Arrestins quench the signaling of a wide variety of G protein-coupled receptors by virtue of high-affinity binding to phosphorylated activated receptors. The high selectivity of arrestins for this particular functional form of receptor ensures their timely binding and dissociation. In a continuing effort to elucidate the molecular mechanisms responsible for arrestin’s selectivity, we used the visual arrestin model to probe the functions of its N-terminal \( \beta \)-strand I comprising the highly conserved hydrophobic element Val-Ile-Phe (residues 11–13) and the adjacent positively charged Lys\(^+\)\(^{14} \) and Lys\(^+\)\(^{15} \). Charge elimination and reversal in positions 14 and 15 dramatically reduce arrestin binding to phosphorylated light-activated rhodopsin (P-Rh*). The same mutations in the context of various constitutively active arrestin mutants (which bind to P-Rh*, dark phosphorylated rhodopsin (P-Rh), and unphosphorylated light-activated rhodopsin (Rh*)) have minimum impact on P-Rh* and Rh* binding and virtually eliminate P-Rh binding. These results suggest that the two lysines “guide” receptor-attached phosphates toward the phosphorylation-sensitive trigger Arg\(^{175} \) and participate in phosphate binding in the active state of arrestin. The elimination of the hydrophobic side chains of residues 11–13 (triple mutation V11A, I12A, and F13A) moderately enhances arrestin binding to P-Rh and Rh*. The effects of triple mutation V11A, I12A, and F13A in the context of phosphorylation-independent mutants suggest that residues 11–13 play a dual role. They stabilize arrestin’s basal conformation via interaction with hydrophobic elements in arrestin’s C-tail and \( \alpha \)-helix I as well as its active state by interactions with alternative partners. In the context of the recently solved crystal structure of arrestin’s basal state, these findings allow us to propose a model of initial phosphate-driven structural rearrangements in arrestin that ultimately result in its transition into the active receptor-binding state.

The first round of signaling by an amazingly diverse superfamily of G protein-coupled receptors is quenched (desensitized) by a uniform two-step mechanism (1). First, the same active receptor conformation that catalyzes GDP/GTP exchange on G proteins is specifically phosphorylated by a G protein-coupled receptor kinase. The active phosphoreceptor is then recognized by a member of the arrestin protein family, which binds tightly to it and makes G protein interaction impossible (apparently, by simple steric exclusion (2)). In photoreceptor cells, visual arrestin dissociates after light-activated rhodopsin decays into opsin (3). Arrestin dissociation allows rhodopsin dephosphorylation and regeneration (3). Nonvisual arrestins bind various components of the internalization machinery of the coated pit with high affinity (4, 5). As a result, in other cells, the complex of nonvisual arrestins with receptors is often internalized via coated pits and can initiate signaling via mitogen-activated protein kinase cascade in the process (6). The loss of active receptor conformation (presumably due to ligand dissociation) facilitates arrestin dissociation, rendering the phosphoreceptor accessible to protein phosphatases. The dephosphorylated receptor can then be recycled back to the plasma membrane (7).

Thus, timely binding and dissociation of arrestin are equally important for high fidelity of this quenching mechanism in all G protein-coupled receptor-driven signaling systems. Both are ensured by a remarkable selectivity of arrestins for the phosphorylated activated form of their cognate receptors. Based on a crystal structure of the basal (inactive) state of visual arrestin (8) and on structure-based targeted mutagenesis (9), we have recently proposed a molecular mechanism for the functioning of arrestin’s main phosphate sensor (8–10). There is an unusual network of buried charged residues in the fulcrum of the two-domain arrestin molecule, which we termed the “polar core” (8, 9). A very delicate charge balance in the polar core due to ion pairing ensures that arrestin is restrained in its basal state. These charged residues include the previously described phosphate-sensitive trigger Arg\(^{175} \) (Arg\(^{169} \) in \( \beta \)-arrestin; Arg\(^{170} \) in arrestin 3) (10–13), which interacts with Asp\(^{296} \) in the polar core. Charge-reversal mutations in the polar core, which disrupt the critical ion pair or upset the charge balance, yield arrestin with enhanced binding to unphosphorylated light-activated rhodopsin (Rh*).\(^{1} \) On the other hand, a combination of these mutations that restores the overall charge balance of the polar core yields mutants virtually as selective for P-Rh* as wild type arrestin (8, 9). Based on these data, we hypothesized that when the receptor-attached phosphate moiety comes close to the polar core, it upsets the charge balance, allowing arrestin to undergo a transition into its active high-affinity receptor-
binding state (8, 9). This mechanism adequately explains how stereoisomerically heterogeneous phosphoreceptor molecules with different numbers of phosphates attached at various sites induce activation of arrestin in the same fashion. However, this mechanism does not fully explain how certain N- and C-terminal mutations (13–18) render arrestin proteins phosphorylation-independent, i.e. allow them to bind with high affinity to activated unphosphorylated receptors. Here we use a visual arrestin-rhodopsin model to further explore the mechanism of arrestin’s strict preference for phosphoreceptors and describe a previously unappreciated phosphate-binding element in the N terminus of arrestin. This two-residue element (Lys14, Lys15) apparently plays a key role in the process of arrestin activation, as well as in phosphate interaction in the active receptor-binding state of arrestin (i.e. after the polar core is rearranged). An adjacent structural element in the N terminus (Val11, Ile13, and Phe14) participates in intramolecular interactions stabilizing the structurally well-defined (8) basal (inactive) conformation and presumably the quite different (14, 19) active conformation of visual arrestin. The two phosphate-binding elements appear to be functionally interdependent. Together, they create an ingenious failsafe mechanism ensuring arrestin’s selectivity for phosphorylated receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP, [3H]leucine, and [14C]leucine were purchased from PerkinElmer Life Sciences. All restriction enzymes were from New England Biolabs. Sepharose 2B and all other chemicals were from the sources described previously (11, 14). Rabbit reticulocyte lysate and SP6 RNA polymerase were prepared as described previously (20). 11-cis-Retinal was generously supplied by Dr. R. K. Crouch (Medical University of South Carolina, Charleston, SC).

**Site-directed Mutagenesis**—Bovine visual arrestin cDNA (21) was a gift from Dr. T. Shinohara (Brigham and Women’s Hospital, Boston, MA). The plasmid pARR-VSP was constructed and modified as described previously (10, 15). This pGEM2-based plasmid encodes bovine wild type arrestin with an “idealized” 5’-untranslated region (20) under the control of the SP6 promoter. Construct pARR-SC (15) was used for all further mutagenesis. All mutations were introduced by polymerase chain reaction using an appropriate mutating oligonucleotide as a forward primer and an oligonucleotide downstream from the tranlation initiation 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 polymerase chain reaction. The resulting fragments were digested with EcoRI/BamHI (N terminus, BamHI/SalI (α-helix I), or BstXI/HindIII (C-tail) and subcloned into appropriately digested pARR-SC. Double and triple mutants were constructed by excising fragments with one mutation and subcloning them into an appropriately digested plasmid carrying another mutation. The sequence of all constructs was confirmed by dyeoxy sequencing.

**In vitro Transcription, Translation, and Evaluation of Mutants’ Stability**—Plasmids were linearized with HindIII. In vitro transcription and translation were performed as described previously (14, 20, 22). All arrestin proteins were labeled by incorporation of [3H]leucine and [14C]leucine, with the specific activity of the mix = 1.5–3 Ci/mmol, resulting in the specific activity of arrestin proteins within the range of 54–90 Ci/mmol (120–200 dpm/fmol). The translation of each of the arrestin mutants used in this study produced a single labeled protein with the expected mobility on SDS-polyacrylamide gel electrophoresis. Two parameters were used for the assessment of mutant phosphorylation: the percentage of wild type arrestin remaining in the supernatant after incubation for 10 min at 37 °C followed by centrifugation. This integral parameter calculated for a mutant was expressed as a percentage of that for wild type arrestin (15). The relative stability of all mutants used in this study exceeds 55%.

**Rhodopsin Preparations**—Urea-treated rod outer segment membranes were prepared, phosphorylated with rhodopsin kinase, and re-generated with 11-cis-retinal as described previously (14, 23, 24). The average stoichiometry of phosphorylation for the rhodopsin preparations used in these studies was 2.4 mol phosphate/mol rhodopsin.

**Arrestin Binding to Rhodopsin**—In vitro translated arrestins (100 fmol) were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl2, 1.5 mM dithiothreitol, and 50 mM potassium acetate at 7.5 pM of the various functional forms of rhodopsin in a final volume of 50 μl for 5 min at 37 °C either in the dark or in room light (14, 22). The samples were immediately cooled on ice and loaded (under dim red light for dark samples) onto 2 ml Sepharose 2B columns equilibrated with 10 mM Tris-HCl, pH 7.5, and 100 mM NaCl. Bound arrestin eluted with the rod outer segments in the void volume (between 0.5 and 1.1 ml). Nonspecific binding determined in the presence of 0.3 μg of liposomes (<1% of the arrestin present in the assay) was subtracted.

**RESULTS**

Three groups of mutations yielding “constitutively active” phosphorylation-independent arrestin proteins have been described thus far (8–18): (a) charge neutralization or reversal mutations in the polar core (8–13), (b) certain mutations and short deletions in arrestin’s C-tail (13–18), and (c) the deletion of residues 2–16 in the N terminus (14, 16). All known activating mutations that do not directly affect the polar core are likely to disturb the three-element interaction that in the basal state involves β-strand I and α-helix I in the N-domain and β-strand XX in the C-tail (8), suggesting that it may be another “hot spot” in arrestin. (Note that here and below, all structural elements are designated according to Ref. 8.)

To find out how structurally diverse mutations yield similar phenotypes, we combined the Δ(2–16) mutation with other activating mutations. Unexpectedly, most of the combinations demonstrated lower P-Rh* and Rh* binding than the parental mutants, with arrestinΔ(2–16)/(R175E) showing the most dramatic 45- and 12-fold reduction in P-Rh* and Rh* binding, respectively (Fig. 1, A and B). Two aspects regarding the binding profile of the combination mutants appear most remarkable: similar to the Δ(2–16) mutant (and in sharp contrast to all other phosphorylation-independent forms of arrestin), they all demonstrate virtually no binding to P-Rh, and, if anything, they prefer Rh* over P-Rh* (Fig. 1B). Thus, the Δ(2–16) mutation works as an “override switch,” making all combination mutants functionally similar to the Δ(2–16) mutant itself. The only exception is the combination of the Δ(2–16) and R175E, where the interaction of two activating mutations yields a protein with remarkable stability (140% of wild type) but very limited propensity for transition into an active rhodopsin-binding state.

Of the 15 residues eliminated by the Δ(2–16) mutation, four (12 through 15 in bovine visual arrestin) are conserved from Caenorhabditis elegans and Drosophila to humans (Fig. 2). The conserved sequence includes two chemically diverse elements: a group of 2 or 3 bulky hydrophobic residues, and 2 positively charged lysines (Fig. 2). To test what part of the N terminus is responsible for the effects of the Δ(2–16) mutation, we constructed arrestins with three shorter deletions: Δ(2–6), Δ(7–10), and Δ(11–16). Not surprisingly, the region 11–16, comprising all conserved residues, appears to be largely responsible for the effects of the Δ(2–16) mutation (Fig. 3A). The two elements in the 11–16 region play essentially opposite roles in arrestin binding (Fig. 3B). The N3A mutation yields a phosphorylation-independent arrestin with somewhat lower potency than R175E, whereas the K14A, K15A double mutation reduces arrestin binding to P-Rh* about 10-fold. This effect cannot be explained by its reduced stability (it is about 60% of wild type, i.e. similar to that of R175E mutant). Adjacent residues Ile14 and His10 do not appear to play a significant functional role (Fig. 3B).

Next we tested the role of selected individual residues within the two elements: Ile12, Lys14, and Lys15 (Fig. 4). The impact of
I12A mutation is minimal, although according to the crystal structure in the basal state Ile 12 interacts with at least five partners in arrestin's C-tail and α-helix I (8). Apparently, either neighboring residues (Val 11 and/or Phe13) can substitute for Ile12 or the stability of the complex is the result of a cooperative multiresidue interaction, as is the case for the C-tail (15), another participant of the three-element interaction. The effects of both K14A and K15A mutations are profound: a 2-fold and nearly 8-fold reduction in P-Rh* binding, respectively (Fig. 4A). In fact, K15A is the most potent loss of function 1-residue mutation characterized thus far in arrestin (9–18). Amazingly, the D(11–16) mutation eliminating both Lys14 and Lys15 (along with the hydrophobic residues 11–13) reduces arrestin binding to P-Rh* by a modest 20% (Fig. 4A), and even a greater Δ(2–16) deletion appears to be less disruptive than K15A alone (Figs. 1 and 4). Conceivably (cf. Fig. 2), the combination of Asn and His placed into this position by the D(11–16) mutation and the amino group placed on the N terminus by the D(2–16) mutation can partially compensate for the loss of Lys 14 and Lys 15.

To gain insight into the mechanism of the N-terminal mutations' action, we combined Δ(11–16) with other activating mutations (11, 12, 15): R175E in the polar core and 3A in the C-tail (Fig. 4A). For comparison, we also combined the N3A mutation with R175E. Mutation Δ(11–16) modestly reduces P-Rh* binding of wild type and the 3A mutant (compare 3A and Δ(11–16)+3A). In contrast, it induces a more than 3-fold reduction of R175E mutant's P-Rh* binding (compare R175E and Δ(11–16)R175E). The N3A mutation has virtually no effect on P-Rh* binding of wild type and R175E arrestin (Fig. 4A). Thus, the elimination of positively charged Lys 14 and Lys 15 appears to be the reason for decreased P-Rh* binding of the Δ(11–16) mutant. The magnitude of this effect is context-dependent and is greatest in combination with R175E, i.e. a mutation that eliminates a positive charge implicated in phosphate binding (8–13). These data suggest that Lys 14 and Lys 15 may also play a role in phosphate binding, but not in arrestin interactions with other parts of rhodopsin, as evidenced by a relatively modest effect of Δ(11–16) mutation on the Rh* binding of combination mutants.

To test this idea, we constructed charge-reversal mutations...
K14E and K15E, as well as the conservative K14R and K15R mutations. If Lys14 and Lys15 participate in phosphate binding, we expected that K→E change would be even more detrimental to P-Rh* and P-Rh binding than K→A mutations, whereas the effects of K→R mutations, if any, would be less significant. That is exactly what we observed (Fig. 4, B and C). Thus, Lys14 and Lys15 apparently participate in phosphate binding, with Lys15 playing a major role in it. The 35% reduction in P-Rh* binding of the K15R mutant also suggests the importance of the exact size and geometry of the side chain in this position and explains the absolute conservation of lysine throughout the arrestin family (Fig. 2). Indirect support for the participation of this element in phosphate binding also comes from a recent study of the ability of arrestin peptides to compete with arrestin and transducin (34). Two peptides that include Lys14 and Lys15 inhibited arrestin binding to P-Rh* much more effectively than transducin binding to Rh*, suggesting that rhodopsin-attached phosphates play an important role in their binding to P-Rh*, whereas other peptides demonstrated comparable effectiveness in both assays (34).

Next we tested the individual K14A and K15A mutations in the context of four different phosphorylation-independent mutants (Fig. 2): arrestin-3A and three structurally distinct types of polar core mutants. The latter include: (a) arrestin(1–378) in which the distal C-tail with a polar core element Arg382 is deleted, but all three bulky hydrophobic residues mutated in 3A are preserved (8, 15); (b) R175E with charge-reversal mutation of the phosphate-binding polar core residue (8, 9, 11);
and (c) another polar core charge-reversal mutant, D296R, which has an intact phosphate-binding Arg<sup>175</sup> (8, 9). All constitutively active mutants bind fairly well not only to P-Rh<sup>*</sup> but also to other functional forms of rhodopsin. This allowed us to independently measure the effects of K14A and K15A mutations on P-Rh binding (which is mediated by phosphate-binding elements) and on Rh<sup>*</sup> binding mediated by an activation recognition site (14–16), where phosphate-binding elements are unlikely to play any role.

The most striking feature of all the K15A mutants is a 85–100% reduction in P-Rh binding (as compared with parental wild type and mutant proteins) (Fig. 4B). In contrast, there is virtually no inhibition of Rh<sup>*</sup> binding. The effect of the K15A mutation on P-Rh<sup>*</sup> binding depends on the context: strong (86%) inhibition is seen in the wild type, moderate (27%) inhibition is seen in the R175E background, and minimum effect is seen in other constitutively active mutants (Fig. 4B). Somewhat weaker effects of K14A mutation follow the same pattern (Fig. 4C). These data strongly support the idea that both Lys<sup>14</sup> and Lys<sup>15</sup> participate in phosphate binding. Both K→A mutations reduce P-Rh<sup>*</sup> binding in the context of wild type protein to a much greater extent than in the context of any phosphorylation-independent mutant. The key difference between these two situations is that rhodopsin’s phosphorylated C-terminal segment needs to reach Arg<sup>175</sup> (localized at the very bottom of the “bowl” formed by the arrestin N-domain; Fig. 5) to activate wild type arrestin (8, 9), whereas in the case of phosphorylation-independent mutants, this is not necessary. Lys<sup>14</sup> and Lys<sup>15</sup> are on the rim of this bowl (Fig. 5). Thus, one of the functions of these two residues is to deliver or guide the phosphate to Arg<sup>175</sup>. This explains the profound effect of neutralizing K→A mutations on P-Rh<sup>*</sup> binding in wild type arrestin and the small to nonexistent effect on P-Rh<sup>*</sup> binding of phosphorylation-independent mutants at the same time. The somewhat greater effect of these mutations on R175E binding to P-Rh<sup>*</sup> along with no effect on its Rh<sup>*</sup> binding suggests that in the active receptor-bound state of arrestin, these lysines still interact with the phosphate. Then the loss of a positive charge on either of these lysines, combined with the mutation R175E (net charge change from +2 to −1), destabilizes the arrestin-phosphoreceptor complex (resulting in lower P-Rh<sup>*</sup> binding) but does not seriously affect the stability of arrestin complex with unphosphorylated Rh<sup>*</sup>. In contrast, when an activating mutation used does not eliminate phosphate-binding positive charges, the effect of K→A mutations on P-Rh<sup>*</sup> binding is negligible (Fig. 4).

Lys<sup>14</sup> and Lys<sup>15</sup> are localized on the short β-strand I next to the three hydrophobic residues 11–13 (Fig. 5). This element stabilizes the basal arrestin conformation (Refs. 8 and 14; Fig. 3B) via a hydrophobic three-element interaction with residues 375–377 (Phe-Val-Phe) of the C-tail and leucines 103, 107, and 111 of the α-helix I (Ref. 8; Fig. 6). A wealth of previous mutagenesis data (14–18) in the context of the three-dimensional structure of arrestin’s basal state (8) suggests that the three-element interaction is disrupted and/or rearranged in the process of arrestin’s transition into the active state.

To shed light on the exact nature of this putative rearrangement, we compared the effects of the triple mutations that eliminate the bulky hydrophobic side chains in each of the three elements: (a) N3A, (b) h3A, and (c) the previously characterized (15) 3A mutation. All three triple mutations were then tested in the context of the wild type protein and in various combinations with each other (Fig. 7A). The crystal structure suggests that all three hydrophobic elements stabilize arrestin’s basal state (8). For some of the residues, this could be their only function. The elimination of such an element can be expected to enhance arrestin binding to P-Rh<sup>*</sup>, P-Rh, and Rh<sup>*</sup>, regardless of whether there are any additional mutations in arrestin. If an element stabilizes both the basal and active states of arrestin, its elimination may either enhance or reduce binding, depending on its relative contribution to the stability of the two states.

Individually, all three triple mutations cause varying degrees of constitutive activity (enhanced binding to P-Rh and Rh<sup>*</sup>) (Fig. 7). However, the constitutive activity of the 3A mutant is very strong, the constitutive activity of N3A is modest, and the constitutive activity of h3A is rather weak. Notably, in terms of binding to all functional forms of rhodopsin (P-Rh<sup>*</sup>, P-Rh, and Rh<sup>*</sup>), the order of potency is the same: 3A > N3A > h3A, suggesting that all three hydrophobic elements are unlikely to be directly involved in phosphate binding or activation recognition. These mutations (similar to other activating mutations in arrestin) enhance even P-Rh<sup>*</sup> binding, with the exception of h3A.

The effects of h3A mutation in various contexts (i.e. h3A versus wild type; h3A+N3A versus N3A alone; or h3A+3A versus 3A, and so forth) are summarized in Fig. 7A. The data suggest a dual role for leucines 103, 107, and 111 in α-helix I. These residues stabilize the basal state (8). Hence, their removal facilitates arrestin’s transition into its active state, thus
Increasing P-Rh and Rh* binding of the h3A mutant (Fig. 7A). This effect is relatively weak and apparent only in the context of the conformationally constrained wild type arrestin. The h3A mutation reduces P-Rh* binding of wild type arrestin and the binding of constitutively active mutants to all functional forms of rhodopsin. Conceivably, leucines 103, 107, and 111 also participate in the interaction(s) stabilizing the active state of arrestin, so that their loss facilitates arrestin’s return to the basal state and its dissociation, thus reducing the binding. This effect of the h3A mutation is also relatively modest (<30%), with two exceptions. When the N-terminal hydrophobic element is missing (i.e., in case of N3A–h3A+N3A and 3A+N3A–h3A+3A+N3A), the h3A mutation results in about a 3-fold drop in P-Rh binding (Fig. 7A).

The effects of the N3A mutation tend to be stronger than the effects of the h3A mutation, but they are just as context-dependent (Fig. 7A). This mutation enhances the binding to all functional forms of rhodopsin in the context of conformationally restrained arrestin (compare N3A and wild type) and decreases it in the context of the “loose” constitutively active mutant (compare 3A and 3A+N3A). In short, it appears that the functions of the hydrophobic element in the N terminus include the stabilization of both the basal and active receptor-bound state of arrestin, similar to α-helix I.

The effects of the 3A mutation on arrestin binding to all functional forms of rhodopsin are remarkably uniform and virtually context-independent: the elimination of the hydrophobic element in the C-tail enhances binding (Fig. 7A). As could be expected, the magnitude of the effect is much greater in the context of the most constrained arrestin (compare wild type and 3A mutant) than in the context of a protein with its basal conformation destabilized by other mutations (e.g., compare N3A and N3A+3A).

Taken together, these data suggest that the only function of the hydrophobic element in arrestin’s C-tail is the stabilization of the basal state and that the overall contribution of this element is considerable. In contrast, hydrophobic elements in the N terminus and α-helix I also participate in the stabilization of the active receptor-bound state of arrestin. The semiadditive inhibitory action of h3A and N3A mutations (most prominent in the case of P-Rh binding; e.g., compare 3A, 3A+N3A, and 3A+N3A+h3A in Fig. 7A) suggests that in the active state these two elements may interact with alternative partners rather than with each other.

In the basal state, hydrophobic three-element interaction “anchors” arrestin’s C-tail (Figs. 5 and 6). The effects of the 3A mutation suggest that the loss of the “anchor” is sufficient for a dramatic increase in arrestin’s propensity to assume its high-affinity receptor-binding state (15) (Fig. 7A). Because partial deletions in arrestin’s C-tail by mutagenesis (14–18) or alternative splicing (26) have similar functional consequences, the 3A mutation apparently induces a release of the C-tail. A similar C-tail release is induced by arrestin binding to P-Rh* (16, 27), i.e., by the normal process of wild type arrestin’s transition into its active state. The displacement of the C-tail likely removes a positively charged residue, Arg382, from the polar core, the neutralization of which per se (R382N mutation) has been shown to enhance arrestin binding to P-Rh*, P-Rh, and Rh* (15). This reasoning is based on the premise that the positioning of Arg382 and the three hydrophobic residues (375–377) in the C-tail is interdependent. If that is so, a change in the length of the “connector” between these elements can be expected to facilitate arrestin’s transition into the active state and yield mutants with reduced selectivity (increased binding to Rh* and P-Rh). There are only 4 residues between the two elements, 3 of which also interact with β-strand I (8). Therefore, to manipulate the length of this connector without introducing additional changes, we either deleted Ala381 or inserted 1, 2, and 3 extra alanines between Ala381 and Arg382. As shown in Fig. 7B, the change of the connector length enhances P-Rh* binding up to 50% and Rh* and P-Rh binding up to 7-fold. The effects of the 1-residue change in either direction are similar and quite substantial, and longer insertions are even more potent. Arrestins with 2 or 3 extra alanines demonstrate a constitutive activity as high as that of the R382N mutant (15), which is, however, lower than that of the 3A mutant. These data suggest that when the distance between the C-tail hydrophobic residues and Arg382 is forcibly changed by mutagenesis, it is Arg382 that likely gets displaced, in line with the idea that Arg382 is held in position by the rest of the C-tail.

**DISCUSSION**

The identification of an additional phosphate-binding element in arrestin and its strategic positioning next to the residues participating in the three-element interaction stabilizing the basal conformation has important implications concerning the mechanism of arrestin’s transition into the active binding-competent state and the number of receptor-attached phosphates necessary to induce it.

**How Many Phosphates Does Arrestin Need for High-affinity Binding?—** In the basal state (8), the distances between residues implicated in phosphate binding (Refs. 10 and 11 and the data presented above; Lys15 and Arg175 as well as between Arg171 and Arg175) are 15 and 14.5 Å, respectively (Fig. 5). Without a conformational rearrangement of arrestin, a minimum of three phosphates are needed to reach all these residues simultaneously. Whereas it is possible (see below) that after phosphate binding Lys15 moves closer to Arg175, Arg171 and Arg175 cannot move much closer to each other without distorting β-strand X (Fig. 5). Considering the size of the phosphate moiety, it appears highly unlikely that one phosphate-attached phosphate can reach all these residues to induce arrestin transition into the active receptor binding state. The number of positively charged arrestin residues implicated in phosphate interaction (Lys14, Lys15, Arg171, Arg175, and Lys176) supports this notion. Direct binding experiments also suggest that visual (14), β-arrestin (17), and arrestin 3 (17) require the stoichiometrical interaction of arrestin with three phosphate groups.
Phosphate-binding Elements in Arrestin

Fig. 7. The effects of the alanine substitution of hydrophobic residues in the N-domain structural elements β-strand 1 and α-helix I and in β-strand XX of arrestin’s C-tail (A). The exact length of the connector between polar core residue Arg382 and hydrophobic residues in the C-tail is crucial for arrestin selectivity (B). In B, Ala381 was deleted or 1, 2, or 3 extra alanine residues (iA, iAA, and iAAA, respectively) were inserted upstream of Arg382. Binding was performed as described in the legend to Fig. 1. The means ± S.D. from two experiments (each performed in duplicate) are shown.

Phosphate-binding Elements in Arrestin

The activation energy of an arrestin splice variant lacking the C-tail is 70 kJ/mol (29), suggesting that about 70 kJ/mol are necessary for arrestin’s transition into the receptor binding state. Elucidating the latter requires determination of at least two phosphates/receptor. It has been reported that in the acute experiments in mice in vivo in which 5–50% of the rhodopsin was bleached, the predominant product is monophosphorylated (at Ser334 or Ser338) rhodopsin (32). The authors concluded that these two serines are the primary targets for rhodopsin kinase and that monophosphorhodopsin is the species likely quenched by arrestin in vivo, whereas polyphosphorylation may be an in vitro artifact (32). However, a recent study of the kinetics of rhodopsin deactivation in transgenic mice expressing various rhodopsin mutants with a decreased number of phosphorylation sites shows that in vivo, at least three phosphorylation sites in the rhodopsin C terminus are necessary for rapid and reproducible inactivation (33). In full agreement with our model of arrestin function (8, 9), it does not matter which sites rhodopsin has, as long as their overall number is three or more (33). Moreover, the rhodopsin with only threonines in the C terminus is quenched rapidly (33). Interestingly, the more phosphorylation sites rhodopsin has, the faster photoresponse is quenched, suggesting that rhodopsin multiphosphorylation is crucial for the fast and reproducible termination of the response (33). One of the possible reasons for this controversy is the high level of rhodopsin bleaching in the experiments reported by Ohguro et al. (32). Conceivably, virtually simultaneous bleaching by a short flash of a large number of rhodopsins makes rhodopsin kinase limiting and results in the predominance of monophosphorylated rhodopsin products. The studies of Mendez et al. (33) were performed at physiologically relevant low intensities of light.

What Changes Can Phosphates Induce in Arrestin Molecule?—The side chain of Lys15 points inward, toward the cavity of the bowl (Fig. 5). In contrast, Lys14 points outward (Fig. 5). The distances between the positive charge of Lys14 and Arg171 and Arg175 (19 and 22 Å, respectively) are far greater than the distances between Lys15 and these arginines, and even the distance between the amino groups of Lys14 and Lys15 is 13 Å (Fig. 5). Thus, to bind the phosphate interacting with Lys15 and/or any other phosphate-binding residue, Lys14 has to flip over. We hypothesize that the phosphate moieties attached to rhodopsin’s C terminus first encounter Lys14 and Lys15 on the rim of the bowl (Fig. 5). As the phosphates keep moving further into the N-domain bowl toward the polar core, Lys15 is dragged along. Either this movement of Lys15 destabilizes β-strand I and allows Lys14 to flip over, or Lys14 itself moves with the phosphate in a similar fashion, but the net result is a serious distortion or a complete meltdown of β-strand I. The consequences of this structural rearrangement are 2-fold. First, it allows Lys14 and Lys15 to attain positions favorable for their interaction with phosphates that have already reached the bottom of the N-domain bowl. Second, the distortion likely spreads through the rest of β-strand I and moves its hydrophobic element Val-Ile-Phe into a position that is unfavorable for its interaction with the hydrophobic element Phe-Val-Phe of the C-tail and presumably favorable for interaction with its alternative partner(s). As a result, arrestin’s C-tail loosens its anchor (8).

The disruption of multiple intramolecular interactions necessary for arrestin’s transition into the receptor binding state explains its unusually high activation energy (140 kJ/mol) (19). The activation energy of an arrestin splice variant lacking the C-tail is 70 kJ/mol (29), suggesting that about 70 kJ/mol are necessary for C-tail displacement. The rest may be expended on the proposed “melting” of β-strand I and other rearrangements of the molecule.

The Model of the Initial Stages of Arrestin’s Activation—To fully understand the mechanism of arrestin function, we need to know the structure of both its basal (8) and active high-affinity receptor binding state. Elucidating the latter requires the crystallization of the arrestin-receptor complex, which has yet to be accomplished. However, we believe that the data presented here allow us to propose a model for the initial stages of arrestin’s transition into the active state and to draw certain conclusions concerning its structure (Fig. 8). It is widely accepted that the cytoplasmic side of rhodopsin molecule first interacts with the cavity of the bowl of arrestin N-domain and that ultimately the phosphates need to reach Arg175 at the bottom of this bowl (8, 9, 25). In the basal state of arrestin (8), 2 of the residues that participate in phosphate binding (10, 11), Arg175 and Lys176, appear to be virtually inaccessible from the cavity of the N-domain bowl. Arg175 is kept in this position primarily by a salt bridge with Asp296 (8, 9). The disruption of this salt bridge by mutagenesis facilitates arrestin transition into its active state (8–11), suggesting that it is disrupted in...
the process of normal arrestin activation by P-Rh*. Therefore, it is tempting to speculate that in the arrestin-phosphorylretinal complex, both Arg<sup>175</sup> and Lys<sup>176</sup> point toward the cavity of the N-domain and the rhodopsin-attached phosphates. Structurally (Ref. 8; Fig. 5), the turn of these two residues in the direction of the cavity would make β-strand X parallel to β-strands V and VI and does not require any extensive re-arrangements in the N-domain.

On the basis of the available data, we hypothesize that receptor-attached phosphates upset the charge balance of the polar core in two ways (Fig. 8). First, phosphates interact with Lys<sup>14</sup> and Lys<sup>15</sup>, inducing a distortion of β-strand I with the ensuing release of the C-tail and resulting removal of a positively charged Arg<sup>382</sup> from the polar core. Second, phosphates neutralize yet another positive charge in the polar core, that of Arg<sup>175</sup> and likely fix this residue in a position unfavorable for the interaction with Asp<sup>296</sup>. This leaves the remaining three negative charges (Asp<sup>390</sup>, Asp<sup>296</sup>, and Asp<sup>303</sup>) in the fulcrum of the two-domain arrestin molecule (8, 9, 25) without any counter ions. Their mutual repulsion likely drives the global rearrangement of the molecule (Fig. 8), involving the movement of the two domains relative to each other (8, 14, 19). This movement appears necessary to bring all arrestin residues implicated in rhodopsin binding (8, 14, 17, 28, 34) into a position favorable for their interaction with a relatively compact structure formed by rhodopsin's cytoplasmic loops and C-terminus (35). The exact nature of this rearrangement and the arrestin and rhodopsin residues involved in the stabilization of the active arrestin conformation and arrestin-receptor complex remain to be elucidated.

Acknowledgments—We are grateful to Dr. J. L. Benovic for purified rhodopsin kinase, Dr. R. K. Crouch for 11-cis-retinal, Dr. T. Shinohara for arrestin cDNA, and Dr. J. A. Hirsch for critical reading of the manuscript and helpful discussions.

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**FIG. 8. The model of arrestin’s interaction with P-Rh*.** We hypothesize that the negatively charged phosphates (red circles) on the C-terminal segment of the receptor interact with Lys<sup>14</sup> and Lys<sup>15</sup> (blue half circles) located on β-strand I and are guided toward the positive potential (blue surface) above the polar core. The charge balance of the polar core is maintained by positive and negative charges contributed by arrestin’s N-domain (pink), C-domain (cyan), and the C-tail (ocher) and is symbolized by overlapping concentric circles indicating positive (blue) and negative (red) charges. The interaction between β-strands I and XX (green broken lines) also stabilizes arrestin’s basal state. The intrusion of the phosphates upsets the charge balance in the polar core and leads to a rearrangement (arrows) of the polar core due to the resulting dominating negative potential (red surface). The conformational changes also include the disruption of the strand-strand interaction and additional rearrangements induced by the binding of P-Rh* to the N- and C-domain. The exact location of the rhodopsin-binding sites remains to be elucidated. Several lines of evidence (8, 14, 17, 28, 34) strongly suggest the participation of both N- and C-domains in the interaction, as depicted.
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