Identification of receptor binding-induced conformational changes in non-visual arrestins

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Background: Non-visual arrestins regulate the signaling of hundreds of GPCRs.

Results: Receptor binding-induced conformational changes in non-visual arrestins partially overlap with those in visual arrestin-1.

Conclusion: Some receptor binding-induced conformational changes are conserved between arrestin-1, -2 and -3.

Significance: Characterization of receptor-induced conformational changes will help identify how the non-visual arrestins interact with hundreds of receptors.

ABSTRACT

The non-visual arrestins, arrestin-2 and arrestin-3, belong to a small family of multifunctional cytosolic proteins. Non-visual arrestins interact with hundreds of G protein-coupled receptors (GPCRs) and regulate GPCR desensitization by binding active phosphorylated GPCRs and uncoupling them from heterotrimeric G proteins. Recently, non-visual arrestins have been shown to mediate G protein-independent signaling by serving as adaptors and scaffolds that assemble multiprotein complexes. By recruiting various partners including trafficking and signaling proteins, directly to GPCRs, non-visual arrestins connect activated receptors to diverse signaling pathways. To investigate arrestin-mediated signaling, a structural understanding of arrestin activation and interaction with GPCRs is essential. Here we identified global and local conformational changes in the non-visual arrestins upon binding to the model GPCR rhodopsin. To detect conformational changes, pairs of spin labels were introduced into arrestin-2 and arrestin-3, and the inter-spin distances in the absence and presence of the receptor were measured by double electron electron resonance (DEER) spectroscopy. Our data indicate that both non-visual arrestins undergo several conformational changes similar to arrestin-1, including the finger loop moving toward the predicted location of the receptor in the complex, as well as the C-tail release upon receptor binding. The arrestin-2 results also suggest that there is no clam-shell-like closure of the N- and C-domains, and that the loop containing residue 136 (homolog of 139 in arrestin-1) has high flexibility in both free and receptor-bound states.

G protein-coupled receptors (GPCRs) represent the largest, most versatile and ubiquitous class of membrane receptors, with more than 800 members in the human genome (1). Upon activation, they regulate a variety of intracellular signaling pathways to produce appropriate cellular responses, such as cell growth, differentiation, metabolism, and mediate smell, vision, and taste (2). Arrestins are a small family of proteins that preferentially bind active phosphorylated GPCRs, block G protein-mediated signaling, and facilitate receptor trafficking (3-6). There are four arrestin subtypes expressed in mammals and they clearly fall into two categories. One is the visual arrestins, which include arrestin-1 and arrestin-4, and are exclusively expressed at high levels in photoreceptors (7-9). Arrestin-1 demonstrates high specificity for its cognate receptor rhodopsin (10-13). The other category is the non-visual arrestins or ²-arrestins, which include arrestin-2 (²-arrestin-1) and arrestin-3 (²-arrestin-2)³. Non-visual arrestins are expressed ubiquitously in all
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cells and tissues with the highest expression levels in mature neurons (14-17). The broad distribution and receptor specificity of non-visual arrestins suggest that these two subtypes recognize and regulate the vast majority of GPCRs (18-20).

The four mammalian arrestin subtypes have greater than 50% amino acid conservation and a similar elongated two-domain structure in the basal state (4,6,14,21-24). The two conserved domains, the N-domain and C-domain, are connected through a hinge region (Fig. 1). In the basal state, the C-terminus folds back toward the N-domain and forms a highly conserved tripartite interaction with the N-domain, involving ²-strand I and ±-helix I (4,25-27). Upon binding to its cognate GPCR rhodopsin, several conformational changes in arrestin-1 have been proposed and confirmed by studies using NMR, fluorescence quenching and site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy techniques (28-35). An EPR study using double electron electron resonance (DEER) combined with RosettaEPR modeling provided a global picture of the phosphorylated activated rhodopsin (PR*)-induced conformational changes in arrestin-1, which involve the release of the C-tail, the movement of the finger loop, the movement of a loop containing residue 139, as well as smaller changes in distal loops containing residues 157 and 344 at the tips of the N- and C-domains (33). Moreover, the DEER study found no inter-domain movement of arrestin-1 induced by PR* binding, which rejects the closing “clam shell” model (33,36). However, a recent crystal structure of pre-activated p44, a naturally occurring variant of arrestin-1 in which the final 35 amino acid residues of the C-terminus are replaced by a single alanine residue, revealed an unexpected 21° twist between the N- and C-domains (37). A model of the receptor-bound state of non-visual arrestins is necessary to elucidate the structural basis of arrestin-mediated signaling. In addition, truncated arrestin-2 was recently co-crystallized with the multi-phosphorylated C-terminus of the vasopressin V2 receptor (38). Although this structure was not obtained by co-crystallization of arrestin-2 with a receptor, the crystal structure of arrestin-2 in complex with an antibody fragment (Fab30) and the phosphorylated peptide (V2Rpp) provided the first glimpse of the possible active form of arrestin-2. Compared to the basal state of arrestin-2, this complex captured a conformation of arrestin-2 that involves a 20° twist between the N- and C-domains and conformational changes in the “finger loop” (residues 63-75), the loop containing residue 136 (residues 129-140) and the “lariat loop” (residues 274-300) (38). The observed consistency of inter-domain rotation between the two crystal structures suggests that the activation mechanism may be conserved among all arrestins. Interestingly, a similar domain rotation was proposed in 2006 by modeling (39) on the basis of earlier findings that hinge deletions in arrestins impede receptor binding (40,41).

Although crystal structures yield atomic details, there are still questions remaining: whether truncated arrestin-2 in complex with V2Rpp exhibits the same conformational changes as WT arrestin-2 in complex with full length receptor, and to what extent did the crystal-packing and the extra antibody fragment (Fab30) contribute to the observed conformational changes (42). Thus, to investigate the proteins under more physiological conditions, we utilized DEER spectroscopy, which provided distance measurements between two attached spin labels in arrestin-2 and arrestin-3 in the presence and absence of bound full-length phosphorylated, light-activated rhodopsin (PR*). DEER spectroscopy is a pulse EPR technique that detects the spin-spin interaction between two spin labeled sites in the range of 20-80Å. Because of the ability to measure long distances, DEER spectroscopy is advantageous to the study of protein conformational transitions and biomolecular associations. In this study, the intra-molecular distance data give a global picture of the receptor-induced conformational changes in the non-visual arrestins. Multiple elements of the non-visual arrestins were specifically investigated and the data revealed essential receptor binding-induced movements.

**EXPERIMENTAL PROCEDURES**

Site-directed mutagenesis - Single cysteine substitutions were introduced into cysteine-less arrestin-2 and arrestin-3 using QuikChange PCR (Stratagene). Double cysteine substitutions were created by introducing one cysteine mutation first and then using the single cysteine vector as a base to introduce the second mutation. All PCR primers were designed by us and purchased from IDT DNA. Mutations were verified by Retrogen Sequencing. Cysteine-less arrestin-2 and arrestin-3 base mutants are fully functional in terms of receptor binding (41).

Protein expression and purification - Arrestin-2 and arrestin-3 were expressed and purified as described (23,30,43). Escherichia coli BL21 (DE3) cells were transformed with plasmids encoding each mutant and grown in Luria broth/ampicillin (100 mg/L) at 30° and induced with 0.1 mM IPTG. After lysis and ammonium sulfate precipitation, the protein was dissolved in buffer and arrestin was purified by sequential chromatography.
on heparin-Sepharose, Q-Sepharose, and SP-Sepharose (GE Life Sciences). Protein purity was verified by 15% SDS-PAGE and concentrations were determined using a BCA assay (Pierce) with bovine serum albumin (BSA) as a standard. Proteins were concentrated to the final desired concentration with YM-10 centrifugal concentrators (Amicon). Rhodopsin was isolated from bovine rod outer segments, as described (44). Phosphorylation of rhodopsin by endogenous GRK1 in purified rod outer segments yielded 2.6 phosphates per rhodopsin (44).

Arrestin-rhodopsin binding assay - Rhodopsin binding of arrestins was tested in the buffer used for the EPR experiments (50 mM MOPS, 100 mM NaCl, pH 7.0) as described (43,45). Purified spin-labeled arrestin mutants (4.5 µM) were incubated with 9 µM phosphorylated rhodopsin. After a 5 min incubation in room light at 37°C, the arrestin/rhodopsin mixture was loaded onto a 100 µL 0.2 M sucrose cushion to separate bound and free arrestin by centrifugation for 20 min at 350,000xg in an ultracentrifuge ( Sorvall). Supernatants were carefully removed and the rhodopsin pellets resuspended in binding buffer (50 mM MOPS, 100 mM NaCl, pH 7.0). Equal amounts of arrestin/PR* input, pellet and supernatant were subjected to 10% SDS-PAGE for direct comparison.

Spin labeling - Purified double arrestin mutants were spin labeled with a 40-fold molar excess of 4-maleimido-TEMPO (MAL-6) spin label, and agitated for 12 hours at 4°C. Excess spin label was removed by extensive dialysis into buffer consisting of 50 mM MOPS, 100 mM NaCl, pH 7.0.

Double electron electron resonance (DEER) spectroscopy - DEER data were collected on a Q-band Bruker ELEXYS E580 equipped with an EN5107D2 resonator with overcoupling at 80K. Samples containing 20% deuterated glycerol as a cryoprotectant and were flash frozen in a dry ice and acetone mixture. Aquired raw data after phase correction were background corrected, plotted and analyzed using DeerAnalysis2011 software freely available at http://www.epr.ethz.ch/ (46) and the LongDistances software program (47) provided by C. Altenbach (University of California - Los Angeles, CA). Distance distributions were determined by fitting the corrected dipolar evolution data using model-free Tikhonov regularization (46) or the algorithms included in the LongDistances program. For distance distributions that are broad or complex, the mean distance values were used in the table to analyze conformational changes. To obtain the mean distances, the distance distributions were integrated and then normalized to the maximum amplitude. The mean distance was estimated as the value at 0.5 of the integrated intensity and the values were reported in Table 1 and Table 2. The upper reliable distance limit for each sample was determined based on the maximum data collection time (t) used in each of the DEER experiments according to the equation d H 5 t/2(13) (48) and is reflected in the x-axis of each distance distribution plot. The expected distance between arrestin-2 pairs was predicted via the PRONOX program (http://rockscluster.hsc.usc.edu/research/software/pronox/pronox.html) (49) by measuring the distances between rotameric configurations of the two spin labels attached to the highest resolution arrestin-2 crystal structure available (50) based on an experimentally derived library of allowed rotameric configurations. For comparison, free and receptor-bound state distances were determined for the free state crystal structure of arrestin-2 (1G4M) and the crystal structure of arrestin-2 in the presence of V2Rpp (4JQI, respectively.

RESULTS

Conformational changes in Arrestin-2 upon Binding to PR* - To identify the conformational changes in arrestin-2 upon receptor binding, 17 pairs of residues were selected for the introduction of spin labels to measure intra-molecular distances. Each double cysteine mutant was purified and spin labeled. The 17 double mutants contain pairs of spin labels that fall into six groups and sample a variety of potential structural changes: three inter-domain pairs, one pair to report on the finger loop, two to report on the C-tail movement, five pairs to report on the 136 loop, two within the N-domain and four within the C-domain (Fig. 1 and Table 1). Since rhodopsin is a prototypical GPCR available as a purified protein in native membranes, PR* was used as the model receptor in these studies. Both non-visual arrestins specifically bind PR* (51). First, the functional consequences of the cysteine mutations and spin labeling of arrestin-2 were assessed by testing the ability of the purified spin-labeled arrestin-2 double mutants to bind to PR*. We found that all spin-labeled arrestin-2 mutants retain the ability to bind PR* at 80-100% of the wild type (WT) level, except 68/167, which binds PR* at approximately 67% of WT, based on densitometry analysis (Fig. 2). In addition, all arrestin proteins tested remained in the supernatant in the absence of PR* (data not shown). Thus, each of the selected double arrestin-2 mutants was able to functionally bind PR* at the molar ratios and concentrations used for the DEER experiments.

DEER data were collected for each double-cysteine arrestin-2 mutant for both the free and PR*-bound states. The distance distributions yield the range of
distances between the two labels and so the width and shape of the distribution contain information on the structural heterogeneity of the protein or the contribution of rotameric equilibria of the spin labeled side chains. For each mutant, the mean distance of the distribution was determined and shown as experimental distances in Table 1. The positive • Distance values indicate a distance increase between the two spin labeled sites of arrestin-2 upon PR* binding, whereas a negative number indicates a decrease in the distance between sites. The results from each of the six groups of mutants are described in detail below.

The release of the C-tail - The most dramatic distance change was observed for the C-tail of arrestin-2, which was probed by the spin labeled double mutant A12C/A392C (Fig. 3A). In arrestin-1, the C-tail was shown to be released from the N-domain upon rhodopsin binding (30,31,33); therefore the 12/392 mutant was chosen to monitor the C-tail movement in arrestin-2. Residue 392 is on the arrestin-2 C-tail, whereas residue 12 is located in β-strand I in the N-domain (Fig. 3F). The distance between positions 12 and 392 in the basal state of free arrestin-2 is short because the C-tail is anchored to the N-domain (50). Upon addition of PR*, there is a 34 Å increase in the distance between 12 and 392, indicating the C-tail is released. Interestingly, the width of the PR*-bound distance distribution is narrow, suggesting that the released C-tail may dock to a specific location elsewhere on the structure. This is in contrast to a very wide distance distribution upon PR* binding between homologous sites in arrestin-1, which suggested that the released arrestin-1 C-tail does not have a fixed position in the receptor-bound form (30,31). To test whether the C-tail of arrestin-2 interacts with the C-domain upon receptor binding, another pair (M192C/A392C) was designed to identify the position of the released C-tail. Residue 192 is located on the edge of the C-domain, 63Å away from 392 in the free state. If the C-tail folds back to the C-domain after its release from the N-domain, the distance between 192 and 392 was expected to become significantly shorter upon PR* binding. The free state DEER distance of 192/392 has a very long component at about 68Å and a short population at 43Å (Fig. 3B; Table 1). Upon PR* binding, we found no dramatic inter-spin distance change between 192 and 392: the DEER distances of the receptor bound state still have similar distributions around 43 Å and a very long distance population at 63 Å. The absence of a dramatic distance decrease between 192 and 392 suggests that the released C-tail does not fold back to the C-domain.

Inter-domain distances - Global conformational changes in arrestin-2 were evaluated by monitoring inter-domain distances between Y47C/E257C, V81C/A339C and V167C/S340C. Spin labels at sites 47, 81 and 167 served as reference points on the N-domain, while sites 257, 339 and 340 were chosen as reference points on the C-domain. The DEER data did not reveal any obvious inter-spin distance change for the 47/257 and 167/340 pairs, suggesting that PR* binding does not induce a dramatic movement of the two domains relative to each other (Fig. 3C and 3E). Moreover, we found slightly increased 81/339 inter-spin distances (Fig. 3D). Thus, the DEER data suggest that the N-domain and the C-domain do not undergo a “clam shell-like” closing movement.

Finger loop extension - As shown in Fig. 4A, a small distance change was observed between L68C and V167C, which is expected to reflect the finger loop movement induced by PR* binding. Site 68 is at the tip of the finger loop, and 167 is a rigid reference point on the N-domain. The free state distance is short because the finger loop is partially folded back onto the N-domain (Fig. 4A). Upon PR* binding, the distance between 68 and 167 was increased by 4 Å, suggesting that the finger loop extends. Despite dislocation of the finger loop, the narrow width of the distance distribution indicates that this loop has a relatively restricted motion upon binding to PR*.

Movement of loop containing 136 - To identify the movement of the loop containing residue 136, several double mutants were analyzed as shown in Fig. 4: L33C/T136C, Y47C/T136C, T136C/V167C, T136C/Y238C and T136C/T246C. Residue 136 is located at the tip of a central loop, adjacent to the finger loop (Fig. 4G), and is predicted to be in a flexible region, especially upon receptor binding. According to the DEER results, the distance distribution between positions 33 and 136 on the N-domain did not change upon PR* binding (Fig. 4B and Table 1). But the distance distributions between positions 47 and 136 and 136 and 167 both demonstrated a small decrease upon PR* binding (Fig. 4C and 4D). There is also a significant broadening of the distance distribution for 136/167, or a second peak at 32 Å. Residue 167 is in the middle of β-strand X in the N-domain, which is expected to be rigid, while 47 is located on a flexible loop at the tip of the N-domain (Fig. 4G). Decreased distances for the 47/136 and 136/167 pairs suggested that the 136 loop slightly moves toward the N-domain and the broad distributions suggest that this loop remains highly flexible upon receptor binding.
Interestingly, the distance distributions associated with 192/339 revealed a 4 Å increase upon addition of PR*. Information. As a result, the mean distances for in order to include all distances and structural well separated, the mean distances are determined for multiple populations. Since these populations are not narrow with the absence and presence of PR*, and both distance populations were found to be increased by 1 Å upon PR* binding. Since residue 246 is located in the loop in the C-domain and adjacent to the 136 loop, the movement between 136 and 246 indicated a slight translation of the 136 loop along with a slight narrowing of the two distance distributions. It was also found that a distance increase of 4 Å occurred between 136 and 238. Taken together, these data suggest a limited movement of the 136 loop towards the N-domain and away from the C-domain upon PR* binding.

Intra-domain distances - Possible conformational changes induced by PR* binding within each domain were investigated using distances between two spin labels located either in the N-domain or in the C-domain. Intra-N-domain conformational changes induced by PR* binding were examined by monitoring the distances between the N-domain loops (Y47C and l158C) and the center of the N-domain (V167C) (Fig. 5G). We detected a 5 Å distance increase between sites 47 and 167 and no change in the distance between sites 158 and 167 (Fig. 5A and 5B). As shown in Fig. 5G, M192C/Y238C, Y238C/A339C and S234C/S340C were selected to measure distances from the tips of the C-domain loops to the center of the C-domain. With reference point 238 at the center of the C-domain, both 192/238 and 238/339 showed longer inter-spin distances upon addition of PR*, with a clear 8 Å and 3 Å increase, respectively, indicating that the C-domain loops move away from 238 upon PR* binding (Fig. 5C and 5D). However, the inter-spin distance between positions 234 and 340 showed a slight decrease upon PR* binding (Fig. 5E). Moreover, the movement between loops of the C-domain was detected by measuring the distance change between 192 and 339, which are located on the tips of adjacent loops (Fig. 5F). The distance distributions for 192/339 in the absence and presence of PR* were found to be broad with multiple populations. Since these populations are not well separated, the mean distances are determined for 192/339 in the absence and presence of PR* (Table 1) in order to include all distances and structural information. As a result, the mean distances for 192/339 revealed a 4 Å increase upon addition of PR*. Interestingly, the distance distributions associated with these C-domain loops (192/238, 238/339 and 234/340) became narrower upon PR* binding, suggesting that receptor binding immobilized these C-domain loop residues. Overall, PR* binding results in small conformational changes in some of the loops within the C-domain and within the N-domain.

Conformational changes in arrestin-3 upon binding to PR* - For comparison, we also tested the other non-visual subtype, arrestin-3, using DEER to detect two possible conformational changes induced by receptor binding. Although arrestin-3 has a largely similar basal structure (23) and has some overlapping functions with arrestin-2 (6), receptor-induced structural changes in the two subtypes were reported to be distinct (52). To identify two key conformational changes upon receptor binding, spin labeled S13C/A392C and D68C/V168C arrestin-3 mutants were used to monitor the movement of the C-tail and finger loop, respectively (Fig. 6B). Since the C-tail release and opening of the finger loop have been found in both arrestin-1 (33) and arrestin-2 (reported here), it is of great interest to determine whether these conformational changes are conserved. As shown in Fig. 6C, both spin-labeled arrestin-3 proteins retained the ability to bind PR*, which was again used as the model GPCR. The DEER data reveals that the inter-spin distance between positions 13 and 392 became longer when arrestin-3 binds to PR*, suggesting the C-tail is released (Fig. 6A). Interestingly, three distinct distance populations coexist between spin labels at 13 and 392 in the receptor-bound conformation, which suggests that the C-tail of arrestin-3 likely adopts several specific conformations, in contrast to arrestin-2 with a single distance (Fig. 3) and to the completely flexible and free C-tail in receptor-bound arrestin-1 (31,33,35). Conformational changes induced by PR* binding in the finger loop were tested using 68/168 arrestin-3. Similar to arrestin-2, a small distance increase of 2 Å between positions 68 and 168 was observed, supporting the model that the finger loop is slightly dislocated upon PR* binding. The relatively broad distance distribution of 68/168 in both free and PR* bound state suggests that the finger loop in arrestin-3 is not fully extended and maintains a flexible range of positions even when it is bound to the receptor. This is similar to the movement of this loop in arrestin-2 (Fig. 4) and arrestin-1 (33).

DISCUSSION

Conformational changes in signaling proteins regulate their interactions with their binding partners. G protein-mediated signaling by the majority of GPCRs is terminated by a conserved two-step mechanism:
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...required for crystallization, crystal packing forces, or all of the above. In addition, here we used full-length arrestin-2, in contrast to the crystal structure, where a truncated arrestin-2-(1-393), which was previously shown to be pre-activated (61), was used for crystallization (38).

Previous EPR studies demonstrated that the C-tail of arrestin-1 becomes dynamically disordered upon receptor binding (30,31,33). Here we show that in both arrestin-2 and arrestin-3 the C-tail is released upon PR* binding. The consistency of this conformational change induced by receptor binding strongly supports that all subtypes of arrestins share the same activation mechanism, since the C-tail is involved in the activation process of arrestins. The arrestin C-tail, which is anchored to the N-domain via the three-element interaction in the free state, is important for constraining the inactive conformation of arrestins (31,65). Here we show directly for the first time that the C-termini of both non-visual arrestins are released upon receptor binding. This makes the release of the C-tail a hallmark of arrestin activation. While EPR (30,31,33) and NMR studies (35,66) of arrestin-1 suggest that the arrestin-1 C-tail becomes dynamically disordered upon PR* binding (30,31,33), the DEER results presented here for arrestin-2 and arrestin-3 suggest that the C-tail may adopt specific conformations after release. Thus, whereas the receptor induced C-tail release is conserved in arrestin-1, -2 and -3, the resulting position of this element in different subtypes is likely distinct. In the case of arrestin-2, upon release the C-tail appears to assume a specific conformation, being located at a fixed distance from β-strand I, which is unlikely to be near the C-domain (Fig. 3) and may instead be located on rhodopsin. In contrast, in arrestin-3 there appear to be at least three distinct positions of the released C-tail (Fig. 6). Regardless of the location of the C-tail after its release, it is likely that this receptor-induced conformational change exposes binding motifs of the C-tail that interact with clathrin (67) and clathrin adaptor AP2 (68) and possibly some signaling proteins. Fixed position(s) of the C-tail in both non-visual subtypes can promote interactions with clathrin and other partners that arrestin-1 does not bind. Interestingly, even the C-tail of arrestin-1 can interact with AP2 upon its release by receptor binding (69).

The N- and C-domains in arrestin are connected by a hinge, the length of which is essential for receptor binding (40,41). As progressive deletions in the inter-domain hinge reduced the ability of all arrestins to bind receptors, the “clam shell” model of arrestin activation, where the two domains move relative to each other closing in on the receptor, was proposed (36,40).
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However, no evidence of such domain movement in arrestin-1 was found by DEER distance measurements (33). Here we also found no evidence of the clam-shell-like motion of the N- and C-domains in arrestin-2 when it binds to PR*. Our data only reveal subtle changes in the inter-domain distances, with only 81/339 showing small distance increases, and both 47/257 and 167/340, revealing no inter-domain distances changes. Notably, 47/257 and 167/340 double mutants bind PR* as well as the wild type arrestin-2 (Fig. 2). Thus, the absence of inter-domain distance changes is not due to a binding deficiency. Domain rotation was earlier predicted based on hinge deletion data by molecular modeling (39). A long hinge provides the flexibility for an inter-domain rotation upon arrestin activation, which can expose additional surfaces for interactions with non-receptor partners (70). Recent crystal structures of a pre-activated short splice variant of arrestin-1 (37) and arrestin-2 in complex with multi-phosphorylated vasopressin receptor peptide (38) support this new possibility of the inter-domain rotation. In the overlay of the free (50) and bound to phosphorylated V2Rpp arrestin-2 crystal structures (38) (Fig. 7), the twisting and translation of the C-domain relative to the N-domain is evident. However, the inter-domain distance changes predicted by these two crystal structures are not supported by the DEER results, since the two spin labeled pairs (81/339 and 167/340) specifically designed to detect the inter-domain twisting revealed different distance changes compared to the crystal structure prediction (Table 1). Arrestins are highly flexible (71), and therefore it is possible that the crystal structure only captured one possible conformation. It is also possible that since bound full-length receptor was absent in both crystal structures of “active” arrestins, they do not represent the physiologically relevant arrestin-receptor complex.

Another conformational change that was detected in both arrestin-2 and arrestin-3 upon PR* binding is the opening up of the finger loop (Tables 1, 2). DEER results for 68/167 of arrestin-2 and 68/168 of arrestin-3 both show relatively small increases in distance upon PR* binding, indicating that the finger loop where 68 is located is extending. Since the finger loop dislocation has also been observed in arrestin-1 upon PR* binding (30,33), this consistency among arrestin subtypes indicates this conformational change is conserved. However, the conformation of the finger loop induced by receptor binding might be distinct in different arrestin subtypes. In the basal state, the finger loops of arrestin-1, -2 and -3 all form a folded conformation, which is confirmed by the shorter distances observed by DEER in the free state (27) (Figs. 4, 6). Specifically, the DEER derived distances for homologous spin labeled pairs in arrestin-1 (72/173) (27), arrestin-2 (68/167) and arrestin-3 (68/168) all manifested similar results in the free state, 19Å, 21Å and 22Å, respectively. However, in the crystal structures of free arrestin-1, arrestin-2, and arrestin-3, the finger loops adopt a fully bent conformation (at least in some protomers in the arrestin-1 crystal tetramer), and the corresponding crystal structure-derived distances are predicted to be 12Å, 14Å and 7Å, respectively. Thus, the DEER measurements suggest the finger loop is not fully bent in the free state, and this is true for arrestin-1, arrestin-2, and arrestin-3. Upon binding to PR*, the arrestin-1 finger loop was observed to move slightly toward the expected location of the receptor and is proposed to form a helical conformation and bind in or near the central cavity of the activated receptor (30,33,34,72). As for arrestin-2, previous continuous wave (CW) EPR studies (12) identified that the finger loop is part of the arrestin-2 receptor binding surface by having more side chain flexibility in the free than in the receptor-bound state (12). This is also confirmed by our DEER data showing a narrower distance distribution in the PR*-bound state. Our DEER data also showed that the arrestin-2 finger loop extends slightly upon PR* binding (Fig. 4), which resembles the slight movement of arrestin-1 finger loop (33). A similar movement was also detected in arrestin-3 finger loop (Fig. 6). This is the first direct detection of the conformational change of the finger loop in arrestin-2 and -3. Small distance changes between the finger loop and the receptor-binding surface of the N-domain revealed by DEER measurements are virtually the same in arrestin-1 (3 Å), arrestin-2 (4 Å) and arrestin-3 (2 Å). Thus, upon PR* binding, the finger loop of arrestin-1, -2 and -3 undergoes a transition from a partially folded to more extended conformation. This conformational change can be visualized in the alignment of the inactive arrestin-2 and V2Rpp-bound arrestin-2 crystal structures (Fig. 7), wherein the finger loop is shown to open up slightly upon V2Rpp binding. Notably, the distance distributions involving finger loops in homologous spin labeled pairs of arrestin-1, -2 and -3 revealed the plasticity of the finger loop. Unlike arrestin-1 and arrestin-2, the finger loop of arrestin-3 is found to be more flexible in both the free and receptor-bound states. Therefore, it is likely that the finger loop of arrestin-1 and -2 is more essential in their interaction with incoming receptors by contacting the receptors directly, whereas the finger loop of arrestin-3 is only slightly affected by the receptor binding and may play a less important role.
The other striking conformational change in arrestins that has drawn a lot of attention is the movement of the central loop containing residues 139 or 136, in arrestin-1 and -2, respectively. In arrestin-1, this loop has been designated as the 139-loop and well investigated by several studies (30,33,73). The 139-loop of arrestin-1 is located in the center of the receptor binding surface, next to several elements directly engaged by PR*. It was proposed that 139-loop likely undergoes a large conformational change in order to facilitate receptor binding by making the finger loop and adjacent elements more accessible (30,33). Notably, there is a remarkable structural conservation of this loop within the arrestin family (4,21,23,24), so it is likely that the central loop in arrestin-2 and -3 also enhances their stability and selectivity. Here we focused on the central loop (136-loop) of arrestin-2 by measuring distance changes between it and different reference sites on both the N- and C-domains in the absence and presence of PR*. Our DEER data for the arrestin-2 136-loop do not show as dramatic structural changes upon PR* binding as were observed in arrestin-1 (33). We found that the 136-loop in arrestin-2 moved slightly towards the N-domain and away from the C-domain upon PR* binding. Moreover, broad distance distributions between 136 and the reference sites in the absence and presence of PR* strongly suggest that this loop has high flexibility in both the basal and active states. The predicted crystal structure distances (38) (Table 1) reveal larger changes at the central loop than the distance changes reflected by the DEER data measured for arrestin-2 in solution. Experimental conditions used to collect DEER data more closely resemble physiological conditions and reveal the dynamic flexibility of the central loop in both the basal and receptor bound state. This kind of flexibility of the central loop may help arrestin-2 to interact with hundreds of GPCRs by fine-tuning its structure (Fig. 4; Table 1).

Finally, the intra-domain conformational changes in arrestin-2 induced by PR* binding were investigated using distances between pairs of spin labeled residues located in the N- or the C-domain. Residues 47, 158, 192, 339 and 340 are all located in plastic loops of arrestin-2 (Fig. 5) that are likely to undergo large conformational changes during arrestin-receptor interaction. Previous CW EPR studies mapped the receptor-binding surface of arrestin-2, which is quite similar to that of arrestin-1, covering the concave sides of both domains (12,13,18,30,74). The distal loops are on the periphery of the receptor-binding surface of both arrestin-2 domains, which suggests that they may not be in direct contact with the receptor. Interestingly, our DEER data revealed dramatic structural changes at the distal tips of both the N-domain (47/167) and C-domain (192/238, 238/339, 192/339 and 234/340) (Table 1). Compared to the V2Rp-bound crystal structure of arrestin-2, the relatively large structural rearrangement of C-domain loops based on the DEER data is also observed in the overlay of the crystal structures (Fig. 7). It is tempting to speculate that these plastic loops in the C-domain might be involved in interactions with other protein partners after the dramatic conformational change upon receptor binding. The movement of the N-domain loops indicated by the DEER data is also observed in the crystal structure overlay (Fig. 7), suggesting that the entire N-domain of arrestin-2 does not need to undergo large structural rearrangement to either facilitate receptor binding or contribute to further interaction with other proteins.

Here we used SDSL and DEER spectroscopy to measure intra-molecular distances in arrestin-2 and arrestin-3 in the basal state and bound to the model GPCR rhodopsin. We provided evidence that arrestin binding to phosphorylated activated rhodopsin induces similar conformational changes in arrestin-2 and arrestin-3, including the C-tail release and partial extension of the finger loop. We did not detect “clam-shell” inter-domain movement or domain twisting in arrestin-2 upon PR* binding. We found that the 136-loop of arrestin-2 undergoes only a small movement towards the N-domain and maintains its structural flexibility upon PR* binding. This may help to fine-tune the arrestin-2 structure to facilitate its binding to a wide variety of GPCRs, in contrast to strict specificity of arrestin-1 for rhodopsin (12,13,51,64). Lastly, our data reveal that the distal loops on the C- and the N-domain, which are unlikely to contact the receptor directly, undergo more significant conformational changes upon PR* binding than those detected in homologous regions of arrestin-1 (33). These changes might prepare the non-visual arrestins for binding numerous non-receptor partners. In summary, DEER data providing dynamic structural information, complemented by the crystal structures, give a comprehensive overview of receptor-induced conformational changes in arrestin-2 and provide a comparison of the receptor binding-induced changes in different members of the arrestin family. Comparisons of conformational changes induced in arrestin-2 or arrestin-3 by their binding to different GPCRs will be necessary to identify how these two non-visual subtypes interact with hundreds of different receptors and dozens of non-receptor partners.
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FOOTNOTES
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3We use the systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (²-arrestin or ²-arrestin1), arrestin-3 (²-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called “arrestin 3” in the HUGO database)
FIGURE LEGENDS

FIGURE 1. Crystal structure of arrestin-2 in the basal conformation (PDB: 1G4M) with the residues studied by DEER spectroscopy shown as red C_α CPK models. The backbone structure of the N domain is shown in gray and the backbone structure of the C domain is shown in black. The major structural features of arrestin-2 are indicated.

FIGURE 2. PR* binding of all spin-labeled arrestin-2 cysteine mutants used in this study. The ability of each arrestin-2 mutant to bind to phosphorylated, light-activated rhodopsin (PR*) was tested. Wild-type arrestin-2 in the presence (wt Arr2) and absence (ctrl) of PR* are shown as positive and negative controls, respectively. Added arrestin-2 (blank), arrestin-2 in the pellet fraction (P), and arrestin-2 in the supernatant (S) fraction are shown for each protein. Arrestin binding to PR* is indicated by the presence of this protein in the pellet, whereas unbound arrestin is found in the supernatant fraction.

FIGURE 3. DEER analysis of the movement of the C-tail and arrestin-2 domains. Fits to the free (black) and PR*-bound (red) background-corrected dipolar evolution data (gray dots) are plotted on the left for the C-tail (A, B) and inter-domain (C-E) mutants to illustrate the data quality and support the distance distribution data. The corresponding distance distributions are shown on the right as overlays for the free (black) and PR*-bound (red) states. F) The free state crystal structure (PDB: 1G4M) of arrestin-2 is labeled with each pair of double mutants (red spheres). Measured distances are shown as dotted lines.

FIGURE 4. DEER analysis of the movement of the finger loop and the loop containing residue 136. Fits to the free (black) and PR*-bound (red) background-corrected dipolar evolution data (gray dots) are plotted on the left for the finger loop (A) and the loop containing residue 136 mutants (B-F) to illustrate the data quality and support the distance distribution data. The corresponding distance distributions are shown on the right as overlays for the free (black) and PR*-bound (red) states. G) The positions of the spin labels are shown as red CPK models on the crystal structure of the free state (PDB: 1G4M) of arrestin-2. Measured distances are shown as dotted lines.

FIGURE 5. DEER analysis of the conformational changes in the N- and C-domains of arrestin-2. Fits to the free (black) and PR*-bound (red) background-corrected dipolar evolution data (gray dots) are plotted on the left for the N-domain (A, B) and the C-domain mutants (C-F) to illustrate the data quality and support the distance distribution data. The corresponding distance distributions are shown on the right as overlays for the free (black) and PR*-bound (red) states. G) The positions of the spin labels are shown as red CPK models on the crystal structure of the free state (PDB: 1G4M) of arrestin-2. Measured distances are shown as dotted lines.

FIGURE 6. A, B. DEER analysis for two arrestin-3 spin labeled double mutants. Fits to the free (black) and PR*-bound (red) background-corrected dipolar evolution data (gray dots) are plotted on the left to illustrate the data quality and support the distance distribution data. The corresponding distance distributions are shown on the right as overlays for the free (black) and PR*-bound (red) states. C. The free state crystal structure of arrestin-3 (PDB ID: 3P2D) is labeled with the double mutants (red spheres) used for the DEER study. D. Pull-down assay results for the spin-labeled arrestin-3 protein pairs.

FIGURE 7. The crystal structures of inactive arrestin-2 (PDB: 1G4M) and V2Rpp-bound arrestin-2 (PDB: 4JQI) overlaid by alignment of the N domains. The inactive structure is shown in grey, and the phosphorylated receptor peptide-bound structure is shown in red. The conformational change of the finger loop is highlighted by the blue dotted circle. The inter-domain rotation observed in the crystal structure is indicated by the blue arrow.
Table 1. Receptor binding-induced changes for 17 inter-spin distances in arrestin-2

| Arrestin-2 doubles | PRONOXY Arr2-Free (Å) | PRONOXY Arr2+V2Rpp (Å) | ΔDistance (Å) | DEER Arr2-Free (Å) | DEER Arr2+PR* (Å) | ΔDistance (Å) |
|-------------------|------------------------|-------------------------|---------------|-------------------|-------------------|---------------|
| C-tail            |                        |                         |               |                   |                   |               |
| 12/392            | 15                     | ND                      | ND            | 21                | 55                | 34            |
| 192/392           | 63                     | ND                      | ND            | 65                | 63                | -2            |
| Interdomain       |                        |                         |               |                   |                   |               |
| 47/257            | 67                     | 67                      | 0             | 65                | 65                | 0             |
| 81/339            | 65                     | 56                      | -9            | 58                | 62                | 4             |
| 167/340           | 48                     | 55                      | 7             | 44                | 44                | 0             |
| 136 loop          |                        |                         |               |                   |                   |               |
| 33/136            | 42                     | 46                      | 4             | 49                | 49                | 0             |
| 47/136            | 39                     | 34                      | -5            | 45                | 44                | -1            |
| 136/187           | 15                     | 18                      | 3             | 24                | 23                | -1            |
| 136/238           | 31                     | 39                      | 8             | 34                | 38                | 4             |
| 136/246           | 31                     | 38                      | 7             | 22, 35            | 23, 36            | 1, 1          |
| Finger loop       |                        |                         |               |                   |                   |               |
| 68/167            | 14                     | 26                      | 12            | 21                | 25                | 4             |
| N-domain          |                        |                         |               |                   |                   |               |
| 47/167            | 29                     | 24                      | -5            | 29                | 34                | 5             |
| 158/167           | 17                     | 17                      | 0             | 24                | 24                | 0             |
| C-domain          |                        |                         |               |                   |                   |               |
| 192/238           | 32                     | 39                      | 7             | 32                | 40                | 8             |
| 192/339           | 17                     | 22                      | 5             | 25                | 29                | 4             |
| 234/340           | 13                     | 19                      | 6             | 27                | 25                | -2            |
| 238/339           | 27                     | 30                      | 3             | 30                | 33                | 3             |

**Table 1.** Summary of receptor binding-induced changes for 17 inter-spin distances in arrestin-2. The expected distances between spin labeled side chains were calculated using PRONOXY and the experimental DEER distances were determined from the best fit to the dipolar evolution curves (see Figs 3, 4, 5). Arr2-free indicates the basal state of arrestin-2, Arr2-V2Rpp represents the crystal structure of truncated arrestin-2 bound to the multi-phosphorylated C-terminal peptide of V2 vasopressin receptor (38) and Arr2+PR* represents arrestin-2 bound to phosphorylated, light-activated rhodopsin. Distance shows the distance change between the two residues upon PR* binding. ND, not determined.
**Table 2. Receptor binding-induced changes for 2 inter-spin distances in arrestin-3**

|                  | PRONOX Arr3-Free (Å) | DEER Arr3-Free (Å) | DEER Arr3+PR* (Å) | ΔDistance (Å) |
|------------------|----------------------|--------------------|-------------------|---------------|
| C-tail           | 15                   | 22                 | 31, 43, 54        | 4, 16, 27     |
| Finger loop      | 7                    | 22, 43             | 24, 43            | 2, 0          |

**TABLE 2.** Distance changes for two double labeled arrestin-3 mutants. Arr3-free indicates the inactive state of arrestin-3 and Arr3+PR* represents arrestin-3 upon binding to light activated and phosphorylated rhodopsin. Distance shows the distance change between the two residues upon PR* binding.
Receptor binding-induced changes in non-visual arrestins

FIGURE 1
Receptor binding-induced changes in non-visual arrestins

**FIGURE 2**

| Mr.    | wtArr2 | ctrl  | 12/392 | 192/392 | 47/257 | 81/339 | 167/340 |
|--------|--------|-------|--------|---------|--------|--------|---------|
|        | P      | S     | P      | S       | P      | S      | P       |

| Mr.    | 68/167 | 33/136 | 136/167 | 47/136 | 136/238 | 136/246 |
|--------|--------|--------|---------|--------|---------|---------|
|        | P      | S      | P       | S      | P       | S       |

| Mr.    | 47/167 | 158/167 | 192/38 | 192/339 | 234/340 | 238/339 |
|--------|--------|---------|-------|---------|---------|---------|
|        | P      | S       | P     | S       | P       | S       |
Receptor binding-induced changes in non-visual arrestins

FIGURE 3

A12C/A392C

M192C/A392C

Y47C/E257C

V81C/A339C

V167C/S340C

F
FIGURE 4

A  L68C/V167C
B  L33C/T136C
C  Y47C/T136C
D  T136C/V167C
E  T136C/Y238C
F  T136C/T246C
G

Receptor binding-induced changes in non-visual arrestins
Receptor binding-induced changes in non-visual arrestins

FIGURE 5

A  Y47C/V167C  B  I158C/V167C

C  M192C/Y238C  D  Y238C/A339C

E  S234C/S340C  F  M192C/A339C

G  [Diagram showing molecular structures]
Receptor binding-induced changes in non-visual arrestins
Identification of Receptor Binding-induced Conformational Changes in Non-visual Arrestins
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