Involvement of distinct arrestin-1 elements in binding to different functional forms of rhodopsin

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Solution NMR spectroscopy of labeled arrestin-1 was used to explore its interactions with dark-state phosphorylated rhodopsin (P-Rh), phosphorylated opsin (P-opsin), unphosphorylated light-activated rhodopsin (Rh*), and phosphorylated light-activated rhodopsin (P-Rh*). Distinct sets of arrestin-1 elements were seen to be engaged by Rh* and inactive P-Rh, which induced conformational changes that differed from those triggered by binding of P-Rh*. Although arrestin-1 affinity for P-Rh* was seen to be low (K_D > 150 μM), its affinity for P-Rh (K_D ~80 μM) was comparable to the concentration of active monomeric arrestin-1 in the outer segment, suggesting that P-Rh generated by high-gain phosphorylation is occupied by arrestin-1 under physiological conditions and will not signal upon photo-activation. Arrestin-1 was seen to bind P-Rh* and P-opsin with fairly high affinity (K_D ~50 and 800 nM, respectively), implying that arrestin-1 dissociation is triggered only upon P-opsin regeneration with 11-cis-retinal, precluding noise generated by opsin activity. Based on their observed affinity for arrestin-1, P-opsin and inactive P-Rh very likely affect the physiological monomer-dimer-tetramer equilibrium of arrestin-1, and should therefore be taken into account when modeling photoreceptor function. The data also suggested that complex formation with either P-Rh* or P-opsin results in a global transition in the conformation of arrestin-1, possibly to a dynamic molten globule-like structure. We hypothesize that this transition contributes to the mechanism that triggers preferential interactions of several signaling proteins with receptor-activated arrestins.

Arrestin-1, also known as visual or rod arrestin, was the first member of the arrestin family discovered (1). Arrestin-1 preferentially binds light-activated phosphorylated rhodopsin (P-Rh*) and prevents further signaling by direct competition with transducin, the visual G protein (2, 3). Arrestin-1 also binds dark phosphorylated (P-Rh) and light-activated unphosphorylated (Rh*) rhodopsin, albeit with much lower affinity (4–5). These interactions, as well as arrestin-1 binding to phospho-opsin (P-opsin), remain largely unexplored, despite their biological importance. Rh* is directly produced by light. P-Rh is generated by high-gain phosphorylation of multiple rhodopsin molecules upon activation of a modest subpopulation (6, 7), and P-opsin is generated by the decay of P-Rh*. Arrestin-1 is the second most abundant protein in rod photoreceptors, expressed at a ~0.8:1 M ratio to rhodopsin (8–10). In dark-adapted rods the bulk of arrestin-1 is localized away from the rhodopsin-containing outer segment. Light induces massive translocation of arrestin-1 into this compartment because of its tight binding to P-Rh* (11, 12). Mammalian arrestin-1 self-associates, and dimers and tetramers predominate at physiological concentrations (13–15). Only the arrestin-1 monomer binds rhodopsin (16). Arrestin-1 monomers, dimers, and tetramers have very different sizes, a fact of importance given that the space between rhodopsin-containing discs in the outer segment is fairly narrow (17). It appears that only monomeric arrestin-1 is small enough to readily diffuse into the interdiscal space, and this has been proposed to be the major factor limiting arrestin concentration in the outer segment under dark conditions (17). The binding of arrestin-1 to any form of rhodopsin affects the arrestin-1 distribution in rod photoreceptors and determines the concentration of the active monomer in the outer segment, which likely contributes to the kinetics of photon-response and recovery (18). Thus, to fully understand receptor signaling it is important to determine the affinities of these interactions and to identify the arrestin-1 residues involved. This determination is necessary for the elucidation of the molecular mechanisms of arrestin-1 function in the visual system and for building a comprehensive model of its behavior in photoreceptors (19). These data are also critical for the design of arrestin-1 mutants capable of compensating for the effects of rhodopsin mutations that cause congenital human disorders (20).

NMR spectroscopy is well suited for the study of dynamic and relatively low-affinity interactions (21), which constitute the majority of biologically important protein–protein and ligand–protein interactions. Here we took advantage of our recent assignment of >140 amide (H, 15N correlation resonances in the NMR spectrum of arrestin-1 (22) to probe its binding interface and affinity for different functional forms of rhodopsin.

Results

Arrestin-1 Retains Native Structure in the Presence of Anionic Bicelles. Rhodopsin must be solubilized in an isotropic model membrane system to investigate arrestin-1 binding by solution NMR. Detergent micelles (23) and lipid-detergent bicelles (24) successfully solubilize functional rhodopsin. However, detergents sometimes disrupt the native structure of arrestins. We examined arrestin-1 structure and stability using near-UV CD in the presence of different types of micelles, bicelles, and amphipols (25). Detergents and amphipols destabilized arrestin-1, whereas three different types of bicelles yielded CD spectra very similar to those observed in buffer only (Fig. S1).

Rhodopsin is stable and functional in anionic bicelles (24). Rhodopsin in bicelle-like nanodiscs containing negatively charged lipids binds arrestin-1 with a physiologically relevant affinity (K_D ~3–4 nM) (26). We acquired a 2D (H, 15N transverse relaxation optimized spectroscopy (TROSY) spectrum of 50 μM-H, 15N-labeled arrestin-1 in the presence of 4.2% (wt/vol) dimyristoylphosphatidylglycerol + diheptanoylphosphatidylcholine (DMPG/D7PC) bicelles containing 20% negatively charged dimyristoylphosphatidylglycerol (DMPG). The NMR spectrum of arrestin-1 in the presence of bicelles was similar to that in buffer (Fig. S2), indicating lack of a bicelle-induced global structural perturbation. Thus, isotropic bicelles are suitable for solution NMR-based studies of arrestin–rhodopsin interactions.

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Fig. 1. Arrestin-1 binding to dark-state P-Rh. (A) Superimposed $^1$H,$^{15}$N-TROSY spectra of 30 μM $^2$H,$^{15}$N-labeled arrestin-1 titrated by P-Rh in 4.2% anionic bicelles, 25 mM Bis-Tris, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT pH 6.5 at 308 K. The bicelles contained DMPC:DMPG = 4:1 (mol:mol), q = 0.3, where q is the mol:mol ratio of the detergent-like D7PC to DMPC + DMPG. All samples in this work were examined in the same buffer and bicelles and at the same temperature. The molar ratio of arrestin-1 to P-Rh was varied: 1:0 (black), 1:1 (green), 1:2 (red), and 1:4 (cyan). (B) Mapping of P-Rh–induced site-specific NMR chemical-shift changes onto the 3D structure of arrestin-1 (PDB ID 1CF1). Red sites are those for which the backbone amide chemical shifts changed by 0.010 ppm or more. Light blue sites are those for which peaks shifted by less than 0.010 ppm, but underwent significant line-broadening in response to P-Rh. Resonances for dark blue sites neither shifted significantly nor broadened. Because the crystal structure does not resolve residues 1–9, 363–373, and 394–404, these segments were modeled into the depicted structure using X-PLOR-NIH (http://nmr.cit.nih.gov/xplor-nih).
**Arrestin-1 Binding to Dark-State Phosphorylated Rhodopsin.** The 2D $^{1}H$–$^{15}N$ correlation NMR [heteronuclear single-quantum coherence (HSQC) or TROSY] has long been used to monitor protein-protein or protein-ligand binding and to map protein residues involved in complex formation. Arrestin-1 binds P-Rh* with high selectivity (27), and arrestin-1 residues engaged by P-Rh* have been extensively mapped (5, 28–34). In vivo, light activation, and subsequent phosphorylation of rhodopsin by GRK1 are essential for high-affinity arrestin-1 binding, which effectively shuts off further signaling (35). Both light-induced Rh* and inactive P-Rh are produced in photoreceptors in vivo (7), but bind arrestin-1 less avidly than P-Rh* (4, 5). However, Rh* and inactive P-Rh remain to be fully elucidated. Here we used NMR titrations to investigate the binding of $^{1}H$,$^{15}N$-arrestin-1 to P-Rh and Rh*.

Fig. 1A shows the titration of NMR-labeled arrestin-1 by dark P-Rh. A number of peaks broadened or shifted in response to complex formation, with many peaks broadening beyond detection at the higher levels of P-Rh. We therefore focused our analysis on the sample with a twofold molar excess of P-Rh over arrestin-1, where most peaks were still observable. The peaks shifted rather than split, which indicates that binding was rapid on the NMR time scale. The set of peaks that uniformly shifted the most in response to complex formation were from the distal C terminus of arrestin-1 (Fig. 1B and Fig. S3), indicating that binding to P-Rh changes the conformation of this element. This finding is consistent with the release of the C terminus previously detected in the presence of chemical mimics of the phosphorylated rhodopsin C terminus, heparin, and hexaphosphoinositol (IP6) (22). A few other peaks also shifted significantly or exhibited line broadening (Fig. 1B and Fig. S3). Those peaks were mainly from the “polar core” and “three-element interaction” that were previously shown to be perturbed by IP6 and heparin (22). For three peaks that exhibited relatively large shifts but did not completely disappear, we plotted the variations in chemical shifts as a function of the P-Rh concentration (Fig. S4). Fitting of the data by a 1:1 binding model (9, 26) yielded a $K_D$ of $80 \pm 30$ μM (Fig. S4) for arrestin-1 binding to P-Rh.

**Binding of Arrestin-1 to Unphosphorylated Light-Activated Rhodopsin.** Visual signal transduction is initiated when rhodopsin absorbs light, which isomerizes the 11-cis-retinal chromophore of rhodopsin into Rh*. Rh* activates transducin and is then rapidly multiphosphorylated at its C terminus (36). The structure of Rh* is significantly different from inactive rhodopsin (37–40). High selectivity of arrestin-1 for P-Rh*, compared with Rh* (5), indicates that arrestin-1 affinity for Rh* must be much lower, although this has not been previously quantitated. Fig. S5A shows overlaid spectra of 30 μM $^{1}H$,$^{15}N$-arrestin-1 with and without 150 μM Rh*. Only a few residues exhibited significant chemical shift changes (Fig. S5B and Fig. S6). Analysis of a full titration (0, 30, 60, and 150 μM Rh*) indicates that binding is relatively weak ($K_D > 150$ μM) (Fig. S7). Most resonances exhibiting significant chemical-shift changes corresponded to residues in the 10–70 and 370–385 ranges. These segments include two of three sets of residues known to be involved in the “three-element interaction” between the N-terminal β-strand I (residues 9–14), α-helix I (residues 103–111), and β-strand XX in the proximal C terminus (residues 373–380) (Fig. S5B). The fact that several of the arrestin-1 resonances involved in the three-element interaction were among the handful of resonances that became sharper upon complex formation with Rh* (Fig. S6) is consistent with an increase in local mobility, implying destabilization upon complex formation. These data suggest that the known destabilization of the three-element interaction by P-Rh* is not solely triggered by the phosphorylated rhodopsin C terminus, but that contributions to this destabilization are also made by elements of rhodopsin that change conformation upon light activation. Given that binding of P-Rh (Fig. 1) or P-Rh* (33, 41) results in release of the distal C terminus (residues 385–404), it is notable that binding of Rh* does not do so.

**High-Affinity Binding of Arrestin-1 to Phosphorylated Light-Activated Rhodopsin.** Arrestin-1 associates with P-Rh* with high affinity (26, 42, 43), blocking transducin binding sites on rhodopsin (2, 3). To probe structural changes that occur in arrestin-1 upon binding, we titrated $^{1}H$,$^{15}N$-labeled arrestin-1 with P-Rh*.

Fig. 2A shows the TROSY spectrum of 30 μM $^{1}H$,$^{15}N$-labeled arrestin-1 in the presence and absence of saturating P-Rh*. Binding of P-Rh* resulted in a dramatic change in the spectrum in which the majority of peaks disappeared (Fig. 2B). The remaining peaks were assigned by tracing the chemical shift changes seen in Fig. 1, which appeared to shift from their positions in free arrestin-1 in the same direction upon complex formation with either P-Rh or P-Rh*. The assignments (Fig. 2B) reveal that the observed arrestin-1 peaks originate from domains shown to be particularly flexible in free arrestin-1 (33, 41), particularly the C-terminal residues 360–404 (see structure in Fig. S8A). Several residues in the body of the N domain (G95, A96,
Rh* was determined to be 49 μM labeled arrestin-1, but no P-Rh*. The light-blue spectrum corresponds to a sample containing both 30 μM labeled arrestin-1 and 90 μM unlabeled P-Rh*.

S97, and G98) were also observed, as well as the N-terminal residues K5, A7, and A10. Resonances from most other peaks disappeared upon complex formation because of extensive line broadening. The high intensity of the peaks from the C terminus after complex formation suggests that binding of arrestin-1 to P-Rh* results in freeing of the C terminus of arrestin-1 from tertiary structural interactions, in agreement with distance measurements between the C terminus and the body of the N domain by electron paramagnetic resonance (EPR) and limited proteolysis (33, 41, 44) and also by NMR paramagnetic relaxation experiments (SI Text and Table S1). P-Rh* induced chemical-shift changes for residues G95, S97, and G98, which are located on a short connector between the body of the N domain and the α-helix I (Fig. S8A). Thus, a structural rearrangement of the N domain takes place upon complex formation. This effect was not seen in the titration of arrestin-1 with IP6 or heparin (22), suggesting a possible shift of the α-helix I resulting from disruption of the three-element interaction. Our data confirm that formation of the complex results in a conformational change in the C terminus that enhances its local motional freedom.

Because light-activated rhodopsin decays faster than equilibrium can be reached, a true $K_D$ cannot be determined in a simple titration experiment. The apparent $K_D$ for arrestin-1 binding to P-Rh* was determined to be 49 ± 11 nM (Fig. 2C), which is similar to the 25- to 50-nM value previously measured using an extra-Meta II assay (42, 43). Some comment on the stoichiometry of the complex is merited. Monomeric rhodopsin was shown to be necessary and sufficient for high-affinity arrestin-1 binding (26, 45). However, recent reports suggest that two types of arrestin-1-rhodopsin complexes are formed upon activation of a high fraction of rhodopsin in native disk membranes, with 1:1 and 1:2 arrestin: rhodopsin stoichiometries (46, 47). In the latter, arrestin-1 apparently binds only one rhodopsin with high affinity, stabilizing its Meta II state (46) and trapping all-transretinal (47), whereas the other molecule is engaged with much lower affinity by distinct arrestin-1 elements localized in the C-domain (47). All forms of rhodopsin used here were solubilized by a large excess of bicelles and most of the perturbations were observed to be in the N-domain of arrestin-1 and in the C terminus that is anchored to this part of the molecule (Figs. 1 and 2). These data point to a 1:1 stoichiometry, making it highly unlikely that “auxiliary” interaction with a second rhodopsin contributed to our results.

Binding to P-Rh* Induces a Major Change in the Global Dynamics of Arrestin-1. Most arrestin-1 peaks disappear upon binding to P-Rh* (Fig. 2B). A priori, this phenomenon could be a result of the large correlation time associated with the ~200-kDa size of the arrestin/P-Rh*/bicelle complex. Alternatively, complex formation might result in a global change of conformational dynamics within arrestin-1, with the induced motions occurring in the intermediate rate regime (msec−1−μsec−1) of the NMR time scale. To determine which line-broadening mechanism actually pertains, we collected 1H,13C-methyl-TROSY spectra of ILV-13C-methyl–protonated arrestin-1 in the presence and absence of P-Rh*. It is well established that high-quality methyl-TROSY spectra can be acquired even for complexes with molecular weights well in excess of 200 kDa (48). However, our data (Fig. 3) revealed the same general disappearance of peaks in the 1H,13C-methyl-TROSY spectrum of backbone amide peaks. This finding indicates that NMR peak disappearance for arrestin-1 upon binding to P-Rh* is most likely a result of widespread conformational exchange in the intermediate rate regime, leading to extensive exchange-broadening. This is not an unusual phenomenon in protein structure and dynamics: widespread exchange-broadening is a hallmark of proteins in molten globule-like conformational states (49). Binding of P-Rh* apparently induces transition of arrestin-1 to such a state, which evidently encompasses both its N and C domains.

Comparison of the Binding Affinity, Structure, and Dynamics of Arrestin-1 Bound to Phosphorylated Opsin (P-Opsin) vs. P-Rh*. The regeneration of rhodopsin following photoactivation requires the release of all-transretinal, which occurs while arrestin-1 is still associated with P-Rh*. Previous studies indicated that arrestin-1 binds retinal-free phosphorylated opsin (5); however, it has not been clear whether the conversion of P-Rh* to P-opsin induces additional changes in bound arrestin-1 or triggers arrestin-1 release of all-transretinal, which occurs while arrestin-1 is still associated with P-opsin. Previous studies indicated that arrestin-1 binds only one rhodopsin with high affinity, stabilizing its Meta II state (42, 43). Thus, a structural rearrangement of the N domain takes place upon complex formation. This effect was not seen in the titration of arrestin-1 with IP6 or heparin (22), suggesting a possible shift of the α-helix I resulting from disruption of the three-element interaction. Our data confirm that formation of the complex results in a conformational change in the C terminus that enhances its local motional freedom.

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that arises from a global intermediate time-scale conformational exchange peak broadening in TROSY spectra, it is unlikely that this result is primarily because of local conformational changes induced by these interactions. Here we used NMR to identify arrestin-1 elements engaged by nonpreferred forms of rhodopsin and to probe the nature of conformational changes induced by these interactions. The fact that these solution NMR studies focused on complex formation between a large soluble protein and a large membrane protein, and were carried out using only 30 μM arrestin-1, also exemplifies the advances in NMR sensitivity in recent years, thanks to the availability of very high-field magnets, exploitation of TROSY spin physics (48, 53), and cryoprobe detectors (54).

We found that binding to Rh* affects the N terminus and proximal C terminus (residues 370–385) of arrestin-1 (Figs. S5 and S6). Rh* binding did not result in the freeing of the distal C terminus (residues 385–404), in contrast to binding of the phosphorylated forms of rhodopsin (Figs. 1 and Fig. S8). This finding provides evidence that a unique conformational change in arrestin is induced by Rh*. Significant chemical-shift changes of residues in the 10–70 and 370–385 ranges, along with sharpening of several peaks upon complex formation (Fig. S6), suggest that active unphosphorylated receptors destabilize the three-element interaction previously believed to be disrupted solely by receptor-attached phosphates (55). The data also indicate that the proximal C terminus can be perturbed without the displacement of the distal C terminus. This possibility has not been previously considered (19). Finally, our estimate of arrestin-1 affinity for Rh* (K_D > 150 μM) is consistent with physiological data that wild-type arrestin-1 cannot effectively quench Rh* signaling (20, 35, 36).

We found that both dark P-Rh and P-opsin displace the distal C terminus of arrestin-1 (Fig. 1 and Fig. S8), similar to P-Rh* (33, 41) and the phospho-mimics, heparin and IP6 (22). These data show that receptor-attached phosphates are necessary and sufficient for this conformational rearrangement. The affinity of arrestin-1 for dark P-Rh measured in this work (K_D ∼ 80 μM) is comparable to the arrestin-1 homodimerization constant (12, 15). This finding suggests that interaction with P-Rh can significantly affect the arrestin-1 monomer-dimer-tetramer equilibrium in photoreceptors. Only the monomeric form of arrestin-1 binds rhodopsin (15). The concentration of arrestin-1 monomer in the outer segments, calculated based on its distribution in rods and its self-association constants (12), yielded values that are insufficient to explain the unexpectedly short lifetime of active rhodopsin (18). Low-affinity binding of arrestin-1 to additional partners, such as nonpreferred forms of rhodopsin, would increase the supply of arrestin-1 monomer available to P-Rh*, reconciling biochemical and physiological evidence.

Complex formation between arrestin-1 and P-Rh* was seen to result in widespread NMR peak disappearance, with the exception of peaks from highly mobile elements (N-terminal 10 residues, sites 95–98, and the distal C terminus). Because peak loss was seen in both 1H,15N-TROSY and in 1H,13C-methyl-TROSY spectra, it is unlikely that this result is primarily because of the size of the complex. A much more likely explanation is intermediate time-scale conformational exchange peak broadening that arises from a global “loosening” of the arrestin-1 conformation.

Although the results do not prove this conclusively, the data are consistent with the notion that the global conformation of arrestin-1 is destabilized by high-affinity binding to P-Rh*, triggering a transition into a molten globule-like structure. High flexibility of both free and rhodopsin-bound arrestin-1 is consistent with rapid H/D exchange observed in both states (28), as well as with the relatively wide distance distributions recently detected in both states by pulsed EPR (57). Based on these observations for arrestin-1, it can be hypothesized that a transition from a flexible but well-ordered structure to a partially disordered ensemble of conformations might be the central mechanism underlying the “activation” of other arrestins (27), which enables association with many signaling proteins. This finding would explain how activation of a given arrestin by a given receptor can result in distinct signaling complexes, leading to different functional outcomes (58, 59).

Previously, receptor binding-induced conformational changes in arrestin have been proposed to involve the movement of the two arrestin domains relative to each other (27), as suggested by progressive reduction of receptor binding by deletions in the interdomain hinge (60). However, the recently reported absence of large changes in the interdomain distances in arrestin-1 upon binding to P-Rh* (57) argues against this model. Our data reveal only localized conformational rearrangements upon binding of arrestin-1 to dark P-Rh (Fig. 1) and Rh* (Fig. S5). For P-Rh* and P-opsin, our data suggest a major change in arrestin-1 dynamics as described above, but does not inform on the nature of the associated conformational changes (Figs. 2 and 3).

Finally, we also found that P-opsin binds to arrestin-1 with relatively high affinity (K_D = 780 nM) and induces changes in its structure and dynamics that are similar to those induced by P-Rh*. These data suggest that it is the regeneration of rhodopsin with 11-cis-retinal, rather than the loss of all-trans-retinal by P-Rh*, as previously proposed (50), that promotes arrestin-1 dissociation. Considering that opsins can activate transducin, delayed arrestin-1 release makes perfect biological sense in rods, where high sensitivity requires efficient noise suppression (61).

Methods

Preparation of Arrestin-1 Samples. Constitutively monomeric arrestin-1 (FFBSA/F197A) was expressed in isotopically labeled form and purified as previously described (22, 62). Near-UV CD spectroscopy of arrestin-1 and preparation of various forms of rhodopsin are described in SI Text.

NMR Spectroscopy of Arrestin-1. 1H,15N-TROSY NMR titrations of 1H,13C-labeled arrestin-1 with various forms of rhodopsin were carried out at pH 6.5 and 308 K in the presence of 100 mM NaCl. Samples with different ratios of arrestin-1 and rhodopsin were made using a constant arrestin-1 concentration of 30 μM. 1H,13C-Me-TROSY NMR spectra (48) were collected of arrestin-1 that was uniformly perdeuterated, except for the side-chain methyl groups of isoleucine, leucine, and valine residues, which were also 13C-labeled. Additional details are provided in SI Text.

Determination of Dissociation Constants. A 1:1 binding model (SI Methods) was fit to NMR titration data to estimate K_D for complex formation of arrestin-1 with various forms of rhodopsin. To determine the affinity by an alternative method in some cases, we used direct binding assay with radiolabeled arrestin-1 generated in cell-free translation, as previously described (4, 62). Increasing amounts of P-Rh* or P-opsin were added to a fixed concentration of arrestin-1 and the amount of bound arrestin was determined upon incubation at 37 °C for 5 min (P-Rh*) or for 30 min (P-opsin). Because true equilibrium cannot be achieved with P-Rh* as a result of its decay, these data yielded an apparent K_D, whereas P-opsin binding was measured at equilibrium, yielding a true K_D.

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Supporting Information

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SI Results

Structural Integrity of Arrestin-1 in Various Media as Determined by Near-UV CD Spectroscopy. Arrestin-1 has 16 phenylalanines, 1 tryptophan, and 14 tyrosines, all of which contribute to its near-UV CD spectrum. The spectrum of arrestin-1 in native conformation in neutral buffer exhibits pronounced negative signals in the 270–290 nm range, whereas the spectrum of denatured protein in 8 M urea is near zero in this range (Fig. S1). We found that the presence of dodecylphosphocholine (DPC) or lyso-myristoylphosphatidylcholine (LMPG) micelles induced rapid precipitation of arrestin-1. Near-UV CD spectra were compared for arrestin-1 under native conditions (Fig. S1, red spectra), upon denaturing with 8 M urea (Fig. S1, green spectra), and in the presence of different membrane mimics. In the presence of 0.1% lyso-myristoylphosphatidylglycerol (LMPG) or amphiphils (Fig. S1 B and C), CD spectra that are vaguely similar to that of arrestin-1 under physiological conditions were obtained, but significant loss of CD signal intensity in the 260–290 nm range was observed, suggestive of destabilization of ordered tertiary structure. In the presence of 0.2% β-n-decyl maltoside (DM) micelles, the CD spectral intensity from 265 to 290 nm actually increased, suggestive of a change or enhancement of tertiary structure. In contrast, three different types of bicelles (Fig. S1 L4) yielded CD spectra that were very similar to the spectrum of arrestin-1 under physiological conditions. These three bicelle systems were: (i) purely zwitterionic 2.5% (wt/vol) dimyristoylphosphatidylcholine + diheptanoylphosphatidylcholine (DMPC/D7PC) bicelles (q = 0.3, where q is the lipid-to-detergent mole:mole ratio, D7PC being the detergent component); (ii) net negatively charged 2.5% (wt/vol) DMPC/D7PC bicelles with 20 lipid-mol% dimyristoylphosphatidylglycerol (DMPG) (DMPC:DMPG = 4:1, q = 0.5); and (iii) positively charged 2.5% DMPC/D7PC bicelles with 20 lipid-mol% hexadecyltrimethylammonium bromide (HTAB) (DMPC:HTAB = 4:1, q = 0.3). Arrestin-1 was equally structurally-ordered in all three bicelle systems (Fig. L4), identifying isotropic bicelles as a suitable medium for solution NMR-based studies of arrestin-1/rhodopsin interactions.

NMR Confirms the Release of Arrestin-1 C-Tail upon Rhodopsin Binding. Both limited proteolysis (1) and electron paramagnetic resonance (EPR) distance measurements (2, 3) indicated that the C-tail of arrestin-1 undergoes major conformational change upon receptor binding. To confirm the structural nature of these changes we collected NMR paramagnetic-relaxation enhancement (PRE) data to provide additional insight. A nitroxide spin label was introduced at site 16 of arrestin-1 and its impact on the intensities (reflected in line widths) of the C-tail NMR resonances was measured before and after binding to phosphorylated light-activated rhodopsin (P-Rh*) (Table S1). In free arrestin-1 the spin label induced significant line broadening for most of the C-tail resonances, indicating that they reside within 25 Å of the side chain of residue 16. However, little or no spin label-induced line broadening was detected following P-Rh* binding, indicating the backbone amides of the C-tail are now more than 25 Å away from the spin label. This result is consistent with the previous finding that the negatively charged C-tail is displaced from its basal association with the basic region in the N domain (4).

SI Methods

Preparation and Spin-Labeling of the I16C Mutant Form of Arrestin-1. A single cysteine was inserted at position 16 (I16C) of the oligomerization-resistant cysteine-less arrestin-1 mutant (C63A, C128S, C143S, F85A, and F197A) using a Qiagen Quickchange kit. The forward primer was 5′-CCAGGTCATCTTAAAAAGTGCTCCCGTGATAATCGTGACCC-3′ and the reverse primer was 5′-GGTCACCGATTTATCACGGGACACTTTTTAAAGATCGTGACGGTG-3′. Purified arrestin-1 samples were stored for short periods at 4°C in Tris buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM DTT, and 1 mM EDTA) and frozen at −80 °C for long-term storage. NMR samples were prepared by changing the buffer to Bis·Tris (25 mM Bis·Tris, 100 mM NaCl, 5 mM DTT, and 0.1 mM EDTA pH 6.5) using an ultrafiltration concentrator [Millipore; molecular weight cut-off (MWCO) = 30 kDa].

For spin labeling of the I16C arrestin-1 single-cysteine mutant, DTT was removed by exchanging the buffer to a degassed reductant-free Bis·Tris solution (25 mM Bis·Tris, 100 mM NaCl, and 0.1 mM EDTA pH 6.5) using a centrifugal ultrafiltration cartridge (Millipore; MWCO = 30 kDa). Protein samples were then diluted using reductant-free Bis·Tris buffer to a final concentration of 0.5 mg/mL, followed by mixing with a 10-fold molar excess of the sulfhydryl-specific spin label reagent 2,2,5,5-tetramethylpyrroline-3-y1-methanethiosulfonate (MTSL; Toronto Research Chemical). The mixture was incubated under argon covered with aluminum foil at 4°C overnight and the unreacted MTSL was removed by buffer exchange using centrifugal ultrafiltration (Millipore filter, MWCO = 30 kDa). The final NMR sample was prepared in the reductant-free Bis·Tris solution, maintaining a low arrestin-1 concentration (30–50 μM) to minimize dimerization. Following collection of a transverse relaxation optimized spectroscopy (TROSY) NMR spectrum, the paramagnetic spin label was reduced to its diamagnetic form by adding ascorbic acid to 5 mM and incubating at 37°C for 2 h. This process was followed by collection of another TROSY NMR spectrum using identical parameters used for the paramagnetic sample. Both paramagnetic and diamagnetic samples were also subjected to continuous wave EPR spectroscopy to confirm the completeness of spin-labeling and of reduction to the diamagnetic form, respectively. Typically, the spin labeling efficiency was >95%, with the efficiency of reduction being ~100%.

Preparation of Rhodopsin, Phosphorylated Rhodopsin, and Phosphorylated Opsin in Bicelles. The preparation of phosphorylated rhodopsin (P-Rh) includes four major steps: isolation of retinal outer segments (ROS), phosphorylation, regeneration, and urea stripping. All operations were carried out under dim red light unless otherwise indicated. ROS were enriched by sucrose gradient from 50 frozen bovine retinas (purchased from W. L. Lawson Co.) as previously described (5). After sonication, the ROS pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4) to a final rhodopsin concentration of 0.5 mg/mL. The rhodopsin suspension was incubated under bright light for 240 min in the presence of 3 mM ATP, 3 mM GTP, and 5 mM MgCl₂ to initiate rhodopsin phosphorylation by endogenous G protein-coupled receptor kinase 1 (GRK1; also known as rhodopsin kinase). This process yielded rhodopsin with high phosphorylation levels (6), which ensures high-affinity arrestin binding (7). Rhodopsin was fully regenerated by 11-cis-retinal (two additions of threefold molar excess) in 100 mM potassium phosphate buffer, pH 7.4, supplemented with 2% (wt/vol) BSA, 1 mM MgCl₂, and 0.5 mM EDTA for 1 h at room temperature (5). After regeneration, 5 M urea was added to remove peripherally associated soluble proteins from the membranes and the
pellets were washed five times with 50 mM Tris•HCl, pH 7.5, 1 mM EDTA, and resuspended in this buffer.

For solubilization with bicelles, regenerated rhodopsin was pelleted by centrifugation at 14,000 × g for 30 min and solubilized by the addition of 12.5% (wt/vol) anionic bicelles (q = 0.3, DMPC:DMPG = 4:1) and incubated for 2 h at 0 °C. The sample was centrifuged at 14,000 × g for 30 min to remove insoluble material. The concentration of dark P-Rh was measured by light absorption at 500 nm (molar extinction coefficient ε = 40,600 M−1 cm−1) using an Olis DW2000 dual-beam spectrophotometer adapted for a dark room. A typical rhodopsin reconstitution was performed by mixing 150 μL 12.5% (wt/vol) anionic bicelles with ROS membrane containing 3 mg rhodopsin. The recovery of rhodopsin was around 90% and the final bicelle concentration was kept within a 6-8% range. The amount of the native lipids solubilized from ROS membranes were considered to be negligible as assessed by 1D proton NMR spectroscopy.

Rhodopsin in bicelles was prepared as described for P-Rh, but without the rhodopsin phosphorylation step and subsequent regeneration. Dark nonphosphorylated rhodopsin in ROS was solubilized with bicelles and mixed with arrestin-1. To generate the light-activated Rh* and P-Rh*, the samples were bleached under bright light until the absorbance peak at ~500 nm completely disappeared. Because of the high concentration of rhodopsin in samples complete bleaching required a number of hours (overnight). Therefore, we cannot exclude the formation of other photoproducts in addition to Meta II, including opsin. However, because rhodopsin was bleached in the presence of arrestin-1, which is known to stabilize Meta II similar to transducin, and because even the C-terminal transducin peptide shifts arrestin-1, which is known to stabilize Meta II similar to transducin, and because even the C-terminal transducin peptide shifts arrestin-1 was also obtained for 50 μM arrestin-1 in Bis-Tris buffer (25 mM Bis-Tris, 100 mM NaCl, 0.1 mM EDTA, and pH 6.5). A spectrum of unfolded arrestin-1 was also obtained for 50 μM arrestin-1 in the same buffer plus 8 M urea. Near-UV CD spectra of 50 μM arrestin-1 were also obtained in the presence of detergents: (i) 0.1% LMPG and (ii) 0.2% DM. Bicelle mixtures tested were: (i) zwitterionic: 2.5% (wt/vol) D7PC/DMPC, q = 0.3 (mole ratio D7PC/DMPC = 33:1); (ii) anionic: 2.5% DMPC/D7PC/DMPG (DMPC:DMPG = 4:1) q = 0.3 [D7PC(DMPC+DMPG) = 33:1]; and (iii) cationic: 2.5% (wt/vol) D7PC/D7PC/HTAB (D7PC:HTAB = 4:1) q = 0.3 [D7PC(D7PC+HTAB) = 33:1]. Amphiphilic polymers (“amphipols”) tested were: (i) 0.2% PMAL-C8, (ii) 0.2% PMAL-C12, and (iii) 0.2% PMAL-C16.

Near-UV CD spectra were acquired after the proteins had been premixed with detergents, bicelles or amphipols for 4–5 d at room temperature. Detergents and amphipols were purchased from Avanti Polar Lipids.

Preparation of Arrestin-1 That Is Perdeuterated Except for Side-Chain Methyl Groups of Leucine, Isoleucine, and Valine, Which Are Also Labeled. Precursors “A” (α-ketovaleric acid salt, 3-methyl-13C, 3,4,4,4-D4) and “B” (α-ketobutyric acid salt, methyl-13C, 3,3-D2) were purchased from Cambridge Isotopes (catalog nos. CDLM-7317–0 and CDLM-7318–0, respectively).

Escherichia coli cells harboring an inducible plasmid that encodes the F85A/F197A (tetramerization-resistant) mutant form of arrestin-1 were used to inoculate 5 mL of a Luria broth (LB) prepared using D-O. The cell were grown overnight at 37 °C. From these cultures we then removed 50 μL and inoculated 5–10 mL of medium that contains perdeuterated glucose and D2O. Cultures were then grown at 30 °C until culture appeared turbid. Each 5-mL culture was then used to inoculate 500 mL (or 1L) of minimal medium that contains perdeuterated glucose, D2O, and precursors A and B (above). Cultures were shaken at 30 °C until OD600 reached 0.6, at which point additional precursors were added (additional A to 75 mg/L and B to 45 mg/L). Shaking of cultures was then continued for 30 min at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 25 μM to induce expression of arrestin-1. Shaking was then continued for 18 h, followed by cell harvesting. Labeled arrestin-1 was then purified and prepared for NMR using the standard procedure.

NMR Experiments. NMR experiments were performed using Bruker Avance 800-MHz and 600-MHz spectrometers equipped with cryogenic triple resonance probes with z-axis pulse-field gradients. A sensitivity-enhanced, phase-sensitive TROSY pulse sequence was used to acquire all 1H–15N correlated 2D spectra (8). Because of the low concentration of the arrestin-1 samples used in this study (30–50 μM), most NMR spectra were acquired using 2,048 × 128 complex points with 200 scans per increment, which result in a typical 20-h acquisition time. For samples containing 15N-arrestin-1 in the presence of excess Rh* or P-Rh*, we conducted experiments in which 2-h TROSY spectra were collected before and after 20-h data collection. In the case of both complexes, the intensity of the arrestin-1 peaks in the 2-h TROSY spectra collected after the 20-h run were weaker than before the 20-h run. Average loss of peak intensity was about 40% for the Rh* complex and about 70% for the P-Rh* complex. However, with a few only exceptions, new peaks did not appear in the “after” spectra relative to the “before” spectra. This result suggests that the arrestin-1 complexes with Rh* or P-Rh* do undergo some sort of time-dependent loss of integrity (most likely formation of very large aggregates). Although this sample loss will attenuate peak intensities in the 20-h TROSY spectra reported in this article, this does not impact peak positions or impact the interpretation of these spectra as presented in the main text or SI Text.

All spectra were acquired at 308 K with temperature pre-calibrated using deuterated methanol. All NMR data were processed using nmrPipe (9) with Gaussian apodization, linear prediction, and zero filling. Peak intensities for determination of paramagnetic relaxation enhancements were measured using Sparky (10).

The 2D methyl-TROSY experiment was run using the 1H,13C-HMOC pulse sequence. The 1,024 × 512 complex points covering spectral widths of 14 × 22 ppm (1H and 13C, respectively) were acquired with 128 scans per each increment, which resulted in a 20-h acquisition time. The acquired spectra were processed with one-time zero filling with a squared sine-bell apodization function.
The 1:1 Binding Model for Fitting NMR Titration Data For the Arrestin-1 Complexes with P-Rh and Rh*.

Binding of P-Rh and Rh* to arrestin-1 occurs on the rapid exchange time scale on the NMR time scale. Under conditions of rapid exchange, complex formation between molecules can result in changes in NMR peak positions for the observed molecule, which reflects the population-weighted average peaks between free and complexed populations. In this case, the observed change in chemical shift (or resonance frequency) in “molecule A” (arrestin-1) induced by complex formation with “molecule B” (P-Rh or Rh*) is:

\[
\text{observed change in chemical shift} = \frac{[B]^\ast \text{ maximum change in chemical shift}}{(K_D + [B])}, \quad [S1]
\]

where \([B]\) is the free concentration of molecule B and the “maximum change in chemical shift” is the change in chemical shift that occurs when molecule A is fully saturated with molecule B. It is often reasonable to assume that the free B concentration equals the total (free + complexed) B concentration. However, that is not the case for the titrations of arrestin-1 (molecule A) with P-Rh (molecule B). In this case the following equation gives the free \([B]\) concentration:

\[
\text{free } [B] = \left\{ \frac{[B]_{\text{total}} - [A]_{\text{total}} - K_D + \left( [B]_{\text{total}} - [A]_{\text{total}} - K_D \right)^2 + 4 \ast K_D \ast [B]_{\text{total}}}{2} \right\}^{1/2} \quad [S2]
\]

For nonlinear least-squares analysis, Eq. [S2] is plugged into Eq. [S1] and the resulting equation is then fit to the experimental data to obtain a best fit and estimates of \(K_D\) and the maximum change in chemical shift for the peak in question.


Fig. S1. Near-UV CD spectra of arrestin-1 in the presence of NMR-compatible model membranes. (A) Arrestin-1 (50 μM) samples were prepared in zwitterionic bicelles (purple), cationic bicelles (blue), and anionic bicelles (black). The reference spectrum for a sample in buffer is colored in red. The spectrum of denatured arrestin-1 in 8 M urea is colored in green. Exact sample compositions are described in SI Methods. (B) The reference spectrum for a sample in buffer-only is colored in red. The spectrum of denatured arrestin-1 in 8 M urea is colored in green. Spectra of arrestin-1 in detergent micelles composed of LMPG (purple) or DM (blue). (C) Spectra of arrestin-1 in zwitterionic amphipols composed of PMAL-C8 (purple), PMAL-C12 (blue), and PMAL-C16 (black). All samples contained 50 μM arrestin-1 at pH 6.5 and 298 K.
Fig. S2. $^1$H,$^{15}$N-TROSY NMR spectra of uniformly-$^2$H,$^{15}$N-labeled arrestin-1 in the absence (black) and presence (red) of 4% (wt/vol) anionic bicelles. Samples were prepared in 10% D$_2$O (in H$_2$O) containing 25 mM Bis-Tris, 100 mM NaCl, 0.1 mM EDTA, and 5 mM DTT, pH 6.5. Under these conditions all amide protons had been back-exchanged from $^2$H to $^1$H. Both spectra were acquired at 308 K.
Fig. S3. Absolute values of changes in TROSY resonance chemical shifts induced by binding of P-Rh to arrestin-1 at 1:2 arrestin-1:P-Rh (mole:mole). At a 1:4 arrestin-1:P-Rh ratio many additional peaks broaden beyond detection (Fig. 1A). Assigned sites where arrestin-1 peaks disappeared in the presence of P-Rh are indicated by a small, negative red bars, whereas sites for which the binding of P-Rh induced significant line broadening are marked with asterisks. The horizontal red line is the cut-off for statistical significance (changes higher than 0.010 ppm were considered to be significant). The samples contained 30 μM 2H,15N-labeled arrestin-1 ± P-Rh in 4.2% (wt/vol) anionic bicelles, 25 mM Bis-Tris, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT pH 6.5 at 308 K. The bicelles in all samples in this work contained DMPC:DMPG = 4:1 (mol:mol), q = 0.3, where q is the mol:mol ratio of the detergent-like D7PC to DMPC+DMPG.

Fig. S4. Estimation of K_D for binding of arrestin-1 to dark state P-Rh. Changes in the TROSY NMR chemical shifts of arrestin-1 (Fig. 1A) for the resonances from K392 (blue), A401 (black), and E399 (red) were plotted as a function of the P-Rh concentration. The K_D for each curve was determined by fitting the model for 1:1 noncooperative binding to the data using nonlinear least squares analysis (see Eqs. S1 and S2). Based on the three individual fits, the K_D is estimated as 80 ± 30 μM.
**Fig. S5.** Arrestin-1 binding to Rh*. TROSY spectra of 30 μM ²H,¹⁵N-labeled arrestin-1 were acquired in the presence of bicelles containing varying Rh*. (A) (black spectrum) Arrestin-1 in absence of Rh*. Only very small chemical-shift changes were observed in the presence of a fivefold molar excess of Rh* (red spectrum). (B) Mapping of Rh*-induced site-specific NMR chemical-shift changes onto the 3D structure of arrestin-1 (PDB ID 1CF1). Red sites are those for which the backbone amide chemical shifts changed by 0.010 ppm or more. Light-blue sites are those for which peaks shifted by less than 0.010 ppm, but underwent significant line-broadening in response to Rh*. Resonances for dark blue sites neither shifted significantly nor broadened.
Fig. S6. Absolute values of the changes in arrestin-1 NMR chemical shifts for assigned residues induced by the presence of a fivefold molar excess of Rh*. The residues exhibiting significant line broadening are labeled with asterisks. The residues exhibiting significant line sharpening are labeled with dots. The horizontal red line is the cut-off for statistical significance (changes higher than 0.005 ppm were considered to be significant). As shown in Fig. S5B, TROSY spectra of 30 μM 2H, 15N-labeled arrestin-1 were acquired in the absence and presence of 5x Rh* (mole:mole) at 308 K. Samples contained 25 mM Bis-Tris, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, and 4% (wt/vol) anionic bicelles, pH 6.5.

Fig. S7. Estimation of $K_D$ for arrestin-1 binding to Rh*. The dose-dependent chemical-shift changes in arrestin-1 peaks positions (from Fig. S5) were plotted as a function of the concentration of Rh* for residues K386 (blue), F375 (red), and H10 (black). The $K_D$ for each curve was determined by fitting the model for 1:1 noncooperative binding to the data using nonlinear least squares analysis (Eq. S1). The large uncertainties associated with two of three fits plus the fact that the lowest of the three $K_D$ was determined to be larger than the highest Rh* concentration reached in the titration leads to the conclusion that $K_D$ is very reliably determined to be larger than 0.15 mM by these data.
Fig. S8. (A) Location of sites in the arrestin-1 structure that yield visible TROSY NMR resonances following formation of a complex with P-Rh*. Red sites in the arrestin-1 structure (PDB ID 1CF1) are those for which resonances are observable in the TROSY NMR spectrum of arrestin-1 following complex formation with P-Rh* (Fig. 2B). (B) Comparison of the NMR spectrum for arrestin-1 complexed with P-opsin versus its spectrum when complexed with P-Rh*. The TROSY spectrum of 30 μM H, 15N-labeled arrestin-1 in the presence of 90 μM P-opsin is shown in red. Samples contained 25 mM Bis-Tris buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 5 mM NH₂OH, and 4% (wt/vol) anionic bicelles pH 6.5. The spectrum was acquired at 308 K. The spectrum of 30 μM arrestin-1 in the presence of 60 μM P-Rh* (black) was collected under identical conditions.
Table S1. Impact of complex formation with P-Rh* on arrestin-1 PREs between spin-labeled residue 16 and the distal C terminus

**Paramagnetic relaxation enhancement of resonances from the arrestin-1 C-tail**

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<th>Residue</th>
<th>No P-Rh*</th>
<th>3x P-Rh*</th>
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<td>E404</td>
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Intensity ratios are given for each TROSY NMR peak under conditions where a nitroxide spin label is present (intensity = $I_p$) at residue 16 relative to conditions in which the spin label was converted to a diamagnetic form (intensity = $I_0$). Measurements were carried out for 30 μM arrestin-1 in bicelles in the absence or presence of 90 μM P-Rh* at pH 6.5 and 308 K. N/A: Measurement could not be made because peak intensity was too low under nonparamagnetic conditions.