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# Elucidation of the active conformation of the amino terminus of receptor-bound secretin using intramolecular disulfide bond constraints

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ABSTRACT

Family B G protein-coupled receptors include several potentially important drug targets, yet our understanding of the molecular basis of ligand binding to and activation of these receptors is incomplete. While NMR and crystal structures exist for peptide ligand-associated amino-terminal domains of several family members, these only provide insights into the conformation of the carboxyl-terminal region of the peptides. The amino-terminal region of these peptides, critical for biological activity, is believed to interact with the helical bundle domain, and is, therefore, unconstrained in these structures. The aim of the current study was to provide insights into the conformation of the amino terminus of secretin as bound to its receptor. We prepared a series of conformationally constrained secretin peptides containing intramolecular disulfide bonds that were predicted by molecular modeling to approximate the conformation of the analogous region of PACAP bound to its receptor that had been determined using transfer-NOE NMR techniques. Secretin peptides with pairs of cysteine residues in positions 2–7, 3–5, 3–6, 4–7, 7–9, and 4–10 were studied as linear and disulfide-bonded forms. The analog with a disulfide bond connecting positions 7–9 had binding affinity and biological activity similar to natural secretin, supporting the relevance of this constraint to its active conformation. While this feature is shared between secretin and PACAP, absence of activity in other constrained peptides in this series also suggest that there are differences between these receptor-bound conformations. It will be critical to extend similar studies to other family members to learn what structural elements might be most conserved in this family.



By first principles, linear peptides tend to have many degrees of conformational freedom. Their inherent flexibility provides a substantial challenge when attempting to elucidate a meaningful conformation of a peptide ligand docked at its receptor. Crystal structures and NMR structures that contain both peptide ligands and receptor molecules can provide useful insights, particularly if there is evidence that the docked conformation reflects normal binding affinity and/or is capable of stimulating biological activity. Unfortunately, many such structures may be more reflective of low affinity, non-specific inter-molecular interactions, such as being driven by hydrophobic interactions between the molecules, which may have limited relevance to natural docking.

Several high-resolution NMR and crystal structures exist for natural peptide ligands or their analogs associated with refolded amino-terminal domains of family B guanine nucleotide-binding protein (G protein)-coupled receptors.[1–6] While these structures provide relatively consistent insights into the docking of the carboxyl-terminal regions of the peptide ligands that typically assume a helical conformation and reside within a binding cleft within the receptor amino terminus,[1–6] unfortunately, none of these structures include the amino-terminal regions of their ligands. This reflects the observation that the amino terminus of the ligands normally interacts with the helical bundle of these receptors,[7–10] and that there is nothing to constrain the structures of that portion of the ligands in these complexes. Indeed, it is the amino-terminal region of these ligands that has been demonstrated to be most responsible for their biological activity.[11,12]

There is only a single structure reported that directly provides insights into the amino-terminal region of a peptide ligand in this family docked at its receptor.[13] This study utilized transfer-NOE NMR techniques that provide information only for the ligand and not for the underlying receptor. This structure (PDB entry 1GEA) represents pituitary adenylate cyclase-activating polypeptide truncated at its carboxyl terminus, PACAP(1–21), bound to the PACAP receptor. The peptide backbone in this structure assumes an extended conformation from residues 1–3, two overlapping β turns, comprised of residues 3–6 and 4–7, and a carboxyl-terminal α-helix from residues 8–21.

Based on the hypothesis that the secretin and PACAP peptides adopt similar receptor-bound conformations, we have used the structure of the amino terminus of PACAP as a template for modeling possible receptor-bound conformations of this region of the secretin peptide in the current project. We have constrained the structure of

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secretin analogs using intramolecular disulfide bonds and have characterized the abilities of these analogs to bind to the intact receptor and to elicit cAMP responses in receptor-bearing cells.

Molecular modeling was used to predict where a disulfide bond could be introduced into the amino-terminal portion of the secretin peptide to achieve a backbone structure similar to that found in the receptor-bound PACAP structure. All modeling was performed using the Internal Coordinate Mechanics (ICM) molecular mechanics program (Molsoft LLC, La Jolla, CA). First, a series of all-atom models was generated for the secretin peptide, incorporating two cysteines and a disulfide bond between them. All possible pairs of cysteine residues in the range of positions 2–11, which were separated by at least two residues in the linear sequence, were examined. Next, harmonic restraints with weight 10 kcal/(mol Å$^2$) were defined between corresponding backbone atoms in the secretin peptide and in the PACAP structure. The secretin peptide has high sequence similarity (43% identity) to PACAP(1–21), providing a confident gapless alignment to infer corresponding residues. The total energy, which is the sum of the physical energy calculated using the ECEPP/3 force field and the harmonic restraint potential, was minimized using the optimal-bias Monte Carlo optimization procedure[14] in ICM. The Metropolis sampling temperature was set at 700 K and each simulation was run for a total of $10^5$ energy evaluations. Simulations were run using each of the 25 deposited structures in the 1GEA PDB entry as backbone templates. The consistency of each of the predicted structures for disulfide-linked secretin was evaluated by its physical energy, disulfide bond geometry, and deviation from the bound PACAP backbone structure. A typical disulfide bond, as inferred from high-resolution X-ray structures, has a $C_\beta$–S–S–$C_\beta$ dihedral angle near 90° and an S–S bond length of approximately 2.0 Å.

The top five disulfide-linked secretin peptides ranked according to these criteria were chosen for experimental testing. A secretin analog incorporating cysteines and a disulfide bond between residues 4 and 10 was also prepared and tested, based on the position of the disulfide bond naturally occurring in calcitonin in the sequence alignment of Neumann et al.,[15] in which a conserved helix-capping structural motif was proposed as playing an important role in natural ligands for family B G protein-coupled receptors. Each of the peptides in the series, except for Cys$^{4,10}$-sec also incorporated a tyrosine in position 10 to replace the natural leucine located in that position for possible radioiodination. This residue replacement has previously been shown to be well tolerated for normal secretin binding and biological activity.[16,17]

The sequences of these peptides ([Cys$^{2,7}$,Tyr$^{10}$]rat secretin-27 (Cys$^{2,7}$-sec), [Cys$^{3,5}$,Tyr$^{10}$]rat secretin-27 (Cys$^{3,5}$-sec), [Cys$^{3,6}$,Tyr$^{10}$]-rat secretin-27 (Cys$^{3,6}$-sec), [Cys$^{4,7}$,Tyr$^{10}$]rat secretin-27 (Cys$^{4,7}$-sec), [Cys$^{7,9}$,Tyr$^{10}$]rat secretin-27 (Cys$^{7,9}$-sec), and [Cys$^{4,10}$]rat secretin-27 (Cys$^{4,10}$-sec)) are shown in Figure 1,[18] with their structural characteristics as predicted by molecular modeling shown in Table 1. Each of these peptides was able to achieve a conformation with backbone root mean square deviation (RMSD) relative to the reference conformation of receptor-bound PACAP[13] that was very small. Each peptide had acceptable length disulfide bonds with acceptable dihedral angles, although the energies varied considerably.

Figure 2 illustrates the receptor binding[19] and biological activity[20] characteristics of each of the peptides. It shows that neither the linear nor disulfide-bonded forms of Cys$^{3,6}$-sec and Cys$^{4,10}$-sec was able to bind to the secretin receptor or to stimulate demonstrable cAMP responses in secretin receptor-expressing CHO-SecR cells. This is also the case for the disulfide-bonded Cys$^{2,7}$-sec analog, while

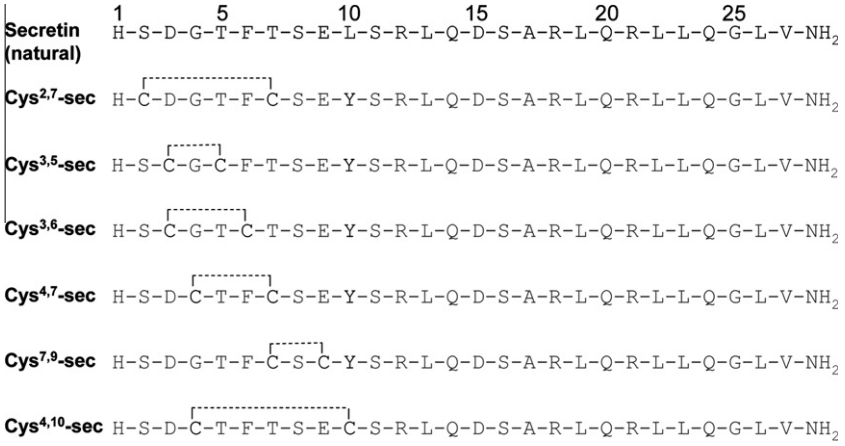


**Figure 1.** Primary structures of secretin analogs used in this study. Shown are the amino acid sequences of natural rat secretin and its dual cysteine-containing analogs. For the analogue sequences, natural residues are illustrated in gray, while modified residues are illustrated in black. Disulfide bonds linking the side chains of the cysteine residues are illustrated with dotted lines.

**Table 1**
Molecular modeling simulation results for the disulfide-bonded secretin analogs studied

| Positions of cysteine residues within secretin analogs | Disulfide bond length (Å) | Disulfide bond dihedral angle (°) | Energy (kcal/mol) | Backbone RMSD from receptor-bound PACAP structure (Å) |
|---|---|---|---|---|
| 2–7 | 2.02 | 102 | −31.6 | 0.20 |
| 3–5 | 1.90 | 96 | −23.7 | 0.24 |
| 3–6 | 1.92 | 102 | −3.63 | 0.20 |
| 4–7 | 2.06 | 98 | −15.3 | 0.32 |
| 7–9 | 1.66 | 85 | −6.97 | 0.28 |
| 4–10 | 1.91 | 55 | −38.8 | 0.20 |

Shown are disulfide bond characteristics of disulfide-bonded secretin analogs, along with their backbone deviations from the structure of receptor-bound PACAP.[13]