformations common to CCK-7 and CCK-4, which do not bond to each other's receptors, and then by selecting conformations in common to CCK-4 and the gastrin-related hexapeptide, which do bind to each other's receptors, we deduce that the biologically active conformation at the gastrin receptor is partly helical and one in which the indole of tryptophan and the aromatic ring of phenylalanine are close to one another while the methionine and aspartic acid side chains point in the opposite direction. These major differences in preferred structures between the common CCK-7/CER-7 peptides and the common CCK-4/little gastrin peptides explain the mutually exclusive activities of these two sets of peptides. We have observed that [Met]enkephalin strongly antagonizes the action of the naturally occurring peripherally active CCK-8 (CCK-7 with an NH_2-terminal aspartic acid residue added). The computed lowest energy structures for this opiate peptide closely resemble key features of the computed CCK-7/CER-7 structure, further supporting the proposed structure.**

Cholecystokinin (CCK) peptides comprise a class of structurally related biologically active molecules that are involved in neural and hormonal regulation of gastrointestinal and central nervous system function. The parent form of CCK is a 114-residue peptide, which is observed principally as its COOH-terminal cleavage fragments of 58, 39, 33, 22, and 8 residues (1). The latter octapeptide is termed CCK-8. Essentially all biological activity on peripheral-type CCK receptors is preserved in the COOH-terminal peptide, termed CCK-7, whose sequence is Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2. A similar level of activity at peripheral receptors is found in the COOH-terminal 7-residue sequence of cerulein, an amphibian decapeptide (2). This sequence is termed CER-7 and is identical to the CCK-7 sequence except that a threonine replaces a methionine at position 2.

In addition, other CCK-like molecules exist that do not interact at all with peripheral CCK receptors but rather selectively interact with gastrin receptors (3). Little gastrin (3) contains the COOH-terminal hexapeptide Tyr-Gly-Trp-Met-Asp-Phe-NH_2 that is identical to the CCK-7 and CER-7 sequences except for the deletion of a residue at position 2. The biological activity of this peptide is quite similar to that of CCK-4 (Trp-Met-Asp-Phe-NH_2); they are exclusively active on gastrin receptors in the gastrointestinal tract.

The fact that the two classes of peptides, those acting on peripheral CCK receptors (CCK-7 and CER-7) and those interacting with gastrin receptors (little gastrin and CCK-4), do not significantly bind to the receptor of the other, despite close similarities in sequence, suggests that major structural differences exist between the peptides in the two classes. Within a given class, however, there must be strong structural similarities between peptides. An exception to these considerations is the forebrain CCK receptor, which does not differentiate between these two classes of peptides (4). We limit the present discussion to the consideration of the gastrointestinal receptors for CCK and gastrin.

In this study, we examine the preferred active conformations of the two sets of peptides in their binding to peripheral and gastrin receptors. In contrast to previous studies on the structures of CCK-7 and the naturally occurring CCK-8 (CCK-7 with an NH_2-terminal aspartic acid residue) that were concerned with the structures of the isolated peptides in solution (5), we examine the most likely biologically active structures of the peptides in a two-step procedure. (*i*) The energetically preferred conformations of each peptide are generated, using conformational analysis (6, 7). (*ii*) All conformations that are the same for the peptides within one class are selected and compared with those found for the peptides in the other class. Any conformations that are common to both classes are excluded, and thus the number of possible active conformations is greatly reduced.

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Abbreviation: CCK, cholecystokinin.

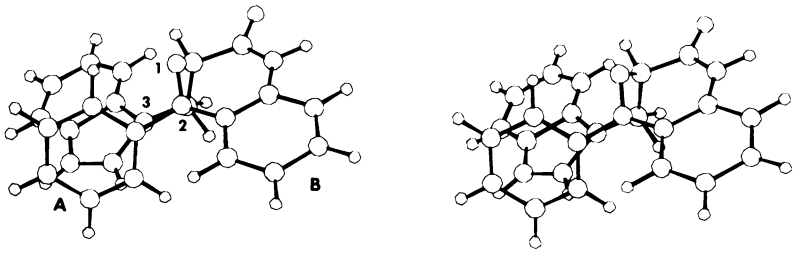

FIG. 1. Stereo view of model-built (using CHEMGRAF, ref. 16) 3-(3-indolylmethyl)benzodiazepine (*R*-configuration). Atoms labeled 1, 2, and 3 are C-3 of the benzodiazepine, the corresponding CH_2 group, and the C-3^i of the indole ring, respectively. The superscript *i* refers to the indole ring. A, pendant aromatic ring; B, fused aromatic ring.

We further take advantage of the discovery that a set of rigid nonpeptide benzodiazepine derivatives has been found to block peripheral CCK receptors with high specificity and affinity (8). This implies that key structural features must be shared between the biologically active conformations of CCK-7 and CER-7 and these antagonists. This observation further narrows the possible biologically active conformations.

We have also observed that [Met]enkephalin antagonizes the biological response of CCK-8 in the isolated pyloric sphincter of the rat through a nonopioid receptor-mediated system with an IC_{50} of 10^{-7} M (R.B.M., J. Gibbs, and G. P. Smith, unpublished data). This antagonism also suggests that conformations of [Met]enkephalin may be related to biologically active conformations of CCK-8 and CCK-7. Several studies, including conformational analysis, have been performed on this peptide (9–11). We, therefore, also compare the conformations of the opiate peptide (11) with those for CCK-7/CER-7 to further narrow the range of biologically active conformations.

METHODS

Conformational energy analysis was carried out on the following peptides: CCK-4, CCK-7, CER-7, and the 6-residue COOH-terminal fragment from little gastrin. All calculations were carried out on the forms of these peptides with the NH_2-terminal amino group blocked by an acetyl group. The COOH-terminal group was blocked by an amino group in all cases. The analysis used a chain build-up procedure as described for polypeptide sequences (12–14). Briefly, the single residue minima (15) for the NH_2-terminal residue of a particular peptide were combined with those for the next residue of the chain, and the resulting conformations were then subjected to energy minimization (7). All of the resulting conformations whose energies lay within 5 kcal/mol (1 cal = 4.184 J) of the energy of the global minimum were selected and combined with the single residue minima for the next amino acid residue in the chain. The resulting conformations were then subjected to energy minimization (11), and the procedures just described were applied iteratively until all amino acid residues of the given peptide were included from the NH_2 to the COOH terminus. A 5 kcal/mol cutoff was used for all peptides.

Conformation of the Peripherally Active Nonpeptide Antagonist, *R*-3-(3-Indolylmethyl)benzodiazepine. A series of indole derivatives of benzodiazepines have been shown to bind to peripheral CCK receptors with high affinity and specificity (8). The parent compound of this series is the 3-indolylmethyl derivative shown in Fig. 1. These derivatives differ from conventional benzodiazepines mainly in an indolyl moiety at position 3 of the benzodiazepine seven-membered ring system. The ring systems of the benzodiazepine portion are conjugated and, therefore, rather rigid. In fact, the only bonds in the 3-indolylmethyl derivative around which rotation can take place are the C-3—CH_2 and CH_2—C-3^i (*i*,

Table 1. Conformations common to CCK-7 and CER-7

Conformer	Conformational state							Relative energy,* kcal/mol	
	Tyr	Xaa†	Gly	Trp	Met	Asp	Phe	CCK-7	CER-7
1	E	D	C‡	F	C	A	A	0.0	1.5
2	E	D	C‡	F	F	A	E	0.4	1.9
3	E	D	C‡	F	C	A	F	0.9	2.5
4	E	D	C‡	F	C	A	D	1.0	2.5
5	E	D	C‡	F	C	A	G	1.0	3.4
6	E	D	C‡	F	C	A	E	1.4	4.7
7	A	C	D‡	A	A	A	A	2.0	0.8
8	E	D	C‡	F	C	A	C	2.5	4.4
9	E	D	C‡	F	F	A	G	2.8	4.3
10	A	C	D‡	A	D	A	E	2.8	3.7
11	E	D	C‡	F	F	A	F	2.8	4.1
12	E	D	C‡	F	F	G	D	2.9	4.3
13	A	C	D‡	A	D	F	E	3.8	5.0
14	A	C	D‡	A	A	A	A†	3.9	2.6
15	E	C	D‡	E	A†	C	E	4.0	2.6

Definitions of conformational states are as follows (see ref. 18): A; $-110 \le \phi \le -40$, $-90 \le \psi \le -10$. C; $-110 \le \phi \le -40$, $50 \le \psi \le 130$. D; $-180 \le \phi \le -110$, $20 \le \psi \le 110$. E; $-180 \le \phi \le -110$, $130 \le \psi \le 180$ or $-180 \le \psi \le -140$. F; $-110 \le \phi \le -40$, $130 \le \psi \le 180$ or $-180 \le \psi \le -140$. G; $-180 \le \phi \le -110$, $-90 \le \psi \le -40$.

*All energies, in kcal/mol, are expressed relative to that of the global minimum. All conformations whose energies are within 3 kcal/mol are shown. The global minimum for CER-7 is not shown, because it does not occur in the low-energy minimum for CCK-7.

†Xaa is methionine in CCK-7 and threonine in CER-7.

‡Obtained by multiplying the corresponding values in the single-letter states by −1 and reversing the inequalities.

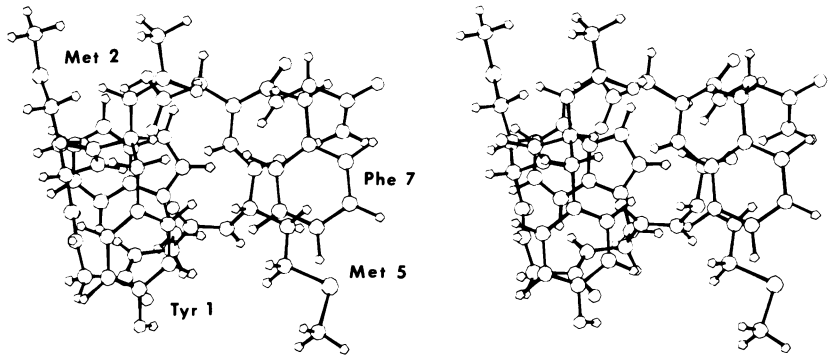

FIG. 2. Stereo view of the lowest energy common conformer for CCK-7 and CER-7 peptides. The peptide shown is CCK-7, corresponding to Table 1, conformer 1.

indole) bonds. A model for this compound was constructed with the molecular graphics program CHEMGRAF (16). The conformation of the benzodiazepine ring was optimized by energy minimization of the graphically constructed model (16, 17). The only change in the structure was a rotation of the pendant aromatic ring (position A in Fig. 1) so that it formed an angle of ≈60° with the fused aromatic ring of the structure (position B in Fig. 1). The rings were thus not coplanar. The low-energy positions for the indolylmethyl groups were then obtained by generating all possible combinations of values for dihedral angles for rotation around the C3—CH_2 and CH_2—C-3′bonds (as defined in Fig. 1) in 30° increments from −180° to +180° and by minimizing the energy for each separate conformation thus generated. A number of low-energy minima resulted, one of which had its indole ring coincident with that for the indole ring of the tryptophan residue of CCK-7 (see below). This conformation is shown in Fig. 1 and also in Fig. 3.

RESULTS AND DISCUSSION

Low-Energy Conformations of CCK-7 and CER-7. CCK-7 and CER-7 have many conformations in common. The common conformations are listed in Table 1. The global minimum for CCK-7 (Table 1, conformer 1) is the lowest energy structure common to CCK-7 and CER-7. This structure for CCK-7 is shown in stereo view in Fig. 2. In this β-bend, the side chains of the tyrosine and phenylalanine residues approach one another; the C^{β} atoms of the two residues are ≈6 Å apart. The global minimum for CER-7 (data not shown) has no counterpart in the computed low-energy conformations for CCK-7. This minimum is also a β-bend with a sharp reverse turn between Gly-3 and Trp-4. The structure is stabilized partly by a long-range hydrogen bond between the side-chain —OH of Thr-2 and the backbone carbonyl oxygen of Phe-7, an interaction that cannot exist in CCK-7 since it does not contain a threonine residue. In this conformation of CER-7, the tyrosine and phenylalanine side-chain aromatic rings lie far apart from one another. The lowest energy conformer common to both peptides, conformer 1 of Table 1, has an energy that is 1.5 kcal/mol higher in energy than that of the global minimum for the CER-7 peptide. Both structures (Table 1, conformers 1) are additionally stabilized by a hydrogen bond between the —OH of Tyr-1 and the carbonyl oxygen of Gly-3 (see Fig. 2).

Superposition of the CCK-7 and CER-7 Structures on That for Indolylmethylbenzodiazepines. As shown in Fig. 1, in the conformation for this derivative, the two phenyl groups must lie close to one another whereas the indole ring must point away from these two rings. Of the common structures for CCK-7 and CER-7 listed in Table 1 only one, entry 1, shown in Fig. 2, has the two phenyl groups in close proximity to one another yet has the indole ring pointing away from these two aromatic rings. As shown in Fig. 3, it is possible to superimpose the CCK-7 structure (Fig. 2) upon the benzodiazepine structure (Fig. 1). This superposition was performed using computer graphics (16). In this figure, the peptide CCK-7 is colored green while the benzodiazepine derivative is colored red. It is seen that the phenolic side chain of Tyr-1 can be made to approximate the pendant aromatic ring of the antagonist; the aromatic ring of Phe-7 can be made to overlie the fused aromatic ring of the benzodiazepine; and the indole side chain of Trp-4 lies near the 3-indolyl moiety of the benzodiazepine derivative. This similarity in orientation of the

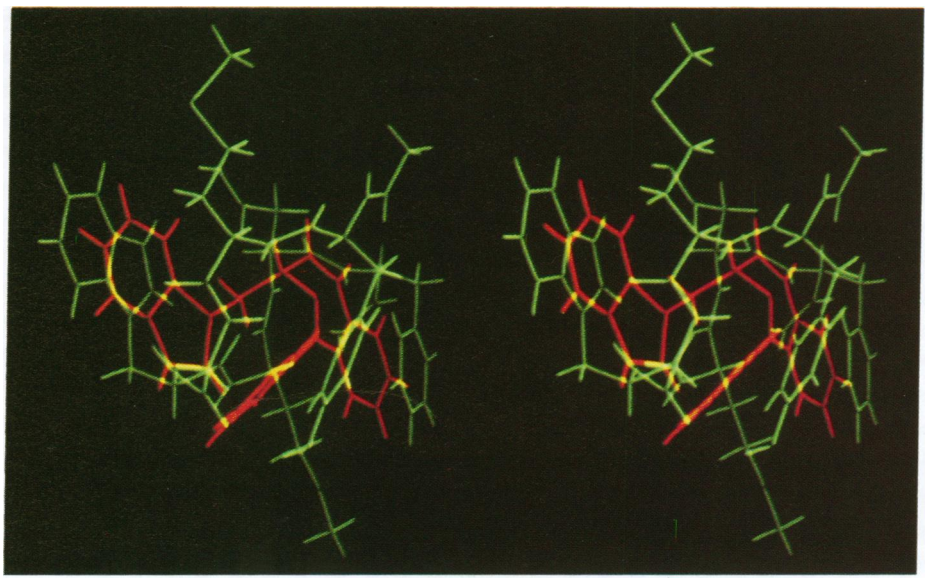

FIG. 3. Color stereo superposition of CCK-7 (Fig. 2) on the benzodiazepine derivative (Fig. 1). The CCK peptide is green, and the benzodiazepine derivative is red. The indole rings are seen on the far left of the figure, Phe-7 lies near the fused benzene ring of the benzodiazepine on the far right, and the Tyr-1 ring and pendant benzene ring of the benzodiazepine derivative lie near one another in the lower center of the figure. The two superimposed molecules are offset slightly with respect to one another to show the identity of the critical residues of each molecule.

critical aromatic rings of CCK-7 and of the benzodiazepine derivative and the fact that the peptide structure in Figs. 2 and 3 is common to both CCK-7 and CER-7 suggests that the conformation of CCK-7 and CER-7 shown in Figs. 2 and 3 may be the biologically active one at peripheral CCK receptors. As will be discussed below, this conformation does not occur in the isolated CCK-4 sequence or in the little gastrin fragment. This is consistent with the observation that these latter two peptides do not bind to peripheral CCK receptors.

It should be noted that, in Fig. 3, the conformation of the indole-ring side chain of the benzodiazepine derivative was only one of several energetically feasible dispositions and was chosen because it was similar to that of the indole ring of Trp-4 of CCK-7. The side-chain conformation (g^+,g^+) for this residue of CCK-7 is stabilized by a hydrogen bond between the —NH of the indole ring and the sidechain C=O of the aspartic acid COOH group. If this interaction is weakened, as for example by the effects of solvation upon either or both groups, then the indole ring of tryptophan would presumably adopt a number of different low-energy conformations. Thus, the superposition in Fig. 3 represents one possible orientation of indole rings. Others may be possible. The essential features of the model, however, are that the two phenyl groups must be proximal and that the indole ring, regardless of its specific orientation, must point away from the two phenyl groups.

Orientation of the Indole Ring of CCK-7 and Biological Activity. CCK-7 and CER-7 alone or in larger peptides naturally contain a sulfated Tyr-1. In this form, these peptides stimulate pancreatic secretion and gallbladder contraction. In the unsulfated form, CCK-8 will block the biological actions of sulfated CCK-8 (8, 19, 20). However, a full pharmacological characterization of this antagonism is lacking. The affinity constant of the sulfated form is roughly 100–1000 times that of the unsulfated peptide. The unsulfated peptide and the benzodiazepine antagonist discussed above bind with a K_d in the range of 0.1–1 μM, whereas the sulfated peptide binds with a K_d in the range of 0.1–1 nM (20).

In the series of benzodiazepine derivatives tested for their peripheral activities, one derivative was found to have a receptor affinity on the order of that for sulfated CCK. This derivative, called L-364,718 (20), is 3-(2-indolylcarboxamido)benzodiazepine. The —NHCO— group replaces the CH_2 group of the 3-(indolylmethyl) parent compound (Figs. 1 and 3) and is somewhat more rigid. A model of this derivative was constructed in a manner similar to that for the 3-(indolylmethyl) derivative, and the two molecules were then superimposed. The results (not shown) indicate that their benzodiazepine rings were fully superimposable. The indole ring of the L-364,718 derivative protruded farther away from the benzodiazepine system than did the indole ring of the 3-(indolylmethyl) derivative shown in Figs. 1 and 3. Nevertheless, the essential structural features postulated to be critical for specificity of binding to peripheral receptors are preserved in this compound.

Because the L-364,718 derivative contains no sulfate or other negatively charged group but has an affinity that is about the same as that of sulfated CCK, it is unlikely that sulfate binding accounts for the different affinities between unsulfated and sulfated CCK peptides. Inspection of the conformation of CCK-7 shown in Figs. 2 and 3 suggests that a sulfate moiety on Tyr-1 would be sufficiently close to the carboxylate group of Asp-6 to repel it. If the side chain of Asp-6 moves significantly so that the favorable hydrogen bond it makes with the —NH of the indole ring of Trp-4 is disrupted, the indole ring would be "free" to adopt other conformations that we find would be directly superimposable on that of the L-364,718 derivative. This explanation presupposes that the presence of the sulfate on the —OH of Tyr-1 would not greatly destabilize the energetically favored conformation shown in Figs. 2 and 3 (see also entry 1, Table 1). Despite the fact that the favorable hydrogen bond between the tyrosine —OH and the backbone C=O of Gly-3 discussed above would be disrupted, we have found by computation that the change in conformational energy would be only 2 kcal/mol. If further extension of the indole ring away from the two phenyl groups results, however, this loss in energy may well be compensated by increased favorable binding to the peripheral CCK receptor.

Table 2. Representative minima for *N*-acetyl-CCK-4

Conformer	Conformational state				Relative energy, kcal/mol
	Trp	Met	Asp	Phe	
1	A	A	A	A	0.0
2	A	A	A	D	1.1
3	A	A	A	E	1.6
4	A	A	A	C	2.0
5	C	A	C	C	2.4
6	C	A	C	A	2.5
7	A	A	G	A	2.7
8	G	A	A	A	2.8
9	A	A	E	A	4.4

See Table 1 for definitions of conformational states and discussion of relative energy. Conformation 9 is included because it is identical to a minimum for the last four residues of the little gastrin hexapeptide (see Table 3, conformer 4).

Biologically Active Structure of Enkephalin. We have obtained results of experimental studies on the isolated rat pylorus in which we examined the possible antagonism of CCK-8 agonist activity by [Met]enkephalin (Tyr-Gly-Gly-Phe-Met) and related opiate peptides (R.B.M., J. Gibbs, and G. P. Smith, unpublished data). These studies were suggested by the homology between the first five residues of the two peptides. The half-maximal dose for antagonist activity is in the vicinity of 10^{-7} M [Met]enkephalin. An exhaustive search for the allowed conformations of enkephalin has been performed (9–11) that has converged upon the set of allowed enkephalin structures. A technique based upon distance geometry theory (21) has been applied to determine the low-energy forms of [Met]enkephalin (11). The global minimum found for this peptide is the lowest found thus far and is quite similar to the global minimum found previously (9, 10). This conformation is (EDC*BE). This structure is similar, though not identical, to the structure of the first five residues of the presently determined global minimum for CCK-7 (Table 1, conformer 1, and Fig. 2). In this structure, the Tyr-1 side chain of enkephalin can be aligned with the Tyr-1 residue of CCK-7 (Fig. 2) whereas the side chain of Phe-5 can be aligned with the side-chain indole ring of Trp-5 of CCK-7 (Fig. 2). None of the other low-energy conformers obtained for enkephalin are so similar both in backbone conformation or in side-chain orientation to the computed

Table 3. Representative low-energy conformations of the little gastrin hexapeptide *N*-acetyl-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

Conformer	Conformational state						Relative energy, kcal/mol
	Tyr	Gly	Trp	Met	Asp	Phe	
1	E	C*	A	A	A	A	0.0
2	E	C*	A	A	A	D	0.8
3	E	C*	A	A	A	E	0.9
4	F	D*	A	A	E	A	1.8
5	E	D*	A	A	A	C	1.9
6	F	C*	A	A	E	E	2.0

See Table 1 for definitions of conformational states and for discussion of relative energy. All conformations whose energies lie within 2 kcal/mol of that of the global minimum are shown.

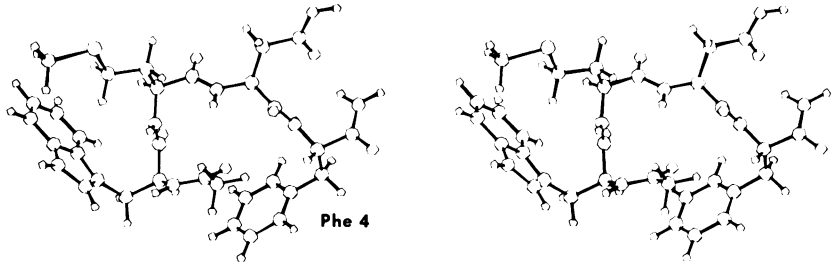

FIG. 4. Possible active conformation for CCK-4 that is also a low-energy conformer of the active hexapeptide of little gastrin.

CCK-7 structure as shown in Figs. 2 and 3. It appears reasonable, therefore, to conclude that this structure (or others similar to it) is the form that is active at peripheral CCK receptors.

Conformations of CCK Peptides Active on Gastrin Receptors: CCK-4 and Little Gastrin Hexapeptide. Exploration of the allowed conformations for CCK-4 resulted in the low-energy minima shown in Table 2. Comparison of the proposed conformations for the last four residues of the CCK-7 and CER-7 peptides (Table 1) with those for CCK-4 (Table 2) shows a significantly different set of structures. CCK-4 tends to adopt predominantly α-helical structures (conformers 1–4 in Table 2), whereas the corresponding residues of CCK-7 and CER-7 are distinctly nonhelical, with the possible exception of conformer 7 in Table 1. This is the only conformer for CCK-7 and CER-7 whose last four residues share a common conformation with CCK-4. This conformation is excluded for CCK-7 and CER-7 because it does not superimpose upon the peripherally active benzodiazepines and because it is common to both sets of peptides. If the all α-helical conformations for CCK-4 were its active form, its binding to gastrin receptors should be inhibited by CCK-7, since CCK-7 can adopt a conformation in which the last four residues are all helical (compare Table 1, conformer 7, with Table 2, conformer 1).

It is possible that the inability of CCK-7 to inhibit the binding of CCK-4 to gastrin receptors is the result of the Tyr-Xaa-Gly sequence that may itself interact unfavorably with the gastrin receptor and thus override the COOH-terminal tetrapeptide of CCK-7 that would tend to bind when in a helical conformation. However, little gastrin, which contains the closely related Tyr-Gly sequence, binds avidly to the gastrin receptor but not to peripheral CCK receptors. Thus, structural differences between CCK-7 and CCK-4 probably account for the differences in biological activity between these two classes of peptides.

To further illuminate the possible structure of CCK-4 bound to the gastrin receptor, the preferred conformations of the active hexapeptide fragment from little gastrin were determined (Table 3). Unlike CCK-7 and CER-7, the lowest energy conformers for this peptide are predominantly α-helical in the COOH-terminal tetrapeptide fragment. No structures with β-turns that bring the aromatic rings of Tyr-1 and Phe-6 into close proximity were obtained. As with CCK-4, the only structures that are similar for CCK-7 (or CER-7) and little gastrin are ones in which the COOH-terminal tetrapeptide residues are α-helical. Thus, deletion of Met-2 or Thr-2 in CCK-7 or CER-7 has significant structural consequences.

Comparison of the low-energy conformations of the gastrin-related hexapeptide (Table 3) with those of CCK-4 (Table 2) shows that, other than the α-helix for the Trp-Gly-Trp-Met-Asp-Phe sequence, only one conformation occurs that is common to both peptides. This conformation is conformer 9 in Table 2 and conformer 4 in Table 3, i.e., the (AAEA) conformation. Because the all-helical conformation is not a likely candidate for the active form of the corresponding residues in CCK-7 and because both CCK-4 and little gastrin bind to the gastrin receptor, we suggest that (AAEA) is a likely conformation for biological activity at the gastrin receptor. This common conformation is shown in Fig. 4 for CCK-4. In this structure, it should be noted that the tryptophan and phenylalanine side chains are in close proximity to one another and on the same "face" of the structure. The aspartic acid and methionine side chains point in the opposite direction. The proximity of tryptophan and phenylalanine side chains is not seen in the CCK-7 or CER-7 structures (Fig. 1). If the proximity of aromatic rings is the principal critical feature that is recognized by the gastrin receptor, it is possible that nonpeptide analogues could be constructed that would exhibit antagonist activity toward gastrin receptors.

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