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# Robust Self-Association Is a Common Feature of Mammalian Visual Arrestin-1

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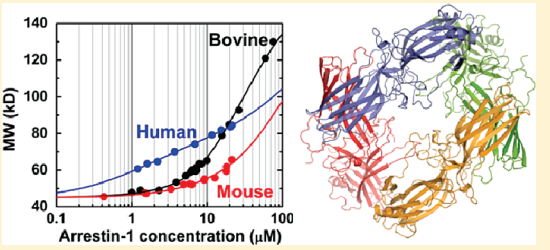
**ABSTRACT:** Arrestin-1 binds light-activated phosphorhodopsin and ensures rapid signal termination. Its deficiency in humans and mice results in prolonged signaling and rod degeneration. However, most of the biochemical studies were performed on bovine arrestin-1, which was shown to self-associate forming dimers and tetramers, although only the monomer binds rhodopsin. It is unclear whether self-association is a property of arrestin-1 in all mammals or a specific feature of bovine protein. To address this issue, we compared self-association parameters of purified human and mouse arrestin-1 with those of its bovine counterpart using multiangle light scattering. We found that mouse and human arrestin-1 also robustly self-associate, existing in a monomer−dimer−tetramer equilibrium. Interestingly, the combination of dimerization and tetramerization constants in these three species is strikingly different. While tetramerization of bovine arrestin-1 is highly cooperative ($K_{D,dim}{}^4 > K_{D,tet}$), $K_{D,dim} \sim K_{D,tet}$ in the mouse form and $K_{D,dim} \ll K_{D,tet}$ in the human form. Importantly, in all three species at very high physiological concentrations of arrestin-1 in rod photoreceptors, most of it is predicted to exist in oligomeric form, with a relatively low concentration of the free monomer. Thus, it appears that maintenance of low levels of the active monomer is the biological role of arrestin-1 self-association.




Arrestin-1[a] binds light-activated rhodopsin phosphorylated by GRK1 (also known as rhodopsin kinase) with high affinity,[1] ensuring the termination of light-induced signaling with subsecond kinetics.[2] Arrestin-1 knockout in mice dramatically slows the photoresponse shutoff in rod[3] and cone[4] photoreceptors. Arrestin-1 deficiency in humans results in Oguchi disease, a form of stationary night blindness.[5] Arrestin-1 is expressed at very high levels in both photoreceptor types, being the second most abundant signaling protein after the corresponding opsin.[4,6,7] Considering that the rhodopsin concentration in rod outer segments (OS) is ~3 mM,[8] the average cytoplasmic concentration of arrestin-1 (which is expressed at an ~0.8:1 ratio with respect to to rhodopsin[6,7,9]) is expected to be >1 mM.[1] Dark-adapted rods are used in most studies of the molecular mechanisms of rod signaling in genetically modified mice (reviewed in refs 10−12). In the dark, ~85% of arrestin-1 resides in the inner segments, cell bodies, and synaptic termini,[6,9,13−15] which brings its concentration in these compartments to >2 mM.[9] While the majority of the functional studies were performed in mice and humans, the biochemical properties of arrestin-1 were mostly studied using bovine protein. Bovine arrestin-1 robustly self-associates,[16,17] cooperatively forming dimers and tetramers.[16,18,19] It is unclear whether this is a peculiar property of bovine protein or a common feature of mammalian arrestin-1 species. In addition, because only monomeric arrestin-1 binds rhodopsin and quenches the signaling,[18] the concentration of the monomer is an important functional parameter; it can be calculated on the basis of total arrestin-1 if the self-association constant(s) are known. In view of the therapeutic potential of "enhanced" mutants that can compensate for deficits of rhodopsin phosphorylation in vivo,[20] characterization of human arrestin-1 is particularly important. Therefore, we explored oligomerization of purified mouse and human arrestin-1 and found that self-association is a common feature of mammalian arrestin-1. Interestingly, we found that while the values of dimerization and tetramerization constants of arrestin-1 from three species are very different, the underlying molecular interactions appear to be similar: the same point mutations render bovine and mouse arrestin-1 constitutively monomeric.

## MATERIALS AND METHODS

**Materials.** [$\gamma$-$^{32}$P]ATP, [$^{14}$C]leucine, and [$^{3}$H]leucine were purchased from Perkin-Elmer NEN. All restriction enzymes were purchased from New England Biolabs. Sepharose 2B and all other chemicals were from sources previously described.[21,22] Rabbit reticulocyte lysate was purchased from Ambion, and SP6 RNA polymerase was prepared as previously described.[23] 11-*cis*-Retinal was generously supplied by R. K. Crouch and the National Eye Institute (Bethesda, MD).

**Site-Directed Mutagenesis.** Bovine, mouse, and human arrestin-1 cDNA was cloned into a pGEM2-based plasmid with an "idealized" 5′-untranslated region[23] under control of a SP6 promoter. All mutations were introduced by polymerase chain reaction (PCR) using an appropriate mutagenizing oligonucleotide

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as a forward primer and an oligonucleotide downstream from the far restriction site to be used for subcloning as a reverse primer. Resulting fragments of various lengths and an appropriate primer upstream of the near restriction site were then used as reverse and forward primers, respectively, for the second round of PCR. Resulting fragments were subcloned back, and all constructs were confirmed by dideoxynucleotide sequencing.

**In Vitro Transcription, Translation, and Evaluation of the Stability of Mutants.** Plasmids were linearized with HindIII before in vitro transcription to produce mRNAs encoding full-length arrestin proteins, as described previously.[22,24] All arrestin proteins were labeled by incorporation of [$^3$H]leucine and [$^{14}$C]-leucine with the specific activity of the mix being 1.5−3 Ci/mmol, resulting in the specific activity of arrestin proteins being in the range of 66−85 Ci/mmol (145−187 dpm/fmol). The translation of every protein produced a single labeled band with the expected mobility on sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The relative stability of all mutants used in this study (evaluated as described in ref 25) exceeds 90% of that of the corresponding wild-type arrestin.

**Rhodopsin Preparations.** Urea-treated rod OS membranes were prepared, phosphorylated with rhodopsin kinase, and regenerated with 11-*cis*-retinal as described previously.[21] The stoichiometry of phosphorylation for the rhodopsin preparations used in these studies was 3.7 mol of phosphate/mol of rhodopsin.

**Direct Binding Assays.** Arrestin-1 binding to rhodopsin was performed, as described previously.[24,26] Briefly, in vitro translated tritiated arrestins (100 fmol) were incubated in 50 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol, and 50 mM potassium acetate with 7.5 pmol (0.3 $\mu$g) of the various functional forms of rhodopsin in a final volume of 50 $\mu$L for 5 min at 37 °C either in the dark or under room light. The samples were immediately cooled on ice and loaded under dim red light onto 2 mL Sepharose 2B columns equilibrated with 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl. Bound arrestin eluted with the disk membranes in the void volume (between 0.5 and 1.1 mL). Nonspecific binding determined in the presence of 0.3 $\mu$g of liposomes was subtracted. Arrestin-1 binding to microtubules (MTs) (purified tubulin polymerized in the presence of paclitaxel) was performed, as described previously.[27] Briefly, 200 fmol of the indicated in vitro translated arrestins was incubated in 50 mM Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, 1.5 mM DTT, 1 mM EGTA, and 50 mM potassium acetate for 20 min at 25 °C with 20 $\mu$g of prepolymerized tubulin. MTs along with bound arrestin were pelleted. MT−arrestin pellets were not washed because of the low affinity (i.e., high off rate) of the interaction. The pellet was dissolved in 0.1 mL of 1% SDS and 50 mM NaOH, and bound arrestin was quantified by liquid scintillation counting. Nonspecific "binding" (arrestin pelleted without microtubules) was subtracted.

**Purification of Arrestin-1 and Analysis of Its Self-Association.** Wild-type (WT) and mutant mouse arrestin-1, WT bovine arrestin-1, and WT human arrestin-1 were expressed in *Escherichia coli* and purified, essentially as described previously.[22] All light scattering measurements were taken with a DAWN EOS detector coupled to an Optilab refractometer (Wyatt Technologies) following gel filtration on a 7.8 mm (inside diameter) × 30.0 cm (length) silica-based column along with its guard column (Wyatt Technologies). The arrestin samples (100 $\mu$L) at different concentrations were incubated in fresh 5 mM DTT for 30 min at room temperature to disrupt covalent inter-arrestin disulfide bonds and injected onto the column at 25 °C, at a flow rate of 0.8 mL/min in 50 mM MOPS and 100 mM NaCl (pH 7.2). The column used did not resolve oligomeric species but simply acted as a filter to remove highly scattering particulates. Light scattering at 18 angles (15−160°), the absorbance at 280 nm, and the refractive index (at 690 nm) for each sample were taken for a slice centered at the peak of the elution profile with a width approximately equal to that of the profile at half-maximum.[18] The experimental weight-average molecular weights were obtained from the protein concentration and light scattering data using ASTRA version 5.3.4.16 (Wyatt Technologies). The weight-average molecular weights were analyzed using the two-step monomer−dimer−tetramer (MDT) model[16]

$$2M \overset{K_{dim}}{\leftrightarrow} D \quad 2M \overset{K_{tet}}{\leftrightarrow} 2D$$

where M, D, and T are monomer, dimer, and tetramer, respectively. Details of the analysis have been previously described.[28] Except where noted, the equilibrium constants are given in terms of the corresponding dissociation constants, $K_{D,dimer}$ ($K_{D,dim}$) and $K_{D,tetramer}$ ($K_{D,tet}$). The errors in equilibrium constants were determined from least-squares fitting of the data to the monomer−dimer−tetramer model (MDT model), taking into account an estimated error of ±1 kDa for the computed values of the average molecular weight.[28]

## RESULTS

The level of arrestin-1 in photoreceptors[4,6,7,9] is 3 orders of magnitude higher than the level of nonvisual arrestins in other neurons.[29,30] Another characteristic feature of arrestin-1 is robust self-association at physiological concentrations.[16−18] Because all previous studies of arrestin-1 self-association were performed with bovine protein, we tested whether WT mouse arrestin-1 uses the same oligomerization mechanism. To this end, we expressed mouse arrestin-1 in *E. coli*, purified it, and tested its self-association by measuring the dependence of the average molecular weight on its concentration by multiangle light scattering. We found that mouse arrestin-1 forms dimers and tetramers (Figure 1A) like its bovine homologue.[18] Although both dimerization ($K_{D,dim}$ = 57.5 ± 0.6 $\mu$M) and especially tetramerization ($K_{D,tet}$ = 63.1 ± 2.6 $\mu$M) constants are higher than the corresponding values for bovine arrestin-1 (37.2 ± 0.2 and 7.4 ± 0.1 $\mu$M, respectively[18]), at physiological concentrations of >2 mM,[1,9] only a small fraction of WT mouse arrestin-1 would be monomeric (Figure 1B). Interestingly, while tetramerization of bovine arrestin-1 was invariably found to be cooperative,[16,18,19] i.e., $K_{D,dim} > K_{D,tet}$, the mouse protein has virtually equal $K_{D,dim}$ and $K_{D,tet}$ values, indicating a lack of cooperativity in self-association (Table 1).

The significant difference between self-association constants of bovine and mouse arrestin-1 prompted us to analyze the human homologue (Figure 1C,D). We found that purified human arrestin-1 also self-associates, with a remarkably low $K_{D,dim}$ of 2.95 ± 0.02 $\mu$M and a relatively high $K_{D,tet}$ of 224 ± 5 $\mu$M (Table 1). Interestingly, these disparate sets of constants in the three mammalian species yield predicted levels of the monomer in the cell body of the dark-adapted rod at a total arrestin-1 concentration of ~2 mM (extrapolating measured mouse values[8,9] to other species) in the relatively narrow range of 30−90 $\mu$M (Table 2). Total tetramer concentrations vary by only 30% (Table 2). The main difference is in the resulting dimer levels, which vary almost 5-fold, from the predicted value of 59 $\mu$M in the bovine rod to 281 $\mu$M in the human rod (Table 2).

The value of the measured $K_{D,dim}$ between human and mouse arrestin-1 shows an ~20-fold difference, and the value of $K_{D,tet}$ between bovine and human proteins differs by ~30-fold. These

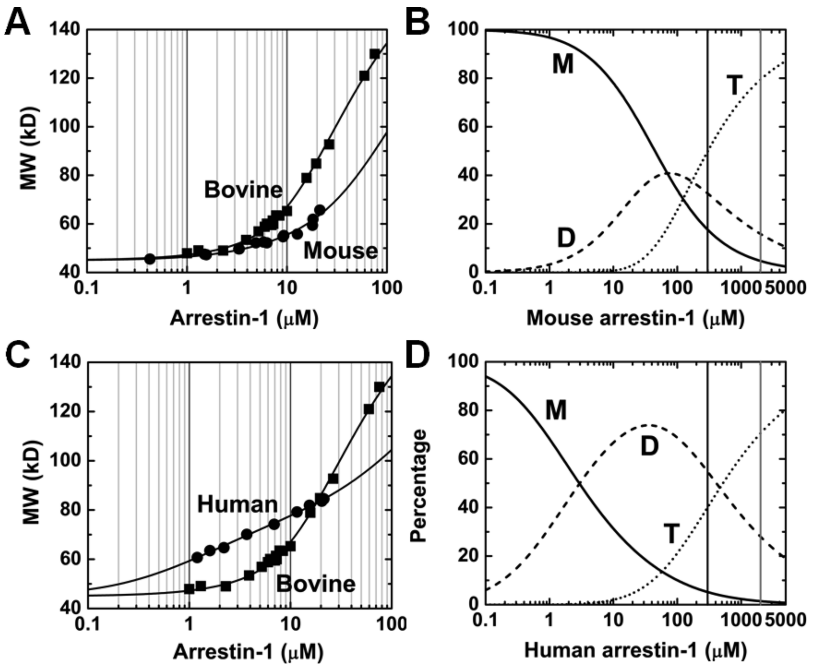


**Figure 1.** Mouse arrestin-1 and human arrestin-1 form dimers and tetramers at physiological concentrations. (A) Average molecular weight of wild-type mouse arrestin-1 as a function of total concentration (●) determined from the light scattering data as described in Materials and Methods. The solid curve is a least-squares fit of the data to the MDT model with a $K_{D,dim}$ of 57.5 ± 0.6 μM and a $K_{D,tet}$ of 63.1 ± 2.6 μM. The data for bovine arrestin-1[18] are shown as squares for comparison. (B) Percentage of mouse arrestin-1 molecules in monomer (M, —), dimer (D, ---), and tetramer (T, · · ·) forms as a function of total arrestin-1 concentration computed for the MDT model and the data in panel A. (C) Average molecular weight of wild-type human arrestin-1 as a function of total concentration (●) determined from the light scattering data. The solid curve is a least-squares fit of the data to the MDT model with a $K_{D,dim}$ of 2.95 ± 0.02 μM and a $K_{D,tet}$ of 224 ± 5 μM. (D) Percentage of human arrestin-1 molecules in monomer (M, —), dimer (D, ---), and tetramer (T, · · ·) forms as a function of total arrestin-1 concentration computed for the MDT model and the data in panel C. Vertical lines in panels B and D correspond to arrestin-1 concentrations in the outer segment (300 μM, black) and cell body (2000 μM, gray) of the dark-adapted rod.

**Table 1. Equilibrium Constants Characterizing Self-Association of Wild-Type and Mutant Mouse, Human, and Bovine Arrestin-1**

| protein | log $K_{dim}$[a] | log $K_{tet}$[a] | $K_{D,dim}$ (μM) | $K_{D,tet}$ (μM) |
|---|---|---|---|---|
| mouse arrestin-1 | 4.24 ± 0.04 | 4.20 ± 0.17 | 57.5 ± 0.6 | 63.1 ± 2.6 |
| mouse arrestin-1(F86A/F198A) | 3.27 ± 0.05 | – | 537 ± 9 | – |
| mouse arrestin-1(F86A/F198A/A349V) | 3.14 ± 0.11 | – | 724 ± 26 | – |
| human arrestin-1 | 5.53 ± 0.03 | 3.65 ± 0.08 | 2.95 ± 0.02 | 224 ± 5 |
| bovine arrestin-1 | 4.43 ± 0.02 | 5.13 ± 0.03 | 37.2 ± 0.2 | 7.4 ± 0.1 |
| bovine arrestin-1(F85A/F197A) | 3.28 ± 0.10 | – | 525 ± 16 | – |

[a] $K_{dim}$ and $K_{tet}$ are the association constants determined from light scattering analysis.

**Table 2. Predicted Concentrations of the Monomer, Dimer, and Tetramer of Mouse, Human, and Bovine Arrestin-1 in the Outer Segment (300 μM) and Cell Body (2000 μM) of Dark-Adapted Rods**

| arrestin-1 | total concn (μM) | [monomer] (μM) (% of total) | [dimer] (μM) (% of total) | [tetramer] (μM) (% of total) |
|---|---|---|---|---|
| bovine | 300 | 27.6 (9.2%) | 20.8 (13.9%) | 57.7 (76.9%) |
| mouse | 300 | 52.8 (17.6%) | 48.8 (32.5%) | 37.4 (49.9%) |
| human | 300 | 15.5 (5.2%) | 82.1 (54.7%) | 30.1 (40.1%) |
| bovine | 2000 | 46 (2.3%) | 59 (5.9%) | 459 (91.8%) |
| mouse | 2000 | 95 (4.7%) | 159 (15.9%) | 397 (79.4%) |
| human | 2000 | 29 (1.5%) | 281 (28.1%) | 352 (70.4%) |

dramatic differences raise the possibility that the three mammalian arrestin-1 species could use distinct interaction interfaces, so that the common self-association phenotype could represent convergent evolution, rather than direct conservation. It has been recently shown that the structure of the solution tetramer of bovine arrestin-1 is different from that of the crystal tetramer.[18] Extensive investigation yielded a model of the solution tetramer in which receptor-binding surfaces are shielded by "sister" subunits, which explains the demonstrated inability of the oligomers to bind rhodopsin.[19] On the basis of this model, a modified bovine arrestin-1 was constructed, where two (F85A and F197A) mutations predicted to disrupt NN (F85A) and CC (F197A)


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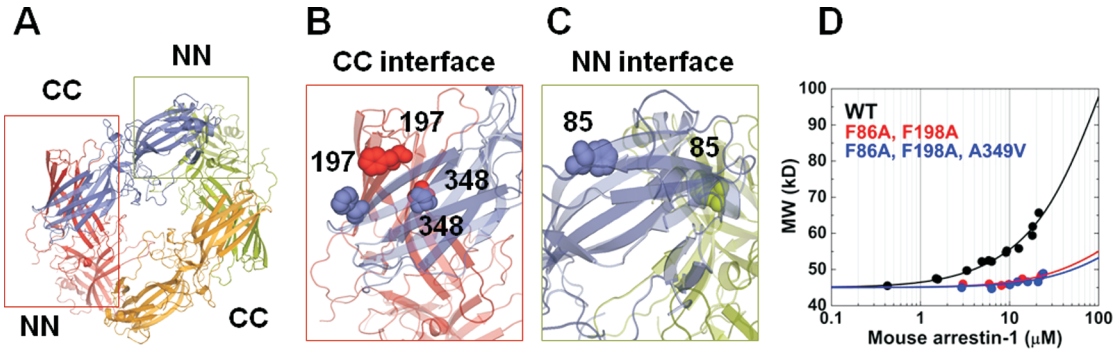


**Figure 2.** Targeted disruption of arrestin-1 self-association. (A) Solution tetramer structure of bovine arrestin-1.[19] Areas enlarged to show intersubunit contacts are indicated. (B) CC interface (between the two C-domains) showing the positions of residues F197 (F198 in mouse) and A348 (A349 in mouse). (C) NN interface (between the two N-domains) showing the position of F85 (F86 in mouse). (D) Average molecular weight of the F86A/F198A (red circles) and F86A/F198A/A349V (blue circles) mouse arrestin-1 mutants as a function of total arrestin concentration determined from the light scattering data (symbols). The fit of the data to the MDT model (solid lines) was obtained as described previously.[18] Note that neither mutant showed detectable tetramerization, so that the resulting fit describes a monomer−dimer equilibrium. The wild-type mouse arrestin-1 data (●) are shown for comparison.

self-association interfaces were introduced (Figure 2A−C).[19] Indeed, this mutant was shown to be essentially monomeric, with a $K_{D,dim}$ of 525 $\mu$M and no detectable tetramer formation,[19] independently confirming the model. To test whether the same subunit arrangement is present in the tetramer of mouse arrestin-1, we introduced homologous mutations (F86A and F198A), expressed this protein, and assessed its self-association. We found that this mutation in mouse arrestin-1 yields the same non-self-associating phenotype as in its bovine counterpart, demonstrating a $K_{D,dim}$ of 537 $\mu$M and no detectable tetramerization (Figure 2D). Further disruption of the CC interface by the addition of an A349V mutation increased the $K_{D,dim}$ to 724 $\mu$M (Figure 2D and Table 1). Thus, homologous mutations in bovine and mouse arrestin-1 affect their self-association in a similar manner, suggesting that the same interfaces are involved in oligomerization of both proteins, and the difference in constants reflects the relative energy of interactions between the subunits, rather than a global difference in the structure of the solution tetramer. Phosphorylated light-activated rhodopsin (P-Rh*) is the main binding target of arrestin-1 in the rod.[31] The amount of rhodopsin present in the OS determines the amount of arrestin-1 that can translocated to this compartment,[7] supporting the idea that rhodopsin binding holds arrestin-1 in the OS in the light.[14] In contrast, in the dark, most arrestin-1 (the estimates for the WT mouse form range from ~85%[9] to 91%[6] to >95%[7,14]) resides in other compartments of the cell,[6,13−15] where it is anchored via low-affinity binding to microtubules[14,32,33] abundant in the inner segment, perinuclear area, and synaptic termini.[34] Therefore, to test whether observed differences in self-association are the result of selective disruption of the interfaces involved, we compared the binding of WT and mutant forms of bovine and mouse arrestin-1 to P-Rh* and in vitro polymerized microtubules (Figure 3). Using fully functional radiolabeled arrestins and standard direct binding assays,[22,27,32,35] we found that mutations that disrupt oligomerization in bovine and mouse arrestin-1 do not appreciably affect the binding to either partner (Figure 3A,B). Arrestin-1 is a highly sensitive molecule in which even small conformational perturbations by mutagenesis result in dramatic changes in its binding to P-Rh*[20,25,26,36−40] and microtubules.[27,32] Thus, virtually wild-type binding to both rhodopsin and microtubules makes it highly unlikely that these mutations induce any global structural changes in the molecule, leaving targeted disruption of the self-association interfaces as the only plausible explanation of their phenotype.

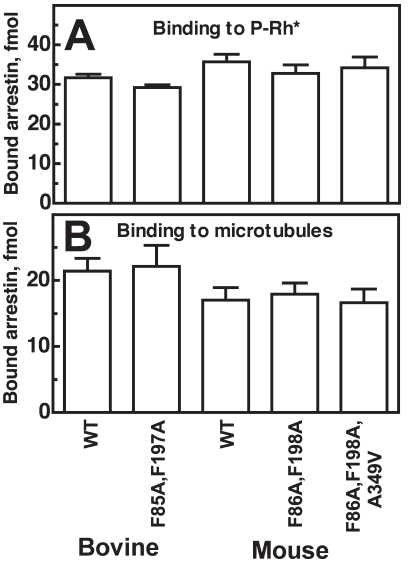


**Figure 3.** Mutations disrupting self-association do not affect the binding of arrestin-1 to P-Rh* and microtubules. Binding of indicated radiolabeled arrestins to P-Rh* (100 fmol/assay) (A) and microtubules (200 fmol/assay) (B) assessed as described in Materials and Methods. Means ± the standard deviation of three experiments performed in duplicate.

## DISCUSSION

Preferential binding of arrestin-1 to P-Rh*[41] and the resulting quenching of rhodopsin signaling[42] were discovered in the mid-1980s, and this remains its least controversial function to this day.[1] The ability of bovine arrestin-1 (under the name of S-antigen) to self-associate was discovered a decade earlier,[43] but no biological function was ascribed to this phenomenon. The interest in arrestin-1 self-association was revived when two crystal structures of the bovine protein[44,45] revealed virtually identical tetramers. Further studies confirmed its self-association in solution,[16−18] although careful examination revealed that the structure of the solution tetramer that forms under much more physiological conditions is dramatically different from that found in the crystal.[18,19] The oligomers were usually discussed as storage forms, an interpretation strongly supported by direct demonstration that only the monomer is capable of binding P-Rh*,[18] yet it remained unclear why of all the signaling

proteins present at enormous (as compared to other neurons) concentrations in the rod,[8] arrestin-1 is the only one that has a special apparently inactive storage form. Because all these studies were performed on bovine protein, it was not even clear whether self-association is a common feature of mammalian arrestin-1 species, which would be the case if it has physiologically relevant function.

Here we compared arrestin-1 from three mammals: (1) bovine, traditionally used for biochemical studies; (2) mouse, the best functionally characterized in vivo; and (3) human, the most therapeutically relevant. In each case, we found that arrestin-1 robustly self-associates at physiological concentrations, which suggests that this feature is biologically important. Surprisingly, the thermodynamics of self-association in the three species are strikingly different. While tetramerization of bovine arrestin-1 is cooperative, in a sense that $K_{D,dim} > K_{D,tet}$, there is no such cooperativity in the mouse form ($K_{D,dim} \sim K_{D,tet}$), whereas in the human form, $K_{D,dim} \ll K_{D,tet}$, being 10 and 20 times lower than the $K_{D,dim}$ values of bovine and mouse arrestin-1, respectively (Table 1). Despite these differences, the structures of the solution tetramers are likely similar, as judged by remarkably uniform effects of mutations destabilizing intersubunit interfaces in bovine and mouse arrestin-1 (Figure 2 and Table 1). Importantly, these mutations do not appreciably affect the binding of arrestin-1 to P-Rh* and microtubules (Figure 3), suggesting that the conservation of the interface residues reflects the need for self-association, rather than being a byproduct of conservation of other arrestin-1 functions. Most of the residues in the self-association interfaces identified in bovine arrestin-1[19] and deduced by homology modeling in mouse and human proteins are conserved, with a few differences possibly responsible for distinct thermodynamics.

Rod photoreceptors function in dim light, becoming saturated even at modest illumination levels.[46] Thus, clues about possible biological functions of arrestin-1 self-association may be revealed through its effects on the state of arrestin-1 at concentrations found in the dark-adapted rod. The distribution of arrestin-1 in dark-adapted rods was quantitatively measured only in the mouse form.[6,7,9,14] However, rod function in different mammals is similar, and arrestin-1:rhodopsin ratios in the OS of dark-adapted mouse[9] and frog[47] rods are remarkably close. Therefore, mouse expression levels were used for all species in the estimates that follow. In the dark, the bulk (85−95%[6,7,9,14]) of arrestin-1 is localized to the cell body, where the expected concentration reaches ~2 mM, with a much lower concentration of ~300 $\mu$M in the OS, where its main target rhodopsin is localized.[9] Using these concentrations and measured values of $K_D$ (Table 1), we estimated the expected equilibrium concentrations of the arrestin-1 monomer, dimer, and tetramer in the dark-adapted rod (Table 2). In all three cases, arrestin-1 self-association makes the fraction of the monomer in the cell body relatively small (1.5−4.7%). However, each species achieves this result in a somewhat different way (Tables 1 and 2). In the case of bovine protein, self-association is cooperative, with a $K_{D,tet}$ that is less than $K_{D,dim}$, so that at 2 mM, the bulk (>90%) of arrestin-1 is stored in the form of tetramer while the fraction of the dimer is very small (<6%). In the mouse form, $K_{D,dim} \sim K_{D,tet}$, so that while the bulk (~80%) is still a tetramer, a much larger fraction (~16%) exists as a dimer. Human arrestin-1 dimerizes more readily than the others but shows less robust tetramerization, so that at the same concentration, only 70% exists as a tetramer whereas as much as 28% is a dimer. In all cases, the monomer concentration is <100 $\mu$M, with ~3-fold differences among species, dimer concentrations vary ~5-fold, and absolute tetramer levels vary by only ~30% (Table 2).

Light exposure induces massive translocation of arrestin-1 from the inner segment and cell body to the OS, which takes up to 30−60 min.[6,13−15,48,49] Considering that the photoresponse, which is normally terminated with subsecond kinetics,[2,50−52] is greatly prolonged in arrestin-1 knockout animals,[3] arrestin-1 present in the OS in the dark must be responsible for timely shut-off. Our analysis of arrestin-1 oligomerization suggests that the level of the monomer, which is the only rhodopsin-binding form,[18] is much higher in the OS (>50 $\mu$M) in nocturnal mice most reliant on rod vision, compared to <30 $\mu$M in bovine rods and just ~16 $\mu$M in human rods. Rod vision is less crucial for these latter diurnal species.

Using the second-order on-rate constant ($k_1$) recently measured for binding of arrestin-1 to P-Rh* (~$10^6$ $M^{-1}$ $s^{-1}$ for P-Rh* in nanodisks[53]) along with the monomer concentration allows one to estimate the expected pseudo-first-order rate constant for an encounter of rhodopsin with arrestin-1 ($k_1$[monomeric arrestin-1]), which in mouse yields a value of ~50 $s^{-1}$. This estimate of the lower limit suggests that each rhodopsin encounters an arrestin-1 molecule on average once every ~20 ms. In other words, arrestin-1 "checks" the functional state of rhodopsin in this time interval and binds tightly when it encounters P-Rh*.[1] Again, this yields an estimate of a lower limit for the active rhodopsin lifetime in mouse rods, which must be >20 ms because of additional time that GRK1 needs to attach three or more phosphates to light-activated rhodopsin necessary for high-affinity arrestin-1 binding.[21,52] This number is consistent with the original experimental estimate of <60 ms[2] and the more recent estimate of ~30 ms,[51] as well as with the modeling of photoresponse dynamics based on these data.[54,55] By the same token, lower monomer concentrations in the OS of bovine and human rods (Table 2) suggest that the lifetime of the active rhodopsin in these species must be longer, >36 and >65 ms, respectively. Because in the OS arrestin-1 diffuses in the cytoplasm with complex geometry[56] while rhodopsin diffuses in two dimensions on the disk membrane, the actual on rate could differ from these estimates, so these predictions need to be tested experimentally.

Several studies showed that light-dependent translocation of arrestin-1 to the OS,[14] as well as the movement of transducin out of the OS,[57] is energy-independent, largely driven by their interactions with nonmoving partners (reviewed in ref 58), whereas others suggested that active transport could be involved.[59,60] Arrestin-1 in photoreceptors is clearly at disequilibrium in the dark and in bright light.[61] Thus, regardless of the mode of transportation, it must be "tethered" in the OS in the light and in the cell body in the dark by other proteins. Otherwise, the diffusion would quickly undo anything that active transport could achieve. Therefore, the concentrations of free monomer, dimer, and tetramer in the OS and cell body are likely equal.[1] Preferential arrestin-1 localization outside of the OS in the dark was reported to be determined by its binding to microtubules,[14,33] abundant in the cell body and sparse in the OS.[34] Other arrestin-1 binding partners, such as *N*-ethylmaleimide-sensitive factor (NSF[62]) and/or enolase,[63] could also serve as anchors in the cell body in the dark. However, the concentration of polymerized tubulin, where each $\alpha\beta$-dimer can bind arrestin-1,[27] by far exceeds those of all other putative anchors combined, suggesting that MTs likely serve as the main binding partner. Thus, the difference in the concentration of each form between these two compartments of the rod largely reflects its MT-associated fraction. The sizes of the rods and their OS are significantly different in different species. Unfortunately, the

volume of the cytoplasm in the OS and other rod compartments, as well as the concentrations of rhodopsin and other signaling molecules, was carefully measured only in mice and frogs.[64] However, arrestin-1:rhodopsin ratios in dark-adapted mouse OS[9] and ~20-fold larger frog OS[47] are remarkably close. On the strength of these findings and the fact that the size of the OS is determined by the amount of rhodopsin present there,[65] the estimates below are based on the assumption that the arrestin-1 concentration in dark-adapted mammalian rods is similar. In this case, in all species, the predicted levels of the monomer, dimer, and tetramer in the cell body exceed those in the OS by 1.7−1.9-, ~3-, and ~10-fold, respectively (Table 2). In bright light, a much higher total arrestin-1 concentration in the OS was shown to be due to its binding to rhodopsin,[6,7,14] as the amount of rhodopsin in the OS clearly limits the molar amount of arrestin-1 that can translocate to this compartment.[7] Similarly, in the dark, the total concentration of each form of arrestin-1 in the cell body likely exceeds the corresponding value in the OS by the amount that is bound to MTs. For example, in the mouse rod, the estimated total concentration of monomer is 52.8 $\mu$M in the OS and 95 $\mu$M in the cell body (Table 2). The latter exceeds the OS concentration by ~42 $\mu$M, suggesting that this amount (~44% of the total monomeric arrestin-1 in this compartment) is bound to MTs. Similarly, calculated differences in the concentrations of monomer, dimer, and tetramer in the OS and the cell body suggest that the fractions of the individual MT-bound forms in the cell body are fairly close in the three mammalian species: monomer (40−47%), dimer (65−71%), and tetramer (87−91%). However, each of the three molecular forms of arrestin-1 apparently has a distinct propensity to associate with MTs, with the following order of potency: tetramer ≫ dimer ≫ monomer.

To conclude, we show here that arrestin-1 self-association is conserved in three mammalian species, indicating that this phenomenon is biologically important. We present evidence that, although the dimerization and tetramerization equilibrium constants are very different in the three species examined, the overall structures of the solution tetramers are likely to be very similar. Despite the differences in the thermodynamics of association, for each species, the concentration of the active monomer is very low, while the bulk of arrestin-1 exists in the form of a tetramer with the best ability to bind microtubules in the cell body. The concentrations of the arrestin-1 monomer in the OS, estimated from the oligomerization equilibrium constants, provide experimentally testable predictions with regard to the lifetime of active rhodopsin in different species. An "enhanced" arrestin-1 mutant with increased affinity for light-activated unphosphorylated rhodopsin was recently shown to have therapeutic potential in genetic disorders with deficient rhodopsin phosphorylation.[20] The biological importance of arrestin-1 self-association indicates that the changes in self-association, inadvertently produced by mutagenesis, may underlie reported deleterious effects of very high levels of expression of this mutant.[20] Thus, the normal ability of any mutant form of arrestin-1 to oligomerize must be ascertained before it can be used for gene therapy.

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### Notes

[a]We use systematic names of arrestin proteins: arrestin-1 (also known as visual or rod arrestin, 48 kDa protein, or S-antigen), arrestin-2 ($\beta$-arrestin or $\beta$-arrestin1), arrestin-3 ($\beta$-arrestin2), and arrestin-4 (cone or X-arrestin).

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## ABBREVIATIONS

$K_{dim}$ ($=1/K_{D,dim}$), dimerization constant, i.e., equilibrium association constant of two arrestin-1 monomers; $K_{tet}$ ($=1/K_{D,tet}$), tetramerization constant, i.e., equilibrium association constant of two arrestin-1 dimers; GRK1, G protein-coupled receptor kinase 1, also known as rhodopsin kinase; P-Rh*, phosphorylated light-activated rhodopsin.

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