Crystal Structure of Hormone-bound Atrial Natriuretic Peptide Receptor Extracellular Domain

ROTATION MECHANISM FOR TRANSMEMBRANE SIGNAL TRANSDUCTION

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A cardiac hormone, atrial natriuretic peptide (ANP), plays a major role in blood pressure and volume regulation. ANP activities are mediated by a single span transmembrane receptor carrying intrinsic guanylate cyclase activity. ANP binding to its extracellular domain stimulates guanylate cyclase activity by an as yet unknown mechanism. Here we report the crystal structure of dimerized extracellular hormone-binding domain in complex with ANP. The structural comparison with the unliganded receptor reveals that hormone binding causes the two receptor monomers to undergo an intermolecular twist with little intramolecular conformational change. This motion produces a Ferris wheel-like translocation of two juxtamembrane domains in the dimer with essentially no change in the interdomain distance. This movement alters the relative orientation of the two domains by a shift equivalent to counterclockwise rotation of each by 24°. These results suggest that transmembrane signaling by the ANP receptor is initiated via a hormone-induced rotation mechanism.

Atrial natriuretic peptide (ANP)* is a hormone produced in the cardiac atrium and secreted into the circulation in response to atrial distension. ANP stimulates salt excretion (1) and dilates arterial vessels (2, 3). Through these activities, ANP plays a major role in the regulation of blood pressure and salt-fluid volume homeostasis. Transgenic animals devoid of the ANP gene develop salt-sensitive hypertension (4), and those lacking the ANP receptor gene develop salt-insensitive essential hypertension accompanied by severe cardiac hypertrophy, fibrosis, and dilatation (5), implicating the ANP and ANP receptor systems in cardiovascular pathophysiology. An analogous hormone, B-type natriuretic peptide (BNP), is also produced and secreted mainly by the heart and has hormonal activities similar to ANP (6). The activities of ANP and BNP are mediated by the ANP receptor or the A-type natriuretic peptide receptor carrying intrinsic guanylate cyclase (GCase) catalytic activity. Binding of the hormone to the receptor stimulates GCase catalytic activity, thereby elevating intracellular cGMP levels. cGMP, in turn, mediates the hormonal actions through cGMP-regulated ion channels, protein kinases, and phosphodiesterases. The ANP receptor occurs as a dimer of a single span transmembrane polypeptide, each containing an extracellular hormone-binding domain and an intracellular domain consisting of a protein kinase-like, ATP-dependent regulatory domain and a GCase catalytic domain (7). The molecular mechanism by which ANP binding to the extracellular domain stimulates the catalytic activity of the intracellular GCase domain is not understood.

A closely related receptor, the B-type natriuretic peptide receptor, mediates actions of C-type natriuretic peptide (CNP), which occurs mostly in the brain. CNP and the B-type receptor are thought to play a role in the central nervous system-mediated control of blood pressure and salt-fluid balance (6, 8, 9). The B-type receptor has ~60% sequence identity with the A-type receptor and has a similar overall molecular topology. It is likely then that the B-type receptor has a signaling mechanism similar to that of the A-type receptor. Yet another related receptor, the natriuretic peptide clearance-receptor, lacks the GCase domain and is not GCase-coupled (10). The clearance-receptor binds ANP, BNP, and CNP as well as some of their biologically inactive fragments with equally high affinity and removes the excesses of these peptides from the circulation (11, 12). The clearance-receptor has not been linked to any of the known hormonal actions of natriuretic peptides.

The GCase-coupled A-type and B-type natriuretic peptide receptors belong to the family of membrane-bound receptor GCases that include guanylin and enterotoxin receptors (13), retinal GCases (14), and olfactory cell GCases (15). These receptor GCases, in turn, belong to the superfamily of single span transmembrane receptors for which the mechanism of transmembrane signaling has not been well defined.

To elucidate the signaling mechanism of the ANP receptor, we have expressed and purified the extracellular hormone-binding domain of the receptor (ANPR) in a soluble form (16) and have characterized its biochemical properties, including the disulfide bond structure (17), glycosyl structure (18), and requirement for chloride ion for its binding with ANP (19). We have also crystallized the ANPR without the hormone (apoANPR) and determined its x-ray structure (20). The apoANPR was originally described as occurring in a tail-to-tail dimer form associated through its membrane-proximal domain (20). However, it was later recognized that the crystal packing...
of apoANPR also contained an alternative dimer pair, a head-to-head dimer, associated through the membrane-distal domain. Both the tail-to-tail and head-to-head dimer interfaces involve a large buried surface area (1,680 and 1,100 Å², respectively) and multiple residue contacts. Thus, from the crystallographic data alone, it has not been possible to distinguish which dimer form represents the physiological ANPR receptor dimer structure. We have recently reported site-directed mutagenesis studies of the residue involved in the two possible dimer interfaces in the full-length ANPR receptor expressed on COS cells. We have found that certain mutations at the head-to-tail dimer interface cause the receptor to become either uncoupled or constitutively GCase active, whereas mutations at the tail-to-tail dimer interface cause no such effect (21). These results strongly suggest that the extracellular domain of the native ANPR receptor on the cell surface assumes the head-to-head dimer structure and that the tail-to-tail apoANPR dimer previously described represents an artificial crystallographic dimer pair occurring only in the crystal packing. The head-to-head dimer structure for the apoANPR is similar to that proposed for the extracellular domain of the natriuretic peptide clearance-receptor (22). In the present study, we have determined the crystal structure of the ANPR complexed with the hormone ANP. The comparison of the complex structure, also occurring in the head-to-head configuration, with the unbound structure reveals a structural change caused by ANP binding and suggests a structural basis for transmembrane signaling by the ANPR receptor.

EXPERIMENTAL PROCEDURES

Crystallization of ANPR-ANP Complex and Data Collection—Expression and purification of the ANPR (16) and crystallization of the ANPR-ANP complex were carried out as described elsewhere (23). Briefly, the ANPR was expressed in Chinese hamster ovary cells and purified by ANP affinity chromatography. The ANPR was obtained N-glycosylated (18). The ANPR was treated with sialidase to reduce heterogeneity in the glycosyl structure and was again purified by ANP affinity chromatography. The complex of the ANPR (10 mg/ml) and an ANP peptide consisting of residues 7–27 (sequence: Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Aas-Ser-Phe-Arg) was crystallized by hanging drop vapor diffusion at room temperature with 1.6–2.0 M ammonium sulfate in 0.1 M MES buffer, pH 6.5, containing 10 mM NaCl. These conditions differ from those used for crystallizing the apoANPR. Crystals were dialyzed against high concentrations of ammonium sulfate solution and were frozen in liquid propane. The crystals had the space group of P6₁, with unit cell parameters a = b = 100.1 Å, and c = 259.8 Å. Two ANPR molecules are present in an asymmetric unit with a V₉ (Matthews coefficient) of 3.9 Å³/dalton. Data were collected at 100 K at Advanced Photon Source beamline 19-ID and National Synchrotron Light Source beamline X4-A and X25. Data were processed and scaled using the HKL package (24).

RESULTS AND DISCUSSION

Overall Structure of the ANPR-ANP Complex—The extracellular hormone-binding domain of the ANPR consisting of residues 1–435 was expressed and purified as described (16). ANPR was crystallized with an ANP peptide with the sequence Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Aas-Ser-Phe-Arg (Cys-7 and Cys-23 are disulfide-bonded) representing ANP residues 7–435 of monomer A and residues 253–256 and 426–435 of monomer B had insufficient electron density to be assigned. The stereochemistry of the final structure was analyzed with PROCHECK (27). Assignment of the secondary structures was done using program DSSP (28).

Crystal Structure of the ANPR with and without bound ANP hormone. a, the structure of the ANPR dimer complexed with ANP (7–27). The ANPR structure is shown in ribbon model; the ANP peptide is shown in green in space-filling model. The ball and stick model shows the carbohydrate structures. b, the structure of the apoANPR dimer. The apoANPR dimer structure was originally proposed in a tail-to-tail dimer configuration (20). However, it has been shown recently by site-directed mutagenesis and chemical modification studies that the ANPR receptor dimer exists in the head-to-head configuration (21). The 2-fold axis relating two ANPR monomers in both ANP-bound and unbound structures runs through the center of the dimer, parallel to the face of the page. Chloride ions are drawn as magenta balls. The figures were drawn with MOLSCRIPT (40) and rendered with RASTER3D (41).
by several α-helices. The membrane-distal domain contains a bound chloride ion (20) necessary for ANP binding (19). The two ANPR monomers, also related by non-crystallographic 2-fold symmetry, form a head-to-head dimer through their membrane-distal domains (Fig. 1a).

**ANP-induced Structure Changes**—To examine the structural basis for ANP receptor signaling, the structure of the ANP-bound ANPR dimer complex (Fig. 1a) was compared with that of the apoANPR dimer (Fig. 1b). The ANP-induced structural change involves a shift in the relative positions of the two ANPR monomers as shown in Fig. 2 (also shown in Supplemental Animation 2). There is no appreciable intramolecular conformational change in each individual ANPR monomer (root mean square deviation of Cα, 0.64 Å) (Fig. 2a). Upon ANP binding, the two ANPR molecules undergo a twist motion centered on a fulcrum point O (Fig. 2). In the front view, ANP binding leads to a seesaw-like motion, centered on point O, that causes the dimer interface to partially open and the membrane-proximal domains to close onto the bound ANP (Fig. 2a). Seen from the side, this motion is accompanied by a counter-clockwise rotation of each monomer, again centered on point O (Fig. 2b). In the juxtamembrane region, this twist motion of the two monomers results in translation of the two membrane-proximal domains by ~10 Å in opposite directions (arrows) (Fig. 2c, bottom view). The distance between the two domains is essentially unchanged. Near the membrane, amino acid residues Pro-417, Cys-423 (and its disulfide-bond counterpart Cys-432), and Phe-425 constitute the juxtamembrane signaling motif (31), a structure that is conserved among receptor-GCases and plays a critical role in transmembrane signaling. The movement of these residues shows that the two juxtamembrane domains essentially unchanged. Near the membrane, amino acid residues Pro-417, Cys-423, and Phe-425 (shown in space-filling model) anchor the juxtamembrane signaling motif invariably found in GCase-coupled receptors (31). Parallel shifts of these residues exemplify the translation of the juxtamembrane domains that alters the relative orientation of the two domains.

**Comparison with the Natriuretic Peptide Clearance-receptor**—The above hormone-induced structural change in the ANP receptor differs markedly from that found in the natriuretic peptide clearance-receptor upon CNP binding (22). In the clearance-receptor, the structural change occurs within each receptor monomer. CNP binding causes each monomer structure to “open” at the flexible intramolecular hinge connecting the membrane-distal and membrane-proximal domains (Fig. 3b). This bending causes the membrane-proximal domain to swing onto the bound ligand, leaving the membrane-distal domain and its dimerized structure essentially unchanged (Supplemental Fig. 3). This motion approximates the two membrane-proximal domains but does not change their relative orientation. Unlike the ANP receptor, the clearance-receptor lacks the GCase domain (10) and is not known to mediate any of the known hormonal activities of natriuretic peptides. The clearance-receptor binds ANP, BNP, and CNP as well as some of their biologically inactive fragments with equally high affinity in order to remove the excess of these peptides from the circulation (11, 12). The difference in the ligand-induced motion in the ANP receptor from that in the clearance-receptor may reflect the fact that the ANP receptor mediates hormonal signaling, whereas the clearance-receptor affects metabolic clearance. Additionally, the conformational flexibility at the intramolecular hinge in the clearance-receptor (22) may be responsible for its broad ligand specificity.

**Dimer Interface Structure**—At the dimer interface in the apoANPR structure (Fig. 4, shown in cyan), intermolecular contacts are made through hydrophobic interaction between Trp-74 of one monomer (monomer A) (residue Trp-74A) and Trp-74B of the other (monomer B), two hydrogen bonds (blue

**Table I**

<table>
<thead>
<tr>
<th>Crystallographic data and refinement statistics</th>
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**Refinement statistics (\(I/\sigma(I) > 1\))**

| Resolution range (Å)                          | 87.71–2.95 |
| Number of reflections                         | 25,503      |
| \(R_{free}\)                                  | 23.9 (26.9) |
| \(R_{merge}\)                                 | 26.9 (30.1) |
| Average B-factors (Å²)                        | 52.4        |
| ANP receptor                                  | 44.0        |
| Oligosaccharides                              | 70.1        |
| Chlorides                                     | 53.1        |
| Root mean square deviation from ideality     |              |
| Bond length (Å)                               | 0.009       |
| Bond angles (°)                               | 1.5         |
| Bonded B-factors (Å²)                         | 1.6, 2.6    |
| Ramachandran plot (%)                        | 84.2, 15.4, 0.4, 0 |

- \(R_{merge} = \sum_{hkl} I_{hkl} - \langle I_{hkl} \rangle / \sum_{hkl} \langle I_{hkl} \rangle\), where \(I_{hkl}\) is the intensity of unique reflection \(hkl\) and \(\langle I_{hkl} \rangle\) is the average over symmetry-related observation of unique reflection \(hkl\).
- \(R_{free} = \sum_{hkl} \left| F_{hkl} - F_{obs} \right| / \sum_{hkl} F_{obs}\), where \(F_{obs}\) and \(F_{calc}\) are the observed and calculated structure factors, respectively.
- \(R_{free}\) is \(R\) with 7.5% of reflections sequestered before refinement.

**Fig. 2.** ANP-induced quaternary structure change in the dimerized ANPR. a, the structure of the ANP-bound complex (orange) superimposed onto the apo structure (cyan) is shown in front view. α-Helices are shown as cylinders and β-sheets as ribbons. ANP is shown as green sticks. Point O is the fulcrum of the movement of the two monomers and is on the axis of 2-fold symmetry. The point O was identified such that the structure of an ANPR monomer in the apoANPR dimer, rotated around point O as the center, superimposes onto that of a corresponding monomer in the ANP-bound structure. b, side view. c, bottom view seen from the membrane. Conserved residues Pro-417, Cys-423, and Phe-425 (shown in space-filling model) anchor the juxtamembrane signaling motif invariably found in GCase-coupled receptors (31). Parallel shifts of these residues exemplify the translation of the juxtamembrane domains that alters the relative orientation of the two domains.
in the natriuretic peptide clearance-receptor extracellular domain. (22). In the clearance-receptor, ligand binding causes each subunit to bend at the flexible intrasubunit hinge structure from “closed” to “open” position, allowing the membrane-proximal domain to swing toward the bound ligand.

dotted lines) between Asp-71A and His-99B and between His-99A and Asp-71B, and hydrophobic interaction between Phe-96A and Phe-96B. Upon ANP binding, the dimer interface partially opens (Fig. 4, shown in orange), breaking the hydrophobic contact Trp-74A-Trp-74B and hydrogen bonds Asp-71A-His-99B and His-99A-Asp-71B. The hydrophobic contact Phe-96A-Phe-96B, on the other hand, remains and apparently contributes to stabilizing the ANP-bound activated receptor complex. We have found that mutation of Asp 71 to Arg (D71R) or W74R in the full-length ANP receptor as expressed on COS cells produced a receptor mutant that is constitutively GCase active (21). These mutations incorporate opposing charges at the dimer interface. The electrostatic repulsion between the charges may force the interface of the apo receptor dimer to become partially open even in the absence of ANP and generate a structure that partially mimics the activated receptor complex. Such an effect may be responsible for the partial constitutive activation of GCase activity. On the other hand, F96D mutation produced an uncoupled receptor mutant that bound ANP but did not cause cGMP stimulation. F96D mutation incorporates two negatively charged residues facing each other near the center of the monomer movement point O. The repulsive force between the two charges may distort the structure of the bound complex and abolish signaling and GCase activation. Thus, the results of mutagenesis studies performed with the full-length ANP receptor expressed on the COS cell surface are consistent with the crystal structures of the apo and ANP-bound ANPR dimer and with the ANP-induced structural change identified from these structures. This finding, in turn, suggests that the hormone-induced structural change identified in this study likely reflects that occurring in the native full-length ANP receptor in the membrane.

ANP-binding Site and Binding Interactions—Binding of ANP is asymmetric, and the binding site in one ANPR molecule (site A) differs from that in the other (site B) (Fig. 5a). Site A interacts mainly with the N-terminal part of the ANP peptide, whereas site B interacts mainly with the C-terminal part (Supplemental Tables 1–3). Buried surface areas at the two sites are nearly equal (1,374 and 1,367 Å² for sites A and B, respectively), suggesting significant contributions of both sites to hormone binding. The surface structures of both sites A and B have a ring-shaped groove lined with polar residues (Fig. 5, b and c). ANP fits closely into the groove at both sites, consistent with the high ligand specificity of the ANP receptor. Upon binding, ANP residue Arg-14 (Arg-14(ANP)) forms hydrogen bonds with Asp-71A and His-99B, and Arg-95A-Asp-62B (orange dotted lines), help stabilize the complex. The figures were drawn with MOLSCRIPT.
this complex closely reflects the structure of the native ANP receptor bound with ANP.

ANP residues contributing to binding to the receptor are all conserved in BNP, including Arg-14, Phe-8, Asp-24 (Asn-24 in ANP), and Arg-24 (Supplemental Fig. 1e). The conservation of these critical residues suggests that the mechanism of BNP binding to this receptor as well as the resulting structural change in the receptor may be similar to those described here for ANP. The ANP receptor binds ANP and BNP with high affinity but shows weak affinity to CNP (16). The weak affinity to CNP, which lacks C-terminal residues (Supplemental Fig. 1e), may be because of its inability to form the C-terminal interaction. The orientation of the bound ANP is consistent with the results of affinity labeling reaction in which ANP peptide analogs containing an electrophilic iodoacetyl group at positions 18 and 29 (peptide derivatives, N_e-acetyl-N_18e-IAc-[Lys18]ANP (4–28)) and N_e-acetyl-N_29e-IAc-[Lys29]ANP (4–29), respectively) reacted with Met-173 and Asp-194, respectively.2

Rotation Mechanism for ANP Receptor Signaling—Fig. 6a illustrates schematically the movement of the two ANPR molecules induced upon ANP binding. Each ANPR molecule is shown by a solid cylinder because ANP binding causes little intramolecular conformational change. Upon ANP binding, the two molecules undergo a twist motion centered on point O and close onto the ligand ANP. In cross-section at the juxtamembrane region (Fig. 6a, arrow) seen from the top (Fig. 6b), this twist motion causes the juxtamembrane domains of the two molecules (depicted by circles) to translocate, by an angle of 24° with respect to point O, from the apo position (circle Capo) to the complexed position (C com).2

This parallel translation alters the relative orientation of the two juxtamembrane domains in the receptor dimer. This change in the orientation is equivalent to rotating each of the two domains by 24° counterclockwise (Fig. 6b, inset). Earlier, Koshland and co-workers (32) postulated models for transmembrane signaling mechanism that depended on the hypothetical motion in the transmembrane region. Their models included association, dissociation, piston, rotation, scissor, and seesaw models. The ANP-induced motion of the juxtamembrane domains in the ANP receptor corresponds closely to the rotation mechanism postulated by these investigators (shown schematically in Fig. 6b, inset).

Signaling by many single span transmembrane receptors is thought to occur by the association (or clustering) mechanism.

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2 K. Misono, manuscript in preparation.
Association of the receptor molecules to a dimer (or an oligomer) brings their intracellular domains close together. The proximity of the intracellular domain, in turn, triggers the actions of the effector-enzyme domains inside the cell. Certain single transmembrane receptors, such as the erythropoietin (EPO) receptor, occur as a preformed dimer in the absence of the hormone. In the crystal structure of the extracellular domain of the EPO receptor, two C termini in the juxtamembrane region are apart by 73 Å, because the two GCase domains are unable to form an active dimer configuration. The GCase domain of ANP receptor, when expressed in a soluble form lacks the transmembrane and intracellular domains and, for this reason, its apo and ANP-bound structures may not precisely reflect those of the native full-length receptor in the cell membrane. However, we showed earlier that the effects of mutations at the dimer interface in the full-length receptor on COS cells are consistent with the crystal structures of the apo and hormone-bound ANPR and with the ANP-induced structural change identified from those structures (21). Additionally, binding interactions found in the complex structure are in agreement with the structure-activity relationship for ANP (30). These findings together suggest strongly that the ANP-induced structural change identified in this study reflects that occurring in the native receptor in the cell membrane and support the proposed rotation mechanism initiating ANP receptor signal transduction. The structures of the intact receptor in the membrane with and without bound ANP should provide direct evidence for the mechanism in the future.

Soluble GCase, which has ~51% sequence identity with the GCase domain of the ANP receptor, is active only as a dimer even though each monomer carries a GCase catalytic site (36). The GCase domain of ANP receptor, when expressed in a soluble form by truncation of the extracellular, transmembrane, and kinase-like domains, spontaneously forms a dimer and is catalytically active (37). It is possible that in the intact ANP receptor dimer the GCase catalytic activity is suppressed because the two GCase domains are close to each other even in the absence of the hormone, and ANP binding causes no appreciable change in the interdomain distance (Figs. 2c and 6b). Instead, ANP binding causes a large 24° rotation of each of the two juxtamembrane domains, altering their relative orientations (Fig. 6b). In the intact ANP receptor, this hormone-induced rotation of the juxtamembrane domains, transduced through the transmembrane helix, may reorient the relative positions (or configuration) of the intracellular domains to cause GCase activation (Fig. 7).

The ANPR expressed in a soluble form lacks the transmembrane and intracellular domains and, for this reason, its apo and ANP-bound structures may not precisely reflect those of the native full-length receptor in the cell membrane. However, we showed earlier that the effects of mutations at the dimer interface in the full-length receptor on COS cells are consistent with the crystal structures of the apo and hormone-bound ANPR and with the ANP-induced structural change identified from those structures (21). Additionally, binding interactions found in the complex structure are in agreement with the structure-activity relationship for ANP (30). These findings together suggest strongly that the ANP-induced structural change identified in this study reflects that occurring in the native receptor in the cell membrane and support the proposed rotation mechanism initiating ANP receptor signal transduction. The structures of the intact receptor in the membrane with and without bound ANP should provide direct evidence for the mechanism in the future.

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brought to an optimal proximity and orientation, thereby giving rise to GCase catalytic activity (Fig. 7).

The rotation mechanism initiating transmembrane signaling by the ANP receptor uncovered in the present study represents, to our knowledge, the first such mechanism identified for any known class of single span transmembrane receptors. At the same time, this finding introduces a new paradigm whereby a transmembrane conformational change involving a rotation, rather than simple association or approximation of the receptor molecules, mediates transmembrane signal transmission.

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