Natriuretic Peptide Receptor A Activation Stabilizes a Membrane-Distal Dimer Interface*

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The abbreviations used are: rANP, rat atrial natriuretic peptide; CNP, C-type natriuretic peptide; NPRA, natriuretic peptide receptor A; ΔKC, NPRA with all cytoplasmic domain truncated; KHD, kinase homology domain; NPRC, natriuretic peptide receptor C; GCAP, guanylyl cyclase activating protein; IBMX, 1-methyl-3-isobutylxanthine; cGMP, cyclic guanosine 3’,5’-monophosphate; DTT, dithiothreitol; NEM, n-ethylmaleimide; oPDM, N,N’-1,2-phenylenedimaleimide; mPDM, N,N’-1,3-phenylenedimaleimide; pPDM N,N’-1,4-phenylenedimaleimide; Cu(OP)₂, Cu-phenanthroline; EDTA, ethylenediaminetetraacetic acid; MTS, methane thiosulfonate.
SUMMARY

We have previously shown that atrial natriuretic peptide (ANP) stabilizes a dimeric form of the natriuretic peptide receptor A (NPRA) by simultaneously interacting with both receptor subunits. However, the first crystallographic study of unliganded NPRA extracellular domain documented a V-shaped dimer involving a membrane-proximal dimer interface and separate binding sites for ANP on each monomer. We explored the possibility of an alternative A-shaped dimer involving a membrane-distal dimer interface, by substituting an unpaired solvent-exposed cysteine for Trp$^{74}$ in the amino-terminal lobe of full length NPRA. The predicted spacing between Trp$^{74}$ from both subunits drastically differs depending on whether the V-shaped (84 Å) or the A-shaped (8 Å) dimer model is considered. In contrast with the expected results for the reported V-shaped dimer, the NPRA$^{W74C}$ mutant was constitutively covalently dimeric. Also, the subunits spontaneously reassociated following transient disulfide reduction by DTT and reoxidation. However, ANP could neither bind to nor activate NPRA$^{W74C}$. Permanent disulfide opening by reduction with DTT and alkylation with NEM rescued ANP binding to NPRA$^{W74C}$. The NPRA mutant could be maintained as a covalent dimer while preserving its function by crosslinking with the bifunctional alkylating agents phenylenedimaleimides (PDM), the ortho-substituted oPDM being more efficient than mPDM or pPDM. These results indicate that the membrane-distal lobe of NPRA extracellular domains are dynamically interfacing in the unliganded state and that ANP binding stabilizes the receptor dimer with more stringent spacing at the dimer interface.
INTRODUCTION

The interaction of the natriuretic peptide ANP with the binding domain of its receptor NPRA is determinant for proper signal transduction leading to cyclic GMP production and to cellular response in the cardiovascular system (1). Natriuretic peptides counterbalance the renin-angiotensin system by lowering blood pressure and by increasing natriuresis and diuresis. This role is exemplified in knockout mice with abrogated or reduced expression of ANP or its receptor NPRA and which are hypertensive (2, 3). Natriuretic peptide receptors are members of the membrane guanylyl cyclase family (4). These receptors are constituted of five domains. The glycosylated extracellular domain is required for binding the activating agents e.g. natriuretic peptides, guanylin and the sea urchin sperm activating peptides (4-6). It is linked through a single transmembrane domain to the intracellular portion which is composed of three domains. First a membrane-proximal domain which is homologous to protein kinase domains and presumably binds ATP but which is lacking catalytic function. This phosphorylated domain serves as a regulatory component for signal transduction (4, 7, 8). It is also a target for the intracellular activating protein GCAP which directly activates retinal and olfactory guanylyl cyclases (9). The kinase homology domain (KHD) is connected through a coiled-coil (10, 11) to the guanylyl cyclase domain effecting the activation process by producing cyclic GMP (11). Initial studies have shown that natriuretic peptide receptors could be documented as constitutively noncovalently dimeric through interaction of both the extracellular (13) and the intracellular domains (10). Photoaffinity derivatives of ANP, with photosensitive substitutions at both ends of the peptide, could specifically crosslink the receptor dimer indicating that the peptide must be interacting with both subunits (14).
Receptor dimerization is essential for the activation of the catalytic domain of retinal guanylyl cyclase since both GTP substrates must interact with each subunit (12). In that model system, the coiled-coil connecting the intracellular domains appears to maintain apart both guanylyl cyclase moieties in the basal state. Activation then appears to involve the release of the constraint imposed by the coiled-coil on the catalytic domain (12). The membrane-proximal kinase homology domain is also determinant in signal transduction. In the absence of ANP the KHD domain maintains the receptor in the basal state (7). The incoming activation stimulus appears to favor ATP binding and to relieve this tonic inhibition through a concerted transmembrane allosteric change, resulting in activation of the catalytic moiety (15). The initial activation step of NPRA is likely to involve a conformational change in the extracellular juxtamembrane region. Indeed it has been reported that ANP activation leads to increased protease sensitivity of the juxtamembrane region (16,17). Site-directed mutagenesis of C423S located in the juxtamembrane region leads both to constitutive activation and to receptor covalent dimerization through the exposed and unpaired Cys\textsuperscript{432} (18). This activation was attributed by Misono et al. to the conformational change occurring in the juxtamembrane region more than to the covalent dimerization process since a double mutation C423S-C432S was shown to be also constitutively active but not covalently dimeric (17). While this interpretation is viable, the occurrence of a constitutive disulfide bridge still indicates the proximity of the extracellular juxtamembrane region of the receptor subunits. The contribution of the extracellular juxtamembrane region in mediating ANP-induced activation is also well documented in a D435C mutant exposing a free cysteine three residues further towards the transmembrane region (19).
This mutant is not covalently dimeric in the basal state but ANP induces disulfide bridge formation through Cys\textsuperscript{435} upon receptor activation.

When the extracellular domain of NPRA is expressed in truncated soluble form, it behaves in solution as a monomer (20). Agonist binding to the soluble extracellular domain induces noncovalent dimerization (20). Misono et al. proposed that ANP is binding to the soluble receptor dimer according to a 2:2 stoichiometric ratio (20). Their results however clearly document that at midpoint of the dimerization process 1 µM of ANP can dimerize 2 µM receptor subunit, implying a 1:2 stoichiometric ratio. This 1:2 ratio is more in agreement with the ratio which we previously documented in full length receptor by comparing ANP binding capacity with immunoassayable receptor subunit density (14).

The NPRA receptor extracellular domain has been crystallized in the unbound form by van den Akker et al. showing that it is constituted of a homodimer (21). Each subunit displays a typical bi-lobed periplasmic protein folding and contains a chloride ion. In this initial report, the receptor structure was presented as a V-shaped dimer with a subunit interface located in the membrane-proximal lobe (Fig. 1). Prediction of the localization of the ANP binding region on this receptor was helped by our previous photoaffinity tagging results (22) and was confirmed by site-directed mutagenesis (21). However the predicted localization of the ANP binding site in the V-shaped dimer was more on the lateral face of each receptor subunit in a position not easily amenable to simultaneous contact of the peptide with both receptor subunits or to any conformational change in the receptor (21). A more recent report on the structure of the CNP-bound NPRC receptor, which is devoid of guanylyl cyclase and which mainly serves for peptide
clearance, indicated an A-shaped dimeric structure with the dimerization interface in the membrane-distal lobe. In that receptor dimer structure a single CNP molecule is binding within the intersubunit cleft, therefore interfacing both receptor subunits (23). The A-shaped dimer structure was also recognized by van den Akker (24) in the original crystal structure of NPRA (Fig. 1). This dimer conformation would conform to the 1:2 stoichiometric ratio of peptide to receptor subunit and would provide, if applicable to the natriuretic peptide A receptor, a more conceivable mechanism for ANP high affinity binding and receptor dimer activation.

In attempting to explore the various conformations of NPRA dimer we noticed that the Trp\textsuperscript{74} residues located in the membrane-distal lobe of the receptor were separated by drastically different alpha-carbon distances in the V-dimer and the A-dimer conformations (82 Å vs 8 Å, Fig. 1). The position of Trp\textsuperscript{74} in NPRA is analogous to that of a cysteine involved in disulfide bridging of eel NPRC dimer (25). We thus explored the ability of a W74C mutant of rat NPRA to form a covalent dimer through either a disulfide bridge or longer spacers provided with bifunctional dimaleimide derivatives. The results indicate that the W74C mutant is constitutively covalently dimeric, confirming the A-shaped dimer and excluding the V-shaped dimer conformation. But the disulfide bridged dimeric mutant is however inactive. However proper binding of ANP and receptor activation can be achieved by maintaining a slightly wider spacing between residues 74 using bifunctional crosslinkers. Reciprocally, ANP-binding hinders disulfide bridge formation in the W74C mutant. Thus, the ANP-bound and activated receptor dimer appears to adopt a more stable conformation than in the unbound state. These results contribute to the understanding of the conformational changes occurring early on during activation of NPRA.
EXPERIMENTAL PROCEDURES

Construction of NPRA mutants—Wild type (WT) rat NPRA clone inserted into pBK-CMV (Stratagene) between sites *NheI* and *KpnI* (26) was used for generating the various mutants. NPRA<sup>W74C</sup> (Fig. 2) was obtained by mutating Trp<sup>74</sup> into Cys according to Clontech kit (Clontech) using the mutagenic primer 5'-GACCTCAAGTGTGACACGCCA-3'. The mutation was checked by sequencing and the fragment encompassing the mutation was subcloned into NPRA<sup>WT</sup>. ΔKC<sup>C423S,C432S</sup> and ΔKC<sup>C423S,C432S,W74C</sup> mutants (Fig. 2) were obtained starting from the deletion mutant ΔKC<sup>WT</sup> lacking all cytoplasmic domain (19) by sequentially mutating Cys<sup>423</sup> and Cys<sup>432</sup> to Ser according to QuikChange kit (Stratagene) using first the oligonucleotide pair 5'-CCTGACGTCCCTAAATCTGGCTTTGACAATGAGG-3' and its complementary, and then the oligonucleotide 5'-GACAATGAGGACCCAGCCTCCAACCAAGACCACCTTTTC-3' and its complementary sequence. The mutation was checked by sequencing and the fragment between sites *EcoRI* and *KpnI* was subcloned into NPRA<sup>WT</sup> and NPRA<sup>W74C</sup> to generate mutants ΔKC<sup>C423S,C432S</sup> and ΔKC<sup>C423S,C432S,W74C</sup>, respectively.

Cell culture and receptor protein expression—HEK 293 cells (American Type Culture Collection) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units of penicillin/streptomycin in a 5% CO<sub>2</sub> incubator at 37°C. Transfection assays were carried out in 100 mm plates (1.2×10<sup>6</sup> cells) using the calcium phosphate co-precipitation technique as previously described (27).
Whole cell guanylyl cyclase stimulation—Cells were replated 48h post transfection in 24-well cluster plates at $10^5$ cells per well and incubated 24h prior to agonist stimulation. The cells were washed twice with serum-free DMEM and incubated at 37°C in quadruplicate wells with varying concentrations of rANP (Peninsula) in the same medium containing 0.5% BSA and 0.5mM IBMX. After a 1h incubation, medium was collected and extracellular cyclic GMP was measured in duplicate by radioimmunoassay as previously described (28).

Preparation of membranes—Membrane preparations of transfected HEK 293 were done according to (18). Essentially cells were harvested 72h post transfection and homogenized in ice-cold buffer (10 mM Tris-HCl pH 7.4, 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 10 μM pefabloc, 1mM EDTA). After centrifugation at 40,000 × g for 30min the pellets were washed twice and finally resuspended in freezing buffer containing 50 mM Tris-HCl pH 7.4, protease inhibitors, 1mM MgCl$_2$ and 250mM sucrose. Membranes were then frozen in liquid nitrogen and kept at –80°C until used. The protein concentration was determined using BCA protein assay kit (Pierce).

In vitro modification of cysteine residues—Membranes (0.6mg/ml) were treated with 5 mM of DTT (Sigma) in Tris-EDTA buffer (50 mM Tris-HCl pH 7.4, 0.2 mM EDTA) at 22°C for 50 min in darkness under argon atmosphere. Tubes containing membranes were then cooled on ice and centrifuged at 12,000 × g for 15 min at 4°C. Membranes were washed with Tris-EDTA buffer alone and pellets were resuspended in the same buffer at a concentration of 0.5 mg/ml. For control, membranes were incubated as described above, except that DTT was omitted. Following the reduction step, membranes were treated with PDM (30) in order to covalently bridge the
cysteine residues. Membrane proteins (100 ug) were incubated in the darkness for 60 min at 4°C with 0.1 mM of oPDM, mPDM or pPDM (Aldrich Chemical Co.) in a final volume of 0.2 ml of Tris-EDTA buffer. Control was obtained with the monovalent maleimide compound NEM (Fisher) at 0.1mM. At the end of incubation, 10 mM NEM was added to all tubes in order to block all free unreacted cysteine residues and to prevent spurious disulfide formation and incubation was continued for 10 min at 4°C. Treated membranes were centrifuged, washed once, resuspended in freezing buffer and kept at –80°C. For reoxidation of cysteine pairs, the DTT-reduced membrane proteins were treated with CuSO₄-orthophenanthroline (Cu(OP)₂) (Fisher) according to Majima et al. (29). Membranes at concentration of 0.5 mg/ml were incubated with or without 25 µM Cu(OP)₂ for 10 min at 4°C. The reaction was stopped by the addition of NEM and EDTA both at 10 mM. After further incubation for 10 min at 4°C, treated membranes were centrifuged and the pellets were washed once in Tris-EDTA buffer. The final pellets were resuspended in freezing buffer and kept at –80°C. When ANP was tested for its ability to interfere with the covalent receptor dimerization, 100 nM rANP was added at 4°C 20 min prior to the treatment with Cu(OP)₂.

Immunoblot analysis—Membrane protein samples (4-10 ug) were solubilized in Laemmli sample buffer (62 mM Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, pH 6.8) without (non-reducing) or with (reducing) 5% β-mercaptoethanol and heated at 100°C for 3 min. Electrophoresis was performed in a 7.5% polyacrylamide gel for the ΔKC mutants and in a 5% polyacrylamide gel for the full length NPRA mutant. Following electrophoresis proteins were transferred to a nitrocellulose membrane (Bio-Rad) using the liquid Mini Trans-Blot System (Bio-Rad). Detection of receptor was achieved using an affinity-purified antibody from a
rabbit polyclonal antiserum raised against the carboxy-terminus sequence of NPRA (18). Specific signal was probed with a horseradish peroxidase-coupled second antibody according to the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

**Receptor binding assays**—$^{125}$I-rANP was prepared using the lactoperoxidase method as described previously (18). The specific activity of the HPLC-purified radioligand was at least 2000 Ci/mmol. Membranes from HEK 293 expressing rat NPRA$^{\text{WT}}$, NPRA$^{W74C}$, ΔKC$^{C423S,C432S}$ or ΔKC$^{C423S,C432S,W74C}$ (0.2-1.0 ug) were incubated in duplicate with 10 fmoles of $^{125}$I-rANP for 20 h at 4°C in 1 ml of 50 mM Tris-HCl buffer pH 7.4 containing 5 mM MgCl$_2$, 0.1 mM EDTA and 0.5% BSA. Non-specific binding was defined by the addition of non-radioactive rANP at 100 nM. Bound ligand was separated from free ligand by filtration on GF/C filters pretreated with 1% polyethylenimine. Filters were washed 4 times and counted in LKB Gamma counter.

**Data analysis**—Dose-response curves were analysed with the program AllFit for Windows based on the four-parameter logistic equation (31).
RESULTS

Covalent dimeric state of the NPRA\textsuperscript{W74C} mutant—The two proposed arrangements of the NPRA extracellular domain as a V-shaped and an A-shaped dimer mainly differ in terms of the localization of the dimer interface in the membrane-proximal and the membrane-distal lobes of the receptor subunits (Fig. 1). The surface-exposed residues Trp\textsuperscript{74} are also widely spaced in the V-dimer (82 Å) while these residues are juxtaposed (8 Å) at the membrane-distal end of the dimer interface in the A-shaped dimer. Therefore substitution of Trp\textsuperscript{74} with Cys should yield a disulfide-bridged dimer only in the case of the A-shaped dimer conformation. When the full length NPRA\textsuperscript{W74C} mutant transiently expressed in HEK293 cells is studied in SDS-PAGE under non-reducing conditions, we observe that the mutant is present almost exclusively as a dimer (Fig. 3, right panel). This dimer can be reduced by treatment with DTT followed by alkylation of free sulfhydryls with NEM (Fig. 3, right panel). However if the receptor mutant is reoxidized in the presence of Cu-orthophenanthroline (Cu(OP)\textsubscript{2}) following reduction with DTT, then the receptor mutant reassociates as a disulfide-bridged dimer (Fig. 3, right panel). Thus the disulfide bridge between the two exposed Cys\textsuperscript{74} is constitutive and is formed spontaneously when reducing conditions are replaced by mild oxidizing conditions. Therefore, residues 74 from both receptor subunits should be spontaneously adjacent, in accordance with the A-shaped conformation and in drastic contrast with the prediction of the V-shaped dimer originally proposed (Fig. 1). In addition, the disulfide bridge, which is likely to occur early on during biosynthesis of the NPRA\textsuperscript{W74C} mutant, does not appear to result from an imposed constraint, since it can be formed again following reduction of the mature and unstimulated receptor protein.
The NPRA<sup>W74C</sup> mutant is inactive in a disulfide-bridged dimeric state—Since the inter alpha-carbon distance (8 Å) of the two Trp<sup>74</sup> residues is slightly longer than the expected distance for disulfides (5-7 Å), we wondered if bridging the two Cys<sup>74</sup> might interfere with the high affinity binding and activation processes of ANP on NPRA<sup>W74C</sup>. Indeed the receptor mutant is almost completely devoid of high affinity for ANP in membrane preparations from HEK293 cells transiently expressing NPRA<sup>W74C</sup> (Fig. 4). The receptor mutant is also almost completely insensitive to ANP-activation with marginal response (Fig. 5). This loss of function is not due to an irreversible alteration by the mutation of receptor folding since reduction and alkylation of the dimer leading to a monomer restores high affinity binding for ANP (Fig. 4). Moreover, reoxidation of the Cys<sup>74</sup> disulfide following reduction leads again to the loss of high affinity binding for ANP (Fig. 4). Thus while Cys<sup>74</sup> disulfide bridge formation is spontaneous for the basal state of the receptor, this tight dimer conformation prevents ANP high affinity binding and functional activation. These results suggest that unlike for the basal state of the receptor, the ANP-bound and active state requires more stringent proximity conditions which the Cys<sup>74</sup> disulfide prevents by slightly constraining the inter alpha-carbon distance of residues 74 to less than 8 Å.

Proper spacing of cross-linked Cys<sup>74</sup> in ΔKC<sup>C423S,C432S,W74C</sup> mutant is required for high affinity ANP binding—In order to verify that proper spacing of residues 74 from both receptor subunits is required for the active state, we looked for various types of bifunctional cross-linking agents specific for surface-exposed sulfydryls. Such agents had to be reacting with Cys<sup>74</sup> following disulfide bridge opening with DTT. Initial attempts to use dual methane thiosulfonate reagents (MTS reagents, Toronto Research Company) were fraught with
difficulties. We observed that preliminary reduction with DTT left other free and reactive cysteines besides Cys$^{74}$. Thus, although MTS compounds could properly cross-link free Cys$^{74}$, additional spurious disulfide formation occurred resulting in multiple receptor oligomers. Also, since MTS compounds form a reducible covalent link with cysteines, SDS-PAGE could not be performed under reducing conditions, precluding elimination of spurious disulfides. We thus resorted to dimaleimide cross-linking reagents which have been used for sizing inter-cysteine distances in proteins (30). oPDM, mPDM and pPDM can efficiently cross-link neighboring exposed cysteines and the resulting dimers can be studied in SDS-PAGE under reducing conditions with the advantage of removing spurious disulfides. In addition, the number of potential free and exposed cysteines was reduced by truncating the cytoplasmic domain of NPRA with many potentially exposed free cysteines and by mutating both Cys$^{423}$ and Cys$^{432}$ which are exposed in the juxtamembrane region, leaving only two buried and unreactive disulfides (Fig. 2). Thus for the $\Delta KC^{C423S,C432S,W74C}$ mutant the additional W74C mutation could provide the only exposed free cysteine, avoiding spurious cysteine reactions.

Following transient reduction with DTT, the $\Delta KC^{C423S,C432S,W74C}$ mutant was efficiently crosslinked as a non-reducible dimer by oPDM and mPDM and somewhat less by pPDM (Fig. 6, right panel). The untreated disulfide-bridged $\Delta KC^{C423S,C432S,W74C}$ mutant was devoid of high affinity ANP binding (Fig. 7). As for the full length NPRA$^{W74C}$ mutant, reduction and alkylation of $\Delta KC^{C423S,C432S,W74C}$ with DTT and NEM could restore ANP binding by cleaving the interchain disulfide (Fig. 7). However crosslinking of the receptor subunits with oPDM completely restored ANP binding (Fig. 7) while maintaining a covalent dimer with a wider spacing of the Cys$^{74}$ from both subunits than was obtained with the disulfide (Fig. 6). mPDM and pPDM were much less
effective, presumably because the inter Cys\textsuperscript{74} spacing imposed by those cross-linking agents was too wide, therefore interfering with optimal positioning of the two subunits at the dimer interface. ANP binding measurements of the cross-linked receptor indicated that those differences were not due to reduction in binding capacity but in binding affinity (data not shown), suggesting that suboptimal spacing of the receptor subunits perturbed the binding interaction of ANP in the binding cleft.

**ANP binding hinders Cys\textsuperscript{74} disulfide bridging of ΔKC\textsuperscript{C423S,C432S,W74C} mutant**—Since disulfide bridging of W74C mutants leads to a slightly constrained inactive dimer, presumably because the dimer conformation does not satisfy the more stringent interface spacing required for high affinity ANP binding, we wondered whether, in reciprocal fashion, ANP binding to the ΔKC\textsuperscript{C423S,C432S,W74C} mutant could prevent Cys\textsuperscript{74} disulfide formation. The receptor mutant was first reduced with DTT (Fig. 8) then incubated with saturating concentrations of ANP before attempting to reoxidize the Cys\textsuperscript{74} disulfide in the presence of Cu(OP)\textsubscript{2}. When studied with SDS-PAGE under non-reducing conditions, the truncated W74C mutant was spontaneously dimeric (Fig, 8, right panel). In analogy with the full length receptor (Fig. 4), this truncated mutant was also devoid of high affinity for ANP (Fig. 9) but peptide binding could be restored by reduction and alkylation. Incubation with ANP inhibited dimer formation following transient reduction with DTT (Fig. 8, right panel). Thus, unlike the basal inactive state of NPRA receptor which allows for spontaneous Cys\textsuperscript{74} disulfide formation presumably due to conformational flexibility and mobility at the dimer interface, the ANP-bound and activated state displays more stringent interface positioning requirements probably because ANP binding stabilizes the receptor dimer and thus reduces the conformational mobility of the subunits interface.
DISCUSSION

We have document that the extracellular domain of the natriuretic peptide receptor adopts an A-shaped dimer conformation with a membrane distal dimer interface. All experiments were based on membrane-anchored receptor therefore ensuring that the conclusions would be representative of native cellular NPRA. The results do not confirm the intial V-shaped dimer conformation proposed for the unbound state of NPRA. According to that originally proposed conformation, the membrane proximal lobes would provide the dimer interface. In addition the V-shaped conformation would allow for one ANP binding surface located on each side of the dimer, therefore resulting in a 2:2 stoichiometric ratio for ANP binding to NPRA (21). While the membrane-proximal localization of the dimer interface is analogous to that for growth hormone receptor (32), it provides little hint about the activation process of NPRA because the ANP molecules would preferentially if not exclusively interface with only one dimer subunit, in contrast with many cases documented so far for growth factor and cytokine receptors (32-34). The ANP-bound NPRA dimer is not likely to adopt the V-shaped conformation since covalent cross-linking of the W74C mutant with oPDM preserves peptide binding and maintains the membrane distal lobes in proximity, albeit more apart than with disulfide bridging, but still considerably closer than what the V-dimer could accomodate.

The A-shaped dimer conformation subsequently proposed by van den Akker (24) for NPRA and documented by Garcia for NPRC (23) is more similar to the conformation of other dimeric receptors e.g. the glutamate metabotropic receptor (35). The constitutively dimeric properties of native NPRA $^{W74C}$ mutant strongly suggest that this A-shaped conformation is
natural and potentially contributes to a loose dimer in basal inactive state of the membrane-anchored receptor. However, as pointed out by van den Akker (24) the membrane-distal dimer interface surface is probably insufficient to maintain by itself the A-dimer conformation, therefore explaining the monomeric state of the unbound extracellular domain in soluble truncated form (20). Indeed ANP binding to an A-shaped NPRA dimer would be expected to significantly contribute to the surface of the dimer interface and therefore to stabilize the dimer. This would explain the observation of ANP-induced dimerization of the extracellular domain (20) which would result from a huge increase of the dimerization constant induced by peptide binding.

While the data presented show that the unbound state of NPRA is characterized by a larger flexibility and mobility at the dimer interface required for allowing for Cys\textsuperscript{74} disulfide formation, the ANP-bound and active state of NPRA displays more stringent intersubunit distance requirements incompatible with Cys\textsuperscript{74} disulfide formation but which could be satisfied by proper spacing with oPDM. Thus, ANP binding is likely to stabilize the NPRA dimer presumably by fitting within the inter subunit cleft below the dimer interface, therefore substantially contributing to the buried surfaces of the dimer interface and restraining its mobility. Reciprocally, binding of ANP within this cleft is likely to tightly retain the bound peptide, resulting in high affinity and slow dissociation rate, in agreement with reported observations. Thus monitoring of residues 74 at the membrane-distal end of the dimer interface is providing a very sensitive assessment of dimer positionning during receptor activation.
While these results fully support the A-shaped dimer conformation involving a dimer interface in the membrane distal lobe of the extracellular domain, they do not exclude the existence of another extracellular domain interface in the juxtamembrane domain. Indeed the CNP-bound NPRC dimer used by Garcia for crystallography included an interchain disulfide in the juxtamembrane portion of the extracellular domain (23). Also, the constitutive formation of a disulfide bridge at Cys$^{432}$ in the NPRA$^{C423S}$ mutant and the observation of an agonist-induced disulfide bridge three residues further in the case of the NPRA$^{D435C}$ mutant both strongly suggest that the juxtamembrane region connecting the bi-lobed periplasmic folded domain with the transmembrane domain is involved in some additional dimer interface, possibly contributing to the signal transduction process from the extracellular to the intracellular domains of the receptor. Crystallographic documentation of the structure of ANP-bound NPRA extracellular domain of the membrane-anchored receptor as well as of the kinase homology domain should provide further insight into the signal transduction mechanism of membrane guanylyl cyclases and should also contribute to better understanding of other single transmembrane domain receptors.
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FIGURE LEGENDS

FIG. 1. Two proposed conformations for NPRA extracellular domain dimer. Two possible structures of rat NPRA non-covalent dimer proposed by van den Akker (5, PDB:1DP4). Each bi-lobed extracellular subunit is grayed differently and both Trp$^{74}$ are tinted as dark grey. Distances between Trp$^{74}$ alpha-carbons are indicated for both the A-dimer and the V-dimer structures.

FIG. 2. Structure of the NPRA mutants used. NPRA$^{WT}$ was used as control for the NPRA$^{W74C}$ mutant exposing an unpaired cysteine (S) at the top of the A-dimer interface (Fig. 1). The ΔKC$^{C423S,C432S}$ mutant was obtained by truncating the cytoplasmic domain (INT) containing unpaired cysteines and by mutating both exposed Cys$^{423}$ and Cys$^{432}$ in the extracellular domain (EXT) close to the transmembrane domain (TM). The ΔKC$^{C423S,C432S,W74C}$ mutant contains an additional unpaired and exposed Cys$^{74}$.

FIG. 3. Covalent dimeric state of NPRA$^{W74C}$. Membranes prepared from HEK293 transiently transfected with NPRA$^{WT}$ (left panel) or NPRA$^{W74C}$ (right panel) were treated when indicated at 22°C with 5 mM DTT, then at 4°C with 5 mM NEM and/or 25 μM Cu(OP)$_2$. 10 μg of membrane proteins were loaded on non-reducing 5% SDS PAGE, and receptor monomers (M) and dimers (D) were detected by Western blotting using a specific antibody as described in Experimental Procedures. The results presented are representative of two replicate experiments.
FIG. 4. Cys\textsuperscript{74} disulfide reduction restores high affinity binding of ANP to NPRA\textsuperscript{W74C}. ANP high affinity binding was measured in NPRA\textsuperscript{WT} (open bars) and NPRA\textsuperscript{W74C} mutant (hatched bars) following reduction and alkylation as described in Fig. 3. The membranes (1 \( \mu \)g) were then incubated with 10 pM \(^{125}\text{I}\text{-ANP, with (nonspecific binding) or without (total binding) an excess of unlabeled ANP for 20h at 4°C. Specific binding was calculated by subtracting nonspecific from total binding and values were normalized to those obtained with DTT + NEM and expressed as mean ± SEM of duplicates. The experiment was repeated twice with similar results.}

FIG. 5. cGMP production is blunted in NPRA\textsuperscript{W74C} mutant. Dose-response curves of ANP on cGMP production was performed on HEK293 cells transiently transfected with NPRA\textsuperscript{WT} (closed circles) or NPRA\textsuperscript{W74C} (open circles), and extracellular cGMP was assayed by radio-immunoassay. Each data point represents the mean ± SEM of four determinations.

FIG. 6. Non-reducible covalent cross-linking of Cys\textsuperscript{74} by PDM in ΔKC\textsuperscript{C423S,C432S,W74C}. Membranes prepared from HEK293 transiently transfected with control ΔKC\textsuperscript{C423S,C432S} (left panel) or mutant ΔKC\textsuperscript{C423S,C432S,W74C} (right panel) were incubated at 22°C with 5 mM DTT then at 4°C with 100 \( \mu \)M NEM, oPDM, mPDM, or pPDM. Membrane proteins (4 \( \mu \)g) were then submitted to 7.5% SDS-PAGE under reducing condition in order to exclude disulfide bridging. Receptor was detected by Western blot using a specific antibody. The position of monomers (M) and dimers (D) are indicated. The figure is representative of duplicate experiments.
FIG. 7. Cross-linking of Cys$^{74}$ with PDM preserves ANP binding to $\Delta KC^{C423S,C432S,W74C}$. Membranes from HEK293 cells transiently transfected with control $\Delta KC^{C423S,C432S}$ control (open bars) and $\Delta KC^{C423S,C432S,W74C}$ mutant (hatched bars) following alkylation with PDM as described in Fig. 6. The membranes (0.2 $\mu$g) were then incubated with 10 pM $^{125}$I-ANP with or without an excess of unlabeled ANP for 20h at 4$^\circ$C. Specific binding is expressed as mean ± SEM of duplicate determinations. This figure is representative of two identical experiments.

FIG. 8. ANP binding to $\Delta KC^{C423S,C432S,W74C}$ inhibits Cys$^{74}$ disulfide formation. Membranes from HEK293 cells transiently transfected with $\Delta KC^{C423S,C432S}$ control (left panel) or $\Delta KC^{C423S,C432S,W74C}$ mutant (right panel) when indicated were reduced at 22°C with 5 mM DTT, then incubated at 4°C with ANP, re-oxidized with 25 $\mu$M Cu(II) and in all cases finally treated with NEM. Proteins (4 $\mu$g) were then loaded on non-reducing 7.5% SDS-PAGE. Receptor was detected by Western blot using a specific antibody. The position of monomers (M) and dimers (D) are indicated. The results presented are representative of duplicate experiments.

FIG. 9. Re-oxidation of Cys74 $\Delta KC^{C423S,C432S,W74C}$ inhibits ANP binding. ANP binding was measured in $\Delta KC^{C423S,C432S}$ (open bars) and in $\Delta KC^{C423S,C432S,W74C}$ mutant (hatched bars) after reduction with DTT and re-oxidation of Cys$^{74}$ disulfide as described in Fig. 8. The membranes (0.5 $\mu$g) were then incubated with 10 pM $^{125}$I-ANP with or without an excess of unlabeled ANP for 20h at 4°C. Specific binding is expressed as mean ± SEM of duplicate determinations. The experiment was repeated twice with similar results.
Fig. 1

V-dimer

A-dimer

W74 82 Å W74

8 Å
|        | NPRA<sup>WT</sup> | NPRA<sup>W74C</sup> |
|--------|-------------------|----------------------|
| DTT    | –                 | –                    |
| NEM    | –                 | –                    |
| Cu(OP)<sub>2</sub> | –                 | –                    |
Fig. 6

ΔKC^{C423S,C432S}

ΔKC^{C423S,C432S,W74C}

MW (KDa)

CTL  NEM  DTT+NEM  DTT+oPDM  DTT+mPDM  DTT+pPDM

CTL  NEM  DTT+NEM  DTT+oPDM  DTT+mPDM  DTT+pPDM

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Fig. 7
ΔKC<sup>C423S,C432S</sup>  

|      | DTT | ANP | Cu(OP)<sub>2</sub> | ΔKC<sup>C423S,C432S,W74C</sup> |
|------|-----|-----|-------------------|----------------------------------|
|      | −   | −   | −                 | −                                 |
|      | +   | +   | +                 | +                                 |
|      | +   | +   | +                 | +                                 |
|      |     |     | −                 |                                   |

Fig. 8
Natriuretic peptide receptor A activation stabilizes a membrane-distal dimer interface
André De Léan, Normand McNicoll and Jean Labrecque

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