Critical Role of the Central 139-Loop in Stability and Binding Selectivity of Arrestin-1*

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Background: Arrestin-1 selectively binds light-activated phosphorhodopsin.

Results: Deletions in the 139-loop or disruptions of its interactions with the body of arrestin-1 greatly reduce arrestin-1 stability and selectivity.

Conclusion: The central 139-loop supports basal arrestin-1 conformation and reduces its binding to non-preferred forms of rhodopsin.

Significance: The central 139-loop is an earlier unappreciated element contributing to the thermal stability and binding selectivity of arrestins.

Arrestin-1 selectively binds active phosphorylated rhodopsin (P-Rh*), demonstrating much lower affinity for inactive phosphorylated (P-Rh) and unphosphorylated active (Rh*) forms. Receptor interaction induces significant conformational changes in arrestin-1, which include large movement of the previously neglected 139-loop in the center of the receptor binding surface, away from the incoming receptor. To elucidate the functional role of this loop, in mouse arrestin-1 we introduced deletions of variable lengths and made several substitutions of Lys-142 in it and Asp-72 in the adjacent loop. Several mutants with perturbations in the 139-loop demonstrate increased binding to P-Rh*, dark P-Rh, Rh*, and phospho-opsin. Enhanced binding of arrestin-1 mutants to non-preferred forms of rhodopsin correlates with decreased thermal stability. The 139-loop perturbations increase P-Rh* binding of arrestin-1 at low temperatures and further change its binding profile on the background of 3A mutant, where the C-tail is detached from the body of the molecule by triple alanine substitution. Thus, the 139-loop stabilizes basal conformation of arrestin-1 and acts as a brake, preventing its binding to non-preferred forms of rhodopsin. Conservation of this loop in other subtypes suggests that it has the same function in all members of the arrestin family.

Rhodopsin is a prototypical G protein-coupled receptor belonging to class A (1–3) that is selectively expressed in rod photoreceptors at extremely high levels, with ~3 mM concentration in the specialized signaling compartment, the outer segment (4). Single photon sensitivity of rods (5) requires a virtually perfect signaling cascade with enormous amplification and very low noise as well as quick and efficient signal termination, which is accomplished by rhodopsin phosphorylation by GRK1 followed by arrestin-1 binding (6). The expression of arrestin-1 is also very high, at an ~0.8:1 molar ratio to rhodopsin (7–9), which is apparently required to completely prevent useless rhodopsin signaling in bright light, when rods no longer function as photoreceptors. Reliable full inactivation of rhodopsin in bright light might be also assisted by the ability of arrestin-1 to engage a second rhodopsin molecule, albeit with much lower affinity (10, 11). In contrast, rapid shutoff of dozens to hundreds of rhodopsin molecules activated by dim light, which is within the working range of rods, can be effectively accomplished by much lower arrestin-1 levels (12). Several mechanisms work in concert to ensure that an enormous stock of arrestin-1 does not inappropriately quench light-activated rhodopsin (Rh*)3 before the signal has a chance to go through and generate cell response. First, the bulk of arrestin-1 in dark-adapted rods is localized in the inner segment, cell body, and synaptic terminals, with a relatively small fraction found in the outer segment (7–9, 13). Second, arrestin-1 robustly self-associates, forming dimers and tetramers at physiological concentrations (14–17), whereas only monomers can bind rhodopsin (15) because the receptor binding surface is shielded by sister subunits (18) in the tetramer and both possible dimers existing in solution. Thus, only a small fraction of arrestin-1 in the outer segment is competent to quench Rh* signaling. Last but not least, arrestin-1 is remarkably selective for active phospho-rhodopsin (P-Rh*), having a much lower affinity for other functional forms (19–21). Moreover, arrestin-1 only swings into action after GRK1 attaches at least three phosphates to Rh* (22, 23), which gives sufficient time for Rh* to sequentially activate multiple molecules of the visual G protein transducin, ensuring high signal amplification (24).

Here we explore a previously unappreciated molecular mechanism that prevents arrestin-1 from binding to unphosphorylated Rh* without adversely affecting P-Rh* binding. We recently found that a loop in the center of the arrestin-1 mole-

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2 We used systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48-kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin-1), arrestin-3 (β-arrestin-2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called arrestin 3 in the HUGO database).

3 The abbreviations used are: Rh*, active rhodopsin; P-ops, phosopho-opsin.
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cule with residue 139 at its tip (139-loop) localized next to several elements directly engaged by P-Rh* and moves to the side of the molecule upon receptor binding (Fig. 1), apparently out of the way of the incoming receptor (25). We demonstrate that the 139-loop has two functions; it is a critical structural element supporting basal arrestin-1 conformation, and it ensures high arrestin-1 selectivity for P-Rh*. Even mild perturbations of this loop are surprisingly detrimental for the thermal stability of the protein and promote arrestin-1 binding to non-preferred functional forms of rhodopsin at the same time. Remarkable structural conservation of this loop in all members of the arrestin family (26–29) suggests that it likely similarly enhances stability and selectivity of other arrestin subtypes.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP, [14C]leucine, and [3H]leucine were from PerkinElmer Life Sciences. All restriction and DNA modifying enzymes were from New England Biolabs (Ipswich, MA). Rabbit reticulocyte lysate was from Ambion (Austin, TX), and SP6 RNA polymerase was prepared as described (30). All other reagents were from Sigma.

Mutagenesis and Plasmid Construction—For in vitro transcription, mouse arrestin-1 (generous gift of Dr. Cheryl Craft, California Science Center) was subcloned into pGEM2 (Promega, Madison, WI) with “idealized” 5-UTR (30) between Ncol and HindIII sites as described (17). Mutations were introduced by PCR using the strategy previously described (31, 32). All constructs were confirmed by dideoxy sequencing. In vitro transcription, translation, and preparation of different functional forms of phosphorylated and unphosphorylated rhodopsin were performed as described recently (31–34).

Direct binding assay was performed as described (32, 33). Briefly, 1 nM arrestin-1 (50 fmol) was incubated with 0.3 μg of various functional forms of rhodopsin in 50 μl of 50 mM Tris-HCl, pH 7.4, 100 mM potassium acetate, 1 mM EDTA, 1 mM DTT for 5 min at 37 °C under room light (P-Rh*, Rh*, or phospho-opsin) or in the dark (P-Rh or Rh). Samples were cooled on ice, and bound and free arrestin-1 were separated at 4 °C by gel filtration on a 2-ml column of Sepharose 2B-CL (under dim light in the case of dark samples). Arrestin-1 eluting with rhodopsin-containing membranes was quantified by liquid scintillation counting. Nonspecific “binding,” measured in samples from which rhodopsin was omitted, was subtracted.

In Vitro Arrestin Stability Assay—Arrestin-1 stability was tested as described (32). Briefly, translated radiolabeled arrestin-1 was incubated for 1–4 h at 39 °C and then cooled on ice. The binding of arrestin-1 in these samples to P-Rh* was compared with that of control sample kept on ice as described above, except that 2 nM arrestin-1 (100 fmol per sample) was used.

RESULTS

Free arrestin-1 in crystal (26, 35) and solution (25) has two adjacent loops in the central crest of the receptor binding surface (Fig. 1, A and B), which was as mapped by numerous groups using a wide variety of methods (10, 11, 21, 25, 33, 36 – 46). One, the “finger loop” in arrestin-1 (38, 47, 48) and arrestin-2 (41), is directly engaged by the receptor. The other, the 139-loop,
appears to make contact with inactive phospho-rhodopsin but not with the preferred arrestin-1 target, P-Rh* (38). In our recent study of the conformation of the receptor-bound arrestin using intramolecular distance measurements in free and P-Rh*-associated states, we found that the 139-loop undergoes large-scale movement toward the N-domain and to the side of the molecule, apparently getting out of the way of the receptor, which then engages the adjacent finger loop (25). Moreover, two short deletions in the 139-loop of bovine arrestin-1 facilitate its binding to non-preferred forms of rhodopsin, Rh* and dark P-Rh, and one of these deletions induces the release of the arrestin-1 C-tail (25), which is a hallmark of its “activation” by bound receptor (38, 40). The 139-loop is fixed in place via several intramolecular interactions in the basal state of arrestin-1 (26). The length, sequence (49) (Fig. 1C), and structure (26–29) of this loop are remarkably conserved in the arrestin family, suggesting that it must be functionally important even though it does not directly participate in receptor binding (25). To elucidate the role of the 139-loop, we performed its systematic structure-function analysis in mouse arrestin-1, which we recently successfully used for targeted engineering of mutants with greatly enhanced binding to Rh* (32). To this end we introduced several 5–6-residue deletions with the addition of 1–3 replacement residues to connect the strands. We also destroyed the putative salt bridge anchoring the 139-loop to the finger loop by substituting Asp-72 in the finger loop or Lys-142 in the 139-loop with residues that eliminate or reverse the charge, comparing them to conservative substitutions that preserve the charge (Fig. 1, B and D).

**Perturbation of the 139-Loop Increases the Binding to Non-preferred Forms of Rhodopsin and Reduces Thermal Stability of Arrestin.—**The deletion of five residues (137–141) with replacement by a single Ser or Ala significantly enhances arrestin-1 binding to its preferred target, P-Rh* (Fig. 2), as could be expected if this loop in its basal position is in the way of the incoming receptor (25). The introduction of a longer linker, Ala-Ser-Ala, to replace deleted residues, which results in net 139-loop shortening by two residues instead of four, has a smaller effect on P-Rh* binding. However, the deletion of six residues (137–142) with a single replacement (Ser), which shortens the loop by five residues, results in a smaller increase in P-Rh* binding than the reduction of its length by four residues (Fig. 2), suggesting that the length of the 139-loop is not the only factor that determines its effectiveness as a “brake” that inhibits receptor binding. Although the most effective deletion in the 139-loop only increases P-Rh* binding by ~50%, the effects on arrestin-1 binding to non-preferred forms of rhodopsin, Rh* and dark P-Rh, are much greater. The elimination of 4–5 residues from the 139-loop increases the binding to dark P-Rh 5–7-fold and significantly enhances Rh* binding, whereas the shortening by only two residues in Δ(137–141)-Ala-Ser-Ala shows no appreciable effect (Fig. 2). More pronounced enabling effects of the 139-loop deletions on the binding to dark P-Rh and Rh* suggest that these forms of rhodopsin cannot move the WT 139-loop out of the way, in contrast to P-Rh*, which can apparently accomplish this fairly effectively. Recently we found that in terms of arrestin-1 binding, phospho-opsin (P-ops) is the second most preferred form, although the affinity of arres-
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tin-1 for P-ops is much lower than for P-Rh* (21). Therefore, we tested the effects of 139-loop deletions on arrestin-1 binding to P-ops. The deletions increase the binding to P-ops 3–4-fold, i.e. to a much greater extent than the binding to P-Rh*, but not as dramatically as the binding to dark P-Rh and Rh* (Fig. 2). As with dark P-Rh and Rh*, Δ(137–141)-Ala-Ser-Ala, which reduces the length of the 139-loop by only two residues, has no effect on P-ops binding. Thus, in agreement with the model where the 139-loop needs to move out of the way to allow receptor binding (25), the reduction of its length facilitates arrestin-1–rhodopsin interactions. Importantly, the magnitude of this effect is modest in the case of the preferred arrestin-1 partner P-Rh*, intermediate in the case of P-ops, and the most dramatic in the case of less preferred functional forms, dark P-Rh and Rh* (Fig. 2). Because P-Rh* can effectively push the WT-sized 139-loop out of the way (25, 38) to enable binding, these data suggest that the effect of the 139-loop deletions is inversely proportional to the inherent ability of different forms of rhodopsin to induce this conformational change in arrestin-1. Thus, P-ops appears to be more effective in this regard than dark P-Rh and Rh*.

The crystal structure suggests that the position of the 139-loop in the basal state of bovine arrestin-1 could be maintained in part by the salt bridge between Asp-71 in the finger loop and Lys-141 in the 139-loop (26) (Fig. 1B). To test the contribution of this putative salt bridge, we replaced corresponding Asp-72 and Lys-142 in mouse arrestin-1 (Fig. 1B) with alanines that completely eliminate interaction potential as well as with residues that either retained charge or H-bonding capability. The majority of these mutations enhance arrestin-1 binding to P-Rh*, with the most effective yielding ~40% gain, comparable to the 139-loop deletions (Fig. 2). In contrast, the effect of these mutations on binding to dark P-Rh and P-ops was much smaller than that of deletions; the increase did not exceed 2-fold even with the most effective mutations (Fig. 2). Among single mutations, only fairly conservative K142R and K142H appreciably increased Rh* binding (Fig. 2). Interestingly, conservative substitutions D72E and K142R increased P-Rh* binding, whereas K142A mutation was the only exception; it did not increase the binding to P-Rh* and, if anything, somewhat decreased the binding to the other forms (Fig. 2). These results do not support the idea that the salt bridge between the two adjacent loops plays an appreciable role in maintaining the basal position of the 139-loop, which impedes receptor binding.

Next, we tested the thermal stability of selected arrestin-1 mutants by incubating these proteins, generated in a cell-free translation (19, 20, 32), at 39 °C for up to 4 h and measuring their ability to specifically bind P-Rh* (Fig. 3). As reported previously (32), WT arrestin-1 remains fully active, whereas the reduction of the length of the 139-loop by four residues in Δ(137–142)-Ser and Δ(137–141)-Ala dramatically reduces protein stability, resulting in the loss of >90 and 50% of activity, respectively, within 1 h and virtually complete inactivation after 4 h at 39 °C (Fig. 3). Interestingly, the reduction of the 139-loop length by two residues in the Δ(137–141)-Ala-Ser-Ala mutant as well as all point mutations tested did not appreciably affect protein stability (Fig. 3). Thus, the relatively long 139-loop stabilizes the basal conformation of arrestin-1. However, a small reduction of its length as well as elimination of the putative salt bridge with the adjacent finger loop is well tolerated. Mutations that increase binding to the non-preferred functional forms of rhodopsin more effectively are the most detrimental to arrestin-1 stability. This correlation is consistent with the idea that receptor first needs to destabilize the basal arrestin-1 conformation to induce its transition into a high affinity receptor binding state (6, 50). Therefore, mutations that “loosen up” the basal state of the molecule facilitate arrestin-1 binding to the receptor and decrease its thermal stability at the same time.

The Detachment of the C-tail Increases the Effects of 139-Loop Mutations on Arrestin-1 Binding to Rhodopsin and Thermal Stability—Recent intramolecular distance measurements in bovine arrestin-1 showed that receptor binding is accompanied by two major conformational rearrangements: the release of the arrestin C-tail and large movement of the 139-loop (25, 38, 40). We previously showed that forçible detachment of the arrestin C-tail by triple alanine substitution of bulky hydrophobic residues that anchor it to the N-domain enhances the binding of all arrestins to the non-preferred forms of their cognate receptors (32, 51–56) by loosening up the basal conformation (57). At least one deletion in the 139-loop of bovine arrestin-1 resulted in partial release of the arrestin-1 C-tail, yielding an equilibrium between populations that have the C-tail attached or detached even in the absence of the receptor (25). To test whether the C-tail release and the movement of the 139-loop affect arrestin-1 function independently or represent obligatory parts of the same mechanism, we introduced the 139-loop deletions and mutations on the background of the mouse arrestin-1–3A (L374A, V375A, F376A) mutant. The binding of arrestin-1–3A to dark P-Rh, P-ops, and Rh* was 3–7 times greater than that of WT arrestin-1 (Fig. 4). None of the loop deletions significantly increased arrestin-1 binding to P-Rh*, P-ops, or dark P-Rh above the level of the 3A mutant (Fig. 4). However, 139-loop deletions enhanced the binding to Rh* up to 2-fold, suggesting that distinct molecular mechanisms might underlie increased binding to different functional forms of the receptor. We also found that on the 3A background, the Δ(137–141)-Ala-Ser-Ala mutation inhibits binding to dark P-Rh, P-ops, and Rh*, similar to its effect on the WT background (Figs. 2 and 4). Apparently the effects of this particular substitution reflect not so much the reduction of 139-loop length but rather the fact that it is unfavorable for the movement of this loop.
Interestingly, virtually all point mutations in the putative salt bridge between Asp-72 and Lys-142 increase binding of the 3A mutant to P-Rh* (Fig. 4). Unexpectedly, two conservative substitutions, K142H and K142R, favored binding to P-Rh* most, increasing it by ~40% over that of the parental 3A mutant (Fig. 4). The same two mutations were the most potent in enhancing the binding of arrestin-1–3A to dark P-Rh, P-ops, and Rh*, whereas the non-conservative D72A and K142A substitutions produced virtually no effect (Fig. 4). Collectively, these data and the effects of point mutations of these two residues on WT background (Fig. 2) suggest that substitutions act via changing the position and/or conformation of the 139-loop rather than by disrupting the salt bridge in the WT protein. Collectively, the binding data show that certain mutations in the 139-loop significantly change the binding pattern of the base 3A mutant, suggesting that these two elements in the arrestin-1 structure, 139-loop and the C-tail, have distinct functions.

Although the mouse arrestin-1–3A mutant is stable enough for successful transgenic expression in vivo (9, 13, 55), it is somewhat less resistant to heat denaturation than WT protein (32). Therefore, to determine whether the anchoring of the C-tail and 139-loop independently contribute to the maintenance of the basal state of arrestin-1, we tested the effect of 139-loop mutations on the thermal stability of arrestin-1–3A (Fig. 5). The longest deletion Δ(137–142)-Ser had the same devastating effect as on the WT background, with complete loss of activity within 1 h (Figs. 3 and 5). However, a shorter deletion in Δ(137–141)-Ala reduced the stability of 3A mutant to a greater extent than that of WT arrestin-1. Even a two-residue reduction in the length of the 139-loop, Δ(137–141)-Ala-Ser-Ala, which had no effect on the WT background (Fig. 3), significantly reduced the stability of arrestin-1–3A (Fig. 5). Similarly, virtually all point mutations that did not destabilize WT arrestin-1 reduced the survival of 3A mutant. The means ± S.D. of two experiments, each performed in duplicate, are shown.

FIGURE 4. The effects of perturbations of the 139-loop and detachment of the C-tail on rhodopsin binding are additive. The binding of mouse arrestin-1–3A (L374A,V375A,F376A) and indicated mutants constructed on the arrestin-1–3A background to five functional forms of rhodopsin (bar patterns are shown in the 3A panel). Note that 139-loop deletions and several mutations enhance rhodopsin binding additively with the 3A mutation. The magnitude of the effects of point mutations in positions 72 (finger loop) and 142 (139-loop) does not correlate with charge conservation. The means ± S.D. of two-three experiments, each performed in duplicate, are shown in all panels.

FIGURE 5. Deletions and substitutions in the 139-loop reduce the thermal stability of the “preactivated” arrestin-1–3A mutant. Indicated translated arrestins were incubated for 60, 120, or 240 min at 39 °C, and their specific binding to P-Rh* was compared with that of controls kept on ice (0 min). Note that the point mutations that did not affect the stability of WT arrestin-1 (Fig. 3) reduced the survival of 3A mutant. The means ± S.D. of two experiments, each performed in duplicate, are shown.
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(Fig. 6). In the latter case the sum total of the binding of each mutant to dark P-Rh, Rh, Rh*, and P-ops was calculated and expressed relative to WT arrestin-1 or base 3A mutant. These indices on WT background, where corresponding binding of WT arrestin-1 was used for comparison (set at 100%), reveal the autonomous effects of 139-loop mutations. The same parameters calculated relative to arrestin-1–3A reveal the effects of 139-loop mutations in addition to a significant change in binding selectivity induced by 3A mutation. We found that on WT background, two deletions (Δ(137–141)-Ala and Δ(137–141)-Ser) and D72E mutation increased the binding to P-Rh* by 40–50%, with the rest of the mutations producing smaller effects (Fig. 6). The binding to non-preferred forms was changed more dramatically; deletions Δ(137–141)-Ala, Δ(137–141)-Ser, and Δ(137–142)-Ser increased it ~5-fold, whereas K142R mutation increased it ~2-fold (Fig. 6). This is consistent with the idea that P-Rh* can push the 139-loop out of the way (25), whereas non-preferred forms of rhodopsin cannot, which indicates that the 139-loop contributes to arrestin-1 selectivity for P-Rh*. This also explains why perturbations of the 139-loop enhance arrestin-1 binding to non-preferred forms to a much greater extent than to its natural target, P-Rh* (Fig. 6). Interestingly, three mutations in the 139-loop, Δ(137–141)-Ala-Ser-Ala, K142A, and K142Q, that either do not affect or slightly increase P-Rh* binding, actually reduce the binding to non-preferred forms by 35–40% (Fig. 6). These results support the idea that the fit of any arrestin-1 mutant with non-preferred forms of rhodopsin is less perfect than with P-Rh*, making the interaction more sensitive to unfavorable mutations.

The results are consistent with the idea that 139-loop perturbations loosen arrestin-1 conformation, as does the 3A mutation. In this case their effects on the background 3A mutant would be expected to be more modest than on WT background. Indeed, in combination with 3A, only K142H and K142R further increase the binding to P-Rh* and non-preferred forms by ~40% (Fig. 6). However, these substitutions produce significant effects in addition to those of 3A mutation, suggesting that although perturbations of the 139-loop act in the same direction as the C-tail release by 3A mutation, the roles that the 139-loop and the arrestin-1 C-tail play in arrestin-1 binding selectivity are non-redundant. Interestingly, the deletion Δ(137–141)-Ala-Ser-Ala comparably reduces the binding to non-preferred forms on both WT and 3A background (Fig. 6), suggesting the changed sequence has a greater negative effect on binding than possible enhancement by a small reduction of loop length.

Two mechanisms can explain observed binding increases; higher energy of the arrestin-1–rhodopsin interaction or lower energy barrier arrestin-1 needs to overcome to engage rhodopsin. Arrestin-1 has a very high Arrhenius activation energy (~163 kJ mol⁻¹) (58), and the deletion of the C-tail reduces it by half (59). Reduction of the energy barrier is easily detected as an increase in binding at low temperatures (59). We showed that the C-tail release by 3A mutation dramatically increases observed binding to P-Rh* even at 0 °C (6). Therefore, to test the underlying mechanism, we compared P-Rh* binding of WT arrestin-1, 3A mutant, and selected forms where the perturbations of the 139-loop on WT background significantly increase the binding: Δ(137–142)-Ser, Δ(137–141)-Ala, and K142R (Fig. 7). The data confirmed that P-Rh* binding of WT arrestin-1 greatly increases with temperature, consistent with high activation energy (58), and revealed a dramatic reduction of activation energy due to 3A mutation (6). We found that the increase in low temperature binding of Δ(137–142)-Ser mutant, where the 139-loop is shortened by five residues, is almost as great as that of arrestin-1–3A. The behavior of the Δ(137–141)-Ala mutant with smaller deletion is intermediate between WT arrestin-1 and its 3A form, whereas K142R mutation yielded only a minor increase in low temperature binding relative to WT (Fig. 7). These results are consistent with the idea that perturbations of the 139-loop reduce the energy barrier of arrestin-1 activation rather than increase the energy of the arrestin-P-Rh* interaction and demonstrate that the magnitude of the effect corresponds to the scale of the perturbation. This mechanism is consistent with greater effects of 139-loop mutations on the binding to non-preferred forms of rhodopsin than to P-Rh* (Fig. 6) as well as with observed correlation between the reduction of selectivity for P-Rh* (Figs. 2 and 4) and thermal stability of the mutants (Figs. 3 and 5).

DISCUSSION

Mammals express four arrestin subtypes (49), all of which preferentially bind active phosphorylated forms of their cognate receptors (60). Arrestins have several positively charged phosphate binding residues (28, 31, 37, 61) and multiple other exposed side chains that recognize the active receptor conformation (33, 39, 41). Both parts of the interaction interface contribute to binding energy and at the same time serve as sensors. Active phospho-receptor is the only form that can engage phosphorylation and activation sensors simultaneously, thereby disrupting stabilizing intramolecular interactions in arrestin and promoting its transition into high affinity receptor binding state (6), which involves several documented changes in its conformation (21, 25, 38, 40). Thus, in their basal state arrestins are poised to undergo a conformational rearrangement that allows high affinity binding to activated phospho-receptors (for review, see Ref. 50).

Several stabilizing interactions that must be disrupted by the receptor to allow tight binding have been identified. The first is the main phosphate sensor, the polar core, an arrangement of five charged residues (Asp-30, Arg-175, Asp-296, Asp-303, Arg-382 in bovine arrestin-1) located between the two arrestin domains (26, 62). It is destabilized due to the neutralization of the positive charge of Arg-175 by receptor-attached phosphates. This effect can be mimicked by mutations that neutralize or reverse the charge of Arg-175 (37, 61, 63) or its salt bridge partner Asp-296 (62), which yield phosphorylation-independent mutants with high binding to all active forms of rhodopsin, P-Rh* or Rh* (26, 62, 63). The second is the three-element interaction between β-strand I and α-helix I in the N-domain and β-strand XX in the C-tail mediated by hydrophobic residues (26). It is also disrupted by the receptor-attached phosphates interacting with Lys-14 and Lys-15 adjacent to the hydrophobic residues in the β-strand I (43). A recent NMR study suggests that it is also destabilized by arrestin-1 interaction with Rh* (21), indicating that the three-element interaction is more than
just a secondary phosphate sensor. Its disruption by alanine substitutions of interacting side chains on any of these three elements also enhances arrestin-1 binding to Rh* (43, 51). Forced detachment of the C-tail of mouse arrestin-1 by a triple alanine substitution of Leu-374, Val-375, and Phe-376 yields a very stable phosphorylation-independent mutant that was shown to partially compensate for the lack of rhodopsin phosphorylation \textit{in vivo} (55). The third is an interdomain interface in arrestin, dominated by hydrophobic interactions with a single salt bridge (39, 60). The disruption of this salt bridge also enhances arrestin-1 binding to non-preferred forms of rhodopsin, dark P-Rh and Rh* (39).

Here we demonstrate that the 139-loop serves the same purpose as these three interactions; it stabilizes the basal conformation of arrestin-1 and suppresses the binding to non-preferred forms of rhodopsin, thereby enhancing arrestin-1 selectivity for P-Rh*. Interestingly, the deletions in the 139-loop reduce arrestin-1 stability even more dramatically than other activating mutations (compare Figs. 3 and 5 and Refs. 25, 32, and 55). We found that even modest perturbations of the 139-loop by substitutions of Lys-142 in it and Asp-72 in the adjacent finger loop (Fig. 1B) have significant effects on arrestin-1 binding and stability (Figs. 2–6). Unexpectedly, conservative substitutions preserving the charge, such as D72E, K142R, or K142H, significantly affect arrestin-1 selectivity, whereas the elimination of the side chain by the K142A mutation is less potent (Figs. 2–6). The effects of different substitutions of Asp-72 and Lys-142 do not support the idea that the putative salt bridge between these two residues in WT arrestin-1 plays an important role in 139-loop function. The simplest explanation of the
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results is that the size and nature of the side chain in both places determine the actual position and conformation of the 139-loop, thereby affecting arrestin-1 selectivity and stability regardless of their propensity to interact with each other.

From the structural perspective, it is easy to understand why the movements of the C-tail and 139-loop are equally necessary to enable high affinity receptor binding. Even though distal C-tail is not resolved in any arrestin structures (26–29, 35, 64), the visible proximal part suggests that it projects into the cavity of the N-domain (Fig. 1A). Because this cavity was identified as the key receptor binding surface (11, 31, 38, 39, 41, 42, 44–46, 61), the basal position of the C-tail would preclude receptor binding. Similarly, the 139-loop in its basal position (Fig. 1, A and B) would impede receptor access to the cavity of the C-domain and adjacent finger loop, both of which directly participate in receptor binding (11, 20, 33, 36, 38, 39, 41, 42, 45, 47, 48). Thus, the movement of both the C-tail and the 139-loop is necessary to allow receptor access to arrestin elements it needs to engage in order to form a high affinity complex. It is likely that evolution created these two structural obstacles, which are remarkably conserved in all members of the family (49), to enhance the selectivity of arrestins for the phosphorylated active forms of their cognate receptors. Even though invertebrate and some vertebrate arrestins bind unphosphorylated active forms of some G protein-coupled receptors (31, 65–68), it appears that certain elements in these receptors (69–71) or possibly negatively charged head-groups of nearby lipids (72) act in lieu of receptor-attached phosphates to induce the same rearrangements in the arrestin molecule.

The effect of “activating” mutations that enable arrestin-1 binding to non-preferred forms of rhodopsin on protein stability (Figs. 3 and 5 and Refs. 32 and 55) is also easy to rationalize. Because receptor binding requires a significant conformational rearrangement in arrestin (21, 25), the molecule has to “jump over” a substantial energy barrier (58). The simplest mechanism whereby a mutation can enhance arrestin binding to non-preferred forms of the receptor is to lower this barrier, which would also be expected to reduce the thermal stability of the protein. Indeed, we found that the greater the 139-loop deletion was introduced, the more it enables the binding at low temperature (Fig. 7). The fact that perturbations in the 139-loop significantly increase arrestin-1 binding even to P-Rh* (Fig. 6) support this interpretation, consistent with the data that high activation energy kinetically controls arrestin-1 binding to P-Rh* (58) and other forms of rhodopsin (59). Although based on the data we cannot definitively exclude that some of the mutations act thermodynamically via increasing the energy of the arrestin-P-Rh* interaction, the reduction in activation energy is the most parsimonious interpretation of the results. Our binding data also demonstrate nearly perfect correlation between the effects of mutations in the 139-loop on arrestin-1 binding to non-preferred forms of rhodopsin and its thermal stability. However, there are certain mutations that similarly increase the binding to Rh* and dark P-Rh without strong adverse effects on arrestin-1 stability (Figs. 2–5). In fact, recent studies show that fairly stable mutants with greatly enhanced Rh* binding can be engineered (32, 55). Apparently, certain mutations act not so much by significantly lowering the energy barrier of the conformational transition of arrestin as by creating an alternative route for this transition that can be used by an unphosphorylated active receptor to successfully induce necessary conformational changes. Enhanced phosphorylation-independent arrestin mutants were suggested as tools with therapeutic potential that can compensate for defects of receptor phosphorylation (73, 74). In addition to revealing the functional role of the 139-loop in arrestins, our study identified K142R as one of these “benign” mutations, enhancing Rh* binding without appreciable detrimental effects on arrestin-1 stability.

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