Constitutive Activation and Uncoupling of the Atrial Natriuretic Peptide Receptor by Mutations at the Dimer Interface

ROLE OF THE DIMER STRUCTURE IN SIGNALING*§

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The crystal packing of the extracellular hormone binding domain of the atrial natriuretic peptide (ANP) receptor contains two possible dimer pairs, the head-to-head (hh) and tail-to-tail (tt) dimer pairs associated through the membrane-distal and membrane-proximal subdomains, respectively. The tt-dimer structure has been proposed previously (van den Akker, F., Zhang, X., Miyagi, M., Huo, X., Misono, K. S., and Yee, V. C. (2000) Nature 406, 101–104). However, no direct evidence is available to identify the physiological dimer form. Here we report site-directed mutagenesis studies of residues at the two alternative dimer interfaces in the full-length receptor expressed on COS cells. The Trp74 to Arg mutation (W74R) or D71R at the hh-dimer interface caused partial constitutive guanylate cyclase activation, whereas mutation F96D or H99D caused receptor uncoupling. In contrast, mutation Y196D or L225D at the tt-interface had no such effect. His59 modification at the hh-dimer interface by ethoxyformic anhydride abolished ANP binding. These results suggest that the hh-dimer represents the physiological structure. Recently, we determined the crystal structure of ANPR complexed with ANP and proposed a hormone-induced rotation mechanism mediating transmembrane signaling (H. Ogawa, Y. Qiu, C. M. Ogata, and K. S. Misono, submitted for publication). The observed effects of mutations are consistent with the ANP-induced structural change identified from the crystal structures with and without ANP and support the proposed rotation mechanism for ANP receptor signaling.

Atrial natriuretic peptide (ANP) is a hormone secreted from the heart in response to atrial distension. ANP plays a major role in the regulation of blood pressure and electrolytes-fluid volume homeostasis through its natriuretic (1) and vasorelaxant activities (2, 3). ANP also has an antitrophic activity, through which it regulates maintenance and remodeling of the cardiovascular system (4–7). Transgenic animals lacking the ANP gene develop salt-sensitive hypertension (8), and those lacking the ANP receptor gene develop salt-insensitive essential hypertension accompanied by severe cardiac hypertrophy (9), implicating the ANP and ANP receptor system in cardiovascular pathophysiology. The hormonal actions of ANP are mediated by a cell membrane receptor carrying intrinsic guanylyl cyclase (GCase) activity. The receptor occurs as a dimer of a single span transmembrane polypeptide containing an extracellular hormone binding domain and an intracellular domain consisting of an ATP-dependent regulatory domain and a GCase catalytic domain (10). ANP binding stimulates GCase activity and elevates intracellular cGMP levels. cGMP, in turn, mediates hormonal actions via cGMP-regulated ion channels, protein kinases, and phosphodiesterases (11). The ANP receptor belongs to the family of GCase-coupled receptors which includes retinal GCases (12), olfactory GCases (13), and guanylin and enterotoxin receptors (14). These receptors have a similar overall molecular topology and may share a common signal transduction mechanism. The signaling mechanism of these receptors, however, remains largely unknown.

Previously, we expressed the extracellular hormone binding domain of ANP receptor (ANPR) in a soluble form and purified by affinity chromatography (15). We have found that the purified ANPR undergoes concentration-dependent self-association to a dimer and that this dimer formation is strongly enhanced by ANP binding. These findings suggest that dimerization of the receptor may play a role in signal transduction. We have also determined the crystal structure of the ANPR without bound ligand (apoANPR) (16). The ANPR contains two domains, the membrane-distal domain and membrane-proximal domain. In the crystal packing, the ANPR molecules can be found in two possible dimer pairs, which are the head-to-head (hh-) dimer associated through the membrane-distal domain and the tail-to-tail (tt-) dimer associated through the membrane-proximal domain. In our earlier report, we proposed the tt-dimer structure for the apoANPR based on the fact that the buried surface area for the tt-dimer interface (1,680 Å²) was larger than that for the hh-dimer interface (1,100 Å²) and that the crystallographic asymmetric unit contained a tt-dimer pair. However, there has been no direct evidence to determine which dimer form represents the true physiological structure.
Recently, He et al. (17) reported the crystal structure of the extracellular domain of the natriuretic peptide clearance receptor with and without the ANP-induced structural change identified from the transmembrane signaling by the ANP receptor. The results of proposed a hormone-induced rotation mechanism mediating the apoANPR hh-dimer structure, we have identified the native ANP receptor dimer structure. Thus, it remains unclear which of the hh- and tt-dimer structures represent the native ANP receptor dimer structure.

In the crystal packing, the molecules make multiple contacts (crystal contacts) other than the true physiological contact. Some of these crystal contacts involve buried surface areas larger than that for the physiological dimer interface. For this reason, the physiological dimer structure cannot be identified from the crystal structure alone. Here, we report site-directed mutagenesis and chemical modification studies of residues involved in the hh- and tt-dimer interfaces which occur in the apoANPR crystal. By examining the effects of these mutations and modifications on the activities of the receptor, we have identified the hh-dimer to be the form that represents the native ANP receptor dimer structure. Remarkably, a single residue mutation in the hh-dimer interface caused constitutive activation of the GCase activity, whereas other mutations caused receptor uncoupling, suggesting a critical role of the dimer interface in ANP receptor signaling. Recently, we have determined the crystal structures of the ANPR bound with the hormone ANP. By comparing this ANP-bound structure with the apoANPR hh-dimer structure, we have identified an ANP-induced structural change in the receptor and have proposed a hormone-induced rotation mechanism mediating transmembrane signaling by the ANP receptor. The results of mutagenesis and chemical modification studies are consistent with the ANP-induced structural change identified from the crystal structures and, hence, support the proposed signaling mechanism for the ANP receptor.

**Experimental Procedures**

**Materials**—The ANP peptide with residues 1–28 (ANP1–28), ANP(4–28), and AN(1–28) were synthesized as described (22). Tosylphenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington (Lakewood, NJ). Ethoxyformic anhydride (EFA) was obtained from Sigma. All other chemicals used were of analytical grade or the highest quality commercially available.

**Site-directed Mutagenesis Studies with the Full-length ANP Receptor**—cDNA encoding the full-length A-type rat ANP receptor was cloned in pCDNA3 (Invitrogen) to obtain a construct designated pCDNA3-natriuretic peptide receptor A (15). Residue mutations were introduced using a QuickChange mutagenesis kit and pCDNA3-ANPR as the template. COS-1 cells were transfected with the plasmid by the DEAE-dextran method. After transfection, the cells were cultured for a period of 2 weeks, harvesting the medium every 2 days. The culture medium was pooled, added with protease inhibitors (0.1 µg/ml aprotinin, 0.1 µg/ml leupeptin, 50 µg/ml phenylmethylsulfonyl fluoride, and 1 mM EDTA), and centrifuged at 8,000 × g for 20 min to remove cell debris. The supernatant was collected, quickly frozen in a dry ice-ethanol bath, and stored at −80 °C until use.

**Expression of Wild Type and Mutant ANP Receptor Extracellular Domain**—cDNA encoding the ANPR, representing amino acid residues 1–28, was cloned in pCDNA3 to obtain a plasmid construct pcDNA3-ANPR (15). Mutations were introduced using the mutagenesis kit and pcDNA3-ANPR as the template. COS-1 cells were transfected with the plasmid by the DEAE-dextran method. After transfection, the cells were cultured for a period of 2 weeks, harvesting the medium every 2 days. The culture medium was pooled, added with protease inhibitors (0.1 µg/ml aprotinin, 0.1 µg/ml leupeptin, 50 µg/ml phenylmethylsulfonyl fluoride, and 1 mM EDTA), and centrifuged at 8,000 × g for 20 min to remove cell debris. The supernatant was collected, quickly frozen in a dry ice-ethanol bath, and stored at −80 °C until use.

**Detection of ANPR Dimerization by HPLC Size-exclusion Chromatography (SEC)**—The conditioned medium from the cells expressing the wild type and mutant ANPR was concentrated 20-fold using a PL-3000 concentrator (Millipore, Bedford, MA). A 0.5-ml aliquot of the concentrated medium was separated on a TSK-G3000SW column (0.75 × 30 cm, Toso-Haas, Montgomeryville, PA) at room temperature in 20 mM sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl at the flow rate of 0.5 ml/min. Fractions of 0.2 ml were collected, of which 100-µl aliquots were used for measuring the ANP binding activity.

**Detection of ANPR Dimerization by LC/MS Analysis**—ANPR (1 µM) was reacted with 0.1 mM EFA in 100 mM sodium phosphate buffer, pH 7.0, at 24 °C. At varying time intervals, 5-µl aliquots were taken and diluted immediately with 95 µl of 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 0.1% bovine serum albumin, and 0.05% bacitracin to stop the reaction. Aliquots were used for ANP binding assay.

**Identification of Histidine Residues Modified by EFA**—The ANPR (1 µM) in 250 µl of 100 mM sodium phosphate buffer, pH 7.0, was reacted with 0.1 mM EFA at 24 °C for 15 min. The reaction was stopped by incubating the reaction mixture onto an TSK-G3000SW exclusion column (Toso-Haas), which was eluted with 100 mM sodium phosphate buffer, pH 7.0. One-fifth of the ANPR fraction was heated at 60 °C for 30 s to denature the protein and treated with 10 units of peptide-N-glycosidase F (Oxford GlycoSystems, Oxford, UK) at 37 °C for 2 h. The fraction was then analyzed by mass spectrometry for the protein weight to determine the number of ethoxyformic acid moieties incorporated into the ANPR. The remaining fraction was digested with 1.0 µg of trypsin at 37 °C for 3 h and used for peptide mapping by LC/MS as follows.

The digest above was separated on a Vydac C18 column (2.1 mm × 250 mm, Hesperia, CA) using a linear gradient of acetonitrile from 2% to 50% in 0.1% trifluoroacetic acid over 80 min at a flow rate of 160 µl/min in a Hewlett-Packard model 1100 HPLC system (Kennent Square, PA). The column effluent was split at 1:9 ratio using a microsplitter valve (Upchurch Scientific, Oak Harbor, WA), and 1/10 of the effluent was passed into a triple quadrupole mass spectrometer.
spectrometer equipped with an electrospray ionization source. The remainder of the effluent was collected manually while monitoring the total ion current on the mass spectrometer. Aliquots of the collected fractions were used for sequencing by tandem mass spectrometry (MS/MS) and by Edman degradation. The ion current was measured in the mass range of m/z 350–2,400 at 35-volt cone voltage and 6-s sweep time in the positive ion mode. The electrospray voltage was 5,500 volts and the source temperature at 70 °C. The nebulizer gas flow and drying gas flow were 10 liters/h and 300 liters/h, respectively. The resolution was set to gain sufficient isotope peak separation for doubly charged ions. These and other mass spectrometric analyses in this study were carried out on a Micromass Quattro II triple quadrupole mass spectrometer (Manchester, UK) at the Cleveland Mass Spectrometry Facility, Department of Chemistry, Cleveland State University. Mass-Lynx 3.02 software (Beverly, MA) was used for data processing.

Amino Acid Sequencing by MS/MS—A peptide sample was introduced directly into the mass spectrometer through a fused silica capillary (100-μm inner diameter) at a flow rate of 2 μl/min. Electrospray and cone voltages were at 3,500 V and 40 V, respectively. The ion source temperature was at 70 °C, the nebulizer gas flow was 10 liters/h, and the drying gas flow at 250 liters/h. Precursor ion was selected by the first quadrupole (Q1), and collision-induced dissociation was effected with argon gas in the second (RF-only) quadrupole (Q2). Product ion spectrum was obtained by scanning in the third quadrupole (Q3) from m/z 50 to up to the molecular mass of the peptide in the positive ion mode.

Edman Sequence Analysis—Edman sequencing was carried out in an Applied Biosystems model 477 protein sequencer (Foster City, CA) at the Macromolecular Structure Analysis Laboratory, Department of Biochemistry, University of Kentucky, Lexington, KY.

Modeling of the Protein Structures—The hh-dimer structure of apoANPR was modeled from the coordinates that were calculated from the tt-dimer coordinates (16) (PDB entry 1DP4) by symmetry operation using the program O (25). The crystal structure of ANP-bound ANPR complex has been determined in our separate study. The buried surface areas were calculated with a 1.4 Å probe radius with CNS 1.1 (26).

**FIG. 1.** Alternative dimer pairs in the crystal packing of apoANPR. A, diagrams showing the hh- and tt-dimer pairs in the crystal packing of apoANPR. The hh-dimer pair is shown in red and the tt-dimer pair in blue. The asymmetric unit of the crystal contains a tt-dimer pair. B and C, the structures of the hh- and tt-ANPR dimer are shown by ribbon model. The hh-dimer structure was constructed by symmetry operation using program O (25) based on the coordinates of the apoANPR tt-dimer structure reported previously (16) (PDB code 1DP4). Amino acid residues contributing to intermolecular contacts are shown by ball-and-stick presentation. Bound chloride ions are shown by magenta balls. The results presented in this report show that the hh-dimer represents the native receptor dimer structure, and the tt-dimer represents an artificial crystallographic dimer pair.

**RESULTS**

Occurrence of Two Alternative Dimer Pairs in the ApoANPR Crystal Packing—In the crystal packing of apoANPR, two alternative dimer pairs can be identified (Fig. 1A). Those are the hh-dimer associated through the membrane-distal domain of the ANPR monomer (Fig. 1B) and the tt-dimer associated through the membrane-proximal domain (Fig. 1C). Previously, we reported the tt-dimer structure for the apoANPR (16). However, there has been no direct evidence to determine which dimer form represents the true physiological structure. Fig. 2, A and B, shows the detailed interface structure for the hh-dimer and tt-dimer, respectively. The intermolecular contacts at the hh-dimer interface include a hydrophobic contact between Trp74 of one monomer (monomer A) and Trp74 of the other monomer (monomer B) (Trp74(1)–Trp74(2)), two hydrogen bonds His99(1)–Asp71B and His99B–Asp71A, and an interaction between Phe36A and Phe36B aligned linearly head-to-head, likely sharing aromatic π-electrons (Fig. 2A). On the other hand, the contacts at the tt-dimer interface include hydrophobic contacts, Tyr196(1)–Tyr196B, Leu200A–Leu200B, and Leu225A–Leu225B; hydrogen bonds, Asp194A–Asn221B, Asp194B–Asn221A, Arg201A–Ser269B, Arg201B–Ser269A, Arg198A–Asn221B, and Arg198B–Asn221A; and ionic interactions, Lys199B–Asp264A and Lys199A–Asp264B (16). The buried surface areas for the hh- and tt-dimer interfaces are 1,100 Å² and 1,680 Å², respectively. Both the hh- and tt-dimer interfaces involve multiple intermolecular contacts and a considerable surface area, making it unclear which dimer represents the native dimer structure.
Role of the Dimer Interface in ANP Receptor Signaling

Partial suppression of ANP-stimulated cGMP production. The double mutation F96D/V70D caused receptor uncoupling similar to F96D mutation, suggesting an essential role of Phe96 in ANP receptor signaling. Uncoupling of the ANP receptor was also observed by H99D mutation (Table I).

Constitutive GCase Activation by Mutations in the hh-dimer Interface—The cells expressing W74R mutant had a basal cGMP level of 24.4 pmol/10 fmol binding site, which was 16-fold higher than that in the cells expressing the wild type (1.5 pmol/10 fmol binding site) (Table I). In the presence of 0.1 μM ANP, the cGMP level increased to 122.6 pmol/10 fmol binding site, a level comparable with that found with the cells expressing the wild type (133.5 pmol/10 fmol binding site). Similar results were obtained with the D71R mutant. The cells expressing D71R mutant had a basal cGMP level at 15.0 pmol/10 fmol binding site, which was 10-fold higher than that in the cells expressing the wild type receptor. ANP increased the cGMP level to 123.2 pmol/10 fmol binding site, a level comparable with that found with the cells expressing the wild type. These results show that either W74R or D71R mutation causes a significant level of constitutive GCase activation.

Mutations at the tt-dimer Interface Cause No Effect—As shown in Fig. 2B, tt-dimer contacts include hydrophobic interactions Tyr196A-Tyr196B and Leu225A-Leu225B. We produced single-residue mutations Y196D and L225D. Each of these mutations introduces two opposing negative charges facing each other in the dimer interface if the receptor assumes the tt-dimer structure. As shown in Table II, neither mutation caused appreciable changes in the basal or ANP-stimulated cGMP levels. These results suggest that the extracellular domain of the native full-length receptor does not form the tt-dimer configuration.

Effects of Mutations in the hh-dimer Interface on ANPR Dimerization—We have shown previously that ANP binding strongly enhances ANPR dimerization (15). This finding suggests a potential role of ANP-induced dimerization in receptor activation. It is possible then that the uncoupling or constitutive activation caused by the mutation in the hh-dimer interface above may have resulted from their effects on receptor dimerization. To test this possibility, we introduced mutations in the ANPR representing the extracellular domain and expressed the ANPR mutants in COS cells. The ANPR mutants expressed in the culture medium were then examined for their dimerization behavior by SEC. When the culture medium from the cells expressing the wild type ANPR was separated by SEC and assayed for ANP binding, the activity peak was eluted at the position corresponding to the monomer ANPR (Fig. 3A). Similar results were obtained with all ANPR mutants (not shown). When the wild type ANPR was first incubated with 125I-ANP at 0 °C for 1 h to allow binding and chromatographed, the 125I radioactivity was eluted in a single major peak at the position corresponding to the ANP-bound ANPR dimer (Fig. 3B). Similar results were obtained with ANPR mutants W74R and D71R (Fig. 3, C and D, respectively). These results suggest that neither W74R nor D71R mutation interferes with ANP-induced ANPR dimerization. On the other hand, with the ANPR mutant F96D, the radioactivity peak was eluted at the position between the complex and monomer positions (Fig. 3E), indicating that the F96D mutation shifted the equilibrium toward the monomer. This result suggests that the F96D mutation makes the ANP-bound dimer complex less stable, thereby abolishing GCase activation by ANP (Table I).

Localization of His99 at the hh-dimer Interface by Chemical Modification Studies—Reaction of the ANPR with EFA caused a complete loss of ANP binding activity, suggesting a critical
role of His residues in hormone binding (Fig. 4A). We determined His residues modified by EFA by LC/MS after tryptic digestion of EFA-modified and inactivated ANPR. Fig. 4B shows the total ion current chromatogram of the tryptic digest. The modification by EFA adds an ethoxyformyl moiety to histidine imidazole group, giving a mass increment of 72 Da. The ethoxyformylated and unmodified tryptic peptides were identified based on their molecular masses (Table III). His-containing peptides identified by LC/MS accounted for all of the 10 His residues in the ANPR.

The peptide assignments were confirmed by sequencing by MS/MS and Edman degradation. For example, peptide T7# was sequenced by MS/MS as shown Fig. 4C. The precursor ion representing T7# (m/z 445.0) was selected and fragmented by collision with argon gas to obtain a series of product ions shown (y-series ions). The ethoxyformylated His99 residue was identified by the mass increment of 210 Da between y3 and y2 ions within the peptide sequence determined (Fig. 4C, inset). Edman degradation of T7# fraction gave an amino acid sequence F-T-A-H-W-R, being consistent with regeneration of His residue during the degradation cycles.

The extent of modification at each histidine residue was estimated from the ion intensities of the unmodified and modified peptides (Table III). His99 was most preferentially modified, followed by His407, His148, and His76. No detectable modification was observed for His130, His141, His185, His195, and His240. At the time of LC/MS analysis, His99 was found to be ~67% modified. The ethoxyformyl linkage to histidine imidazole is spontaneously hydrolyzed to regenerate His residues (half-life of 2 h at pH 2 and 55 h at pH 7 (30)). We estimate the extent of His99 modification nearly complete at the end of 15-min reaction with EFA. Incubation of the cells with and without 1\(^{125}\)I-ANP at 0 °C for 1 h and chromatographed. The \(1^{25}\)I radioactivity in the fractions was counted. C-E, experiments similar to E were performed with the ANPR mutants W74R, D71R, and F96D, respectively. The arrow a indicates the void volume of the column. The arrows b and c show the elution positions of the purified wild type ANPR and ANP-bound ANPR dimer complex (15) used as the standards.

**Table I**

Site-directed mutagenesis studies of the residues at the head-to-head dimer contact

The wild type and mutated full-length receptors were expressed on COS-1 cells. The dissociation constant for ANP (\(K_d\)) was estimated by competition binding with ANP using \(1^{25}\)I-ANP as the tracer using intact cells in culture dishes at 4 °C. The amount of cGMP was measured after incubation of the cells with and without 1 \(\mu\)M ANP at 37 °C for 15 min. The values are normalized by the receptor density and expressed as pmol of cGMP produced/10 fmol equivalent of ANP binding sites found on the transfected COS cells. Each entry is the mean ± S.D. (n = 12).

| Receptor expressed | K_d for ANP (nM) | cGMP produced (pmol/10 fmol binding site) | Effects on signaling |
|--------------------|-----------------|----------------------------------------|---------------------|
|                    | \(K_d\)         | –ANP        | +ANP        |                      |
| Wild type          | 0.28            | 1.5 ± 0.3  | 133.5 ± 27.0 | Uncoupling          |
| F96D               | 0.21            | 1.3 ± 0.6  | 1.7 ± 0.8   | Suppression         |
| V70D               | 0.19            | 0.8 ± 0.3  | 51.9 ± 20.7 | Uncoupling          |
| F96D/V70D          | 0.34            | 2.3 ± 1.2  | 2.9 ± 1.0   | Uncoupling          |
| H99D               | 0.26            | 1.6 ± 0.3  | 3.0 ± 0.7   | Uncoupling          |
| W74R               | 0.52            | 24.4 ± 9.9 | 122.6 ± 39.3| Constitutive activation |
| D71R               | 0.22            | 15.0 ± 6.4 | 123.2 ± 25.5| Constitutive activation |

**Table II**

Site-directed mutagenesis studies of the residues at the tail-to-tail dimer contact

The experiments were performed in a manner similar to that described in Table I. Each entry is the mean ± S.D. (n = 6).

| Receptor expressed | K_d for ANP (nM) | cGMP produced (pmol/10 fmol binding site) | Effects on signaling |
|--------------------|-----------------|----------------------------------------|---------------------|
|                    | \(K_d\)         | –ANP        | +ANP        |                      |
| Wild type          | 0.29            | 1.4 ± 1.1  | 97.1 ± 19.1 | No significant effect |
| Y196D              | 0.36            | 1.5 ± 0.9  | 93.4 ± 9.1  | No significant effect |
| L225D              | 0.30            | 1.4 ± 1.2  | 100.9 ± 17.5| No significant effect |

FIG. 3. SEC of the wild type and mutant ANPR in the absence and presence of the hormone. A, the conditioned culture medium containing the wild type ANPR was separated by SEC, and the ANP binding activity in the fractions was measured using \(1^{25}\)I-ANP as the tracer. B, the culture medium containing the wild type ANPR was first incubated with \(1^{25}\)I-ANP at 0 °C for 1 h and chromatographed. The \(1^{25}\)I radioactivity in the fractions was counted. C-E, experiments similar to E were performed with the ANPR mutants W74R, D71R, and F96D, respectively. The arrow a indicates the void volume of the column. The arrows b and c show the elution positions of the purified wild type ANPR and ANP-bound ANPR dimer complex (15) used as the standards.
or tt-dimer interface. His\textsuperscript{185} and His\textsuperscript{195} occur in the ANP binding site region but were not modified (Table III). Therefore, it is likely that the ethoxyformylated His\textsuperscript{99} residue prevents formation of a stable ANP-bound ANPR dimer complex, thereby abolishing the ANP binding activity.

**DISCUSSION**

Lines of evidence presented in this study show that the hh-dimer structure represents the physiological receptor dimer structure. The evidence include: 1) H99D or F96D mutation in the hh-dimer interface causes receptor uncoupling, and W74R or D71R mutation causes a significant level of constitutive activation; 2) mutations in the tt-dimer interface had no such effect; 3) chemical modification of His\textsuperscript{99}, localized in the hh-interface, by EFA inhibits ANP binding. The hh-dimer structure is also consistent with the crystal structure of ANPR dimer in complex with ANP, which we have determined recently.\textsuperscript{2} In the ANP-bound complex structure, two ANPR molecules...
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The tryptic peptides and their elution time, calculated and observed molecular masses, and relative abundance are shown.

| Position of His residue | Peptide no. | Amino acid residues | Retention time | Molecular mass | Relative amount |
|------------------------|------------|---------------------|----------------|----------------|----------------|
|                        |            |                     | min            | Calculated     | Observed       | %              |
|                        |            |                     |                | Da             | Da             |                |
| 76                     | T5 + 6º    | 48–95               | 34.5           | 4789.4         | 4789.2         | 82.8           |
|                        | T5 + 6ººº  | 48–95               | 37.0           | 4861.5         | 4861.5         | 17.2           |
| 99                     | T7         | 96–101              | 23.8           | 816.9          | 816.9          | 32.8           |
|                        | T7ººº      | 96–101              | 29.4           | 889.0          | 889.0          | 67.2           |
| 110                    | T10        | 126–132             | 4.5            | 724.8          | 725.0          | 100.0          |
|                        | T10ººº     | 126–132             | ND             | 796.9          | ND             | 0.0            |
| 141                    | T11        | 133–142             | 28.9           | 1128.3         | 1128.6         | 100.0          |
|                        | T11ººº     | 133–142             | ND             | 1200.4         | ND             | 0.0            |
| 148                    | T13        | 144–157             | 32.8           | 1670.9         | 1671.1         | 72.7           |
|                        | T13ººº     | 144–157             | 38.5           | 1743.0         | 1743.3         | 27.3           |
| 185,195                | T16 + 17ºº | 177–198             | 29.0           | 2637.9         | 4448.4         | 100.0          |
|                        | T16 + 17ººº| 177–198             | ND             | 2782.0         | ND             | 0.0            |
| 240                    | T24        | 221–249             | 51.4           | 3226.8         | 3227.0         | 100.0          |
|                        | T24ººº     | 221–249             | ND             | 3298.8         | ND             | 0.0            |
| 322                    | T35º        | 315–351             | 37.2           | 3943.4         | 6010.4         | 85.0           |
|                        | T35ººº     | 315–351             | 37.2           | 4015.5         | 6080.8         | 15.0           |
| 407                    | T41º        | 390–408             | 28.9           | 2119.4         | 3336.3         | 51.2           |
|                        | T41ººº     | 390–408             | 31.2           | 2191.5         | 3490.8         | 48.8           |

* Peptides are numbered in order from the N terminus of the protein.

° The mass values (monoisotopic mass) were calculated based on the amino acid sequence of the ANPR deduced from its cDNA sequence and specificity of trypsin cleavage.

* T5 + T6 is connected by a disulfide linkage.

°, The peptides containing an ethoxyformyl group(s).

° ND, not determined.

° The peptide is a product of incomplete tryptic digestion.

° The peptide is a glycopeptide. The calculated molecular mass of the peptide is the one of nonglycosylated form.

**Fig. 5.** The positions of His residues shown in the apoANPR hh-dimer. His residues are shown in yellow. The two ANPR monomers (green and blue) are in 2-fold symmetry by the axis that runs parallel to the face of the page. His99 occurs at the hh-dimer interface. The two ANPR monomers undergo a twist motion altering their relative orientation. This change in the orientation is equivalent to rotating each domain by 24° counterclockwise. Based on these structural analyses, we have proposed that a structural change involving a rotation mechanism mediates transmembrane signaling by the ANP receptor.2 Remarkably, the effects of mutations in the hh-dimer interface observed in this study are consistent with the structural change identified from the apo and complex crystal structures and, therefore, support the proposed rotation mechanism for ANP receptor signaling.

In the apoANPR-dimer, the dimer interface is stabilized by a hydrophobic contact Trp74A-Trp74B, two hydrogen bonds His99A-Asp71B and His99B-Asp71A, and a hydrophobic contact Phe96A-Phe96B (Fig. 6B). The uncoupling or constitutive activation of the receptor by the mutations at the dimer interface suggests a critical role of the dimer structure in ANP receptor signaling. The comparison of the ANP-bound complex structure with the apo-hh-dimer structure reveals a large quaternary structure change in response to ANP binding but without appreciable intramolecular conformational change.2 Upon ANP binding, the two ANPR monomers undergo a twist motion altering their relative positions (Fig. 6A and Supplemental Animation 1). This twist motion causes the membrane-proximal domains of the ANPR dimer to close onto the bound ANP and the dimer interface to open partially (Fig. 6B and Supplemental Animation 2). This motion, at the same time, causes the two juxtamembrane domains of the dimer complex to undergo parallel translocation, altering their relative orientation. This change in the orientation is equivalent to rotating each domain by 24°. Based on these structural analyses, we have proposed that a structural change involving a rotation mechanism mediates transmembrane signaling by the ANP receptor.2 Remarkably, the effects of mutations in the hh-dimer interface observed in this study are consistent with the structural change identified from the apo and complex crystal structures and, therefore, support the proposed rotation mechanism for ANP receptor signaling.
Role of the Dimer Interface in ANP Receptor Signaling

The hydrophobic contact Phe96A-Phe96B remains and apparently contributes to stabilizing the ANP-bound complex. We have found that F96D mutation in the full-length ANP receptor causes it to become uncoupled. The F96D mutation incorporates two negative charges facing each other at the interface near the center of the bound complex. The electrostatic repulsion between the two negative charges may distort the structure of the ANP-bound receptor complex and thereby abolish receptor signaling. We have also found that the F96D mutation causes the dimer interface to open partially, breaking the hydrophobic contact Trp74A-Trp74B and hydrogen bonds Asp71A-His99B and His99A-Asp71B. The hydrophobic contact Phe96A-Phe96B remains and likely contributes to stabilizing the ANP-bound complex.

Fig. 6. Structural change caused by ANP binding. A, the crystal structure of hh-apoANPR dimer (shown in blue) is overlaid with that of the ANP-bound complex (orange) in backbone presentation. B, close-up view of the dimer interface in the apoANPR dimer (blue) and in the ANP-bound complex (orange). α-Helices are shown by cylinders and β-sheets by ribbons. ANP is shown in green by space-filling model. In the apo-dimer, dimer contacts are made by hydrophobic contact Trp74A-Trp74B, hydrogen bonds Asp71A-His99B and His99A-Asp71B, and hydrophobic contact Phe96A-Phe96B. ANP binding causes the dimer interface to open partially, breaking the hydrophobic contact Trp74A-Trp74B and hydrogen bonds Asp71A-His99B and His99A-Asp71B. The hydrophobic contact Phe96A-Phe96B remains and likely contributes to stabilizing the ANP-bound complex.

The hydrophobic contact Phe96A-Phe96B remains and apparently contributes to stabilizing the ANP-bound complex. We have found that F96D mutation in the full-length ANP receptor causes it to become uncoupled. The F96D mutation incorporates two negative charges facing each other at the interface near the center of the bound complex. The electrostatic repulsion between the two negative charges may distort the structure of the ANP-bound receptor complex and thereby abolish receptor signaling. We have also found that the F96D mutation causes the ANPR monomer-dimer equilibrium strongly to the monomer even in the presence of ANP, supporting the notion that F96D mutation renders the ANP-bound complex structure unstable.

The reaction of EFA with ANPR resulted in rapid modification of His99 with a concomitant loss of ANP binding activity. His99 is localized in the hh-dimer interface (Fig. 5) near the center of the ternary complex (Fig. 6B). All other His residues occur outside of the hh- or tt-dimer interfaces. Two of such His residues occur in the ANP binding site region but were not modified. These findings suggest that the hh-interface is the native dimer interface and that His99 modification in this interface may make the complex structure unstable, thereby abolishing the ANP binding and signaling. This interpretation was corroborated by the mutagenesis experiment, in which H99D mutation caused the ANPR receptor to become uncoupled (Table I).

On the other hand, mutations W74R and D71R caused constitutive GCase activation. These mutations incorporate opposing charges at the distal section of the dimer interface (Fig. 6B). The electrostatic repulsion between the charges may force the interface of apo-receptor dimer to become partially open even in the absence of ANP and may generate a structure that partially mimics the structure of the activated receptor complex, thereby causing a partial activation of GCase activity. Additionally, we found that W74R and D71R mutations had no effect on ANP-induced ANPR dimerization. The partially open dimer interface structure in the ANP-bound complex is consistent with the lack of their effect.

Thus, the results of mutagenesis studies presented in this study are consistent with the structural change identified from the crystal structures of the apo- and ANP-bound ANPR complex. These findings, in turn, suggest that the ANP-induced structural change identified from the crystal structures reflects that occurring in the native, full-length receptor in the cell membrane and support the structural mechanism we propose for ANPR receptor signaling.

Recently, De Lean and associates (31) have reported that the ANPR receptor with W74C mutation forms a constitutively disulfide-linked dimer that is incapable of binding ANP. The ANP binding activity is rescued by cleaving the disulfide bond by reduction and alkylation. A disulfide bond between the substituting Cys residues (Cys74A and Cys74B) is likely to prevent the movement of the extracellular domains, leaving the binding site pocket for ANP in an “open” state and unable to capture an ANP molecule. These investigators have also found that insertion of a longer linker space between Cys74A and Cys74B using a bifunctional cross-linker phenylene-bismaleimide preserves the ANP binding activity. This finding is consistent with the partial opening of the dimer interface upon ANP binding as revealed by the crystal structure of the complex. Thus, the results of mutagenesis reported by these investigators are also consistent with the ANP-induced structural change identified from the crystal structures and with the proposed signaling mechanism for the ANP receptor.

In summary, the results of site-directed mutagenesis and chemical modification studies strongly suggest that the hh-dimer structure reflects the physiological ANP receptor dimer structure. The results of mutagenesis studies are consistent with the ANP-induced structural change identified by the crystal structures of the apo- and ANP-bound ANPR complex, and strongly support the proposed rotation mechanism for transmembrane signaling by the ANP receptor.

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Note Added in Proof—The EFA-modified ANPR was analyzed by LC/MS tryptic mapping directly without deglycosylation because of the known chemical instability of ethoxyformyl linkages. In Table III, tryptic peptides, T16 + 17, T35, and T41 had observed mass values larger than the calculated peptide masses because of glycosylation at Asn180, Asn347, and Asn395, respectively (Miyagi, M., Zhang, X., and Misono, K. S. (2000) Eur. J. Biochem. 267, 5758–5768). Peptide T16 + 17 (residues 177–198; amino acid sequence ERLNITVNHQEFVEGDP–5768). Peptide T16 + had an observed mass of 4448.4.
This value is close to the mass of the corresponding peptide from a tryptic digest of unmodified ANPR (Miyagi, M., Zhang, X., and Misono, K. S. (2000) *Eur. J. Biochem.* **267**, 5758–5768, page 5763, Table II, raw 7; observed mass 4447.8, calculated mass 4448.5 based on the saccharide composition HexNAc5Hex4DeoxyHex1 deduced from the mass value). This result suggests that His185 and His195 in T16 ride composition HexNAc5Hex4DeoxyHex1 deduced from the mass value. The absence of His185 and His195 modification is not immediately clear. The reactivity of His residues may be influenced by local environment such as the orientation and polar interactions with neighboring residues.

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