How Does Arrestin Respond to the Phosphorylated State of Rhodopsin?

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Visual arrestin quenches light-induced signaling by binding to light-activated, phosphorylated rhodopsin (P-Rh*). Here we present structure-function data, which in conjunction with the refined crystal structure of arrestin (Hirsch, J. A., Schubert, C., Gurevich, V. V., and Sigler, P. B. (1999) Cell, in press), support a model for the conversion of a basal or “inactive” conformation of free arrestin to one that can bind to and inhibit the light activated receptor. The trigger for this transition is an interaction of the phosphorylated COOH-terminal segment of the receptor with arrestin that disrupts intramolecular interactions, including a hydrogene-bonded network of buried, charged side chains, referred to as the “polar core.” This disruption permits structural adjustments that allow arrestin to bind to the receptor. Our mutational survey identifies residues in arrestin (Arg175, Asp30, Asp296, Asp303, Arg382), which when altered bypass the need for the interaction with the receptor’s phosphopeptide, enabling arrestin to bind to activated, nonphosphorylated rhodopsin (Rh*). These mutational changes disrupt interactions and substructures which the crystallographic model and previous biochemical studies have shown are responsible for maintaining the inactive state. The molecular basis for these disruptions was confirmed by successfully introducing structure-based second site substitutions that restored the critical interactions. The nearly absolute conservation of the mutagenically sensitive residues throughout the arrestin family suggests that this mechanism is likely to be applicable to arrestin-mediated desensitization of most G-protein-coupled receptors.

The visual amplification cascade (light-activated rhodopsin (Rh*) → transducin → cGMP phosphodiesterase) is an archetypal signaling pathway initiated by a G protein-coupled receptor (1). A single Rh* can activate sequentially hundreds of transducin molecules. Active cGMP phosphodiesterase hydrolyzes hundreds to thousands of cGMP molecules. Thus, the potential for signal amplification is enormous, providing for a very high sensitivity and requiring mechanisms to moderate the response within a vast dynamic range.

Visual signal transduction has a special requirement, since the lifetime of activated rhodopsin is far longer than the required time resolution. Hence, a molecular mechanism is necessary to rapidly shut down the receptor. This is accomplished by the combined effect of rhodopsin kinase and arrestin on light-activated rhodopsin; the former quickly phosphorylates the COOH-terminal segment of Rh* and arrestin then binds to P-Rh*, blocking further transducin activation (2, 3). Arrestin appears to stay bound until metarhodopsin II decays into opsin, but ultimately it must dissociate to allow the dephosphorylation of phosphoprotein (4).

It has been proposed that free arrestin exists in an inactive state and is activated only by interaction with the phosphorylated form of Rh* (5, 6). Inactive arrestin has a bipartite molecular architecture with an N- and C-domain each comprised of a β-sandwich (9, 20). In addition, it has an extended carboxyl-terminal chain called the C-tail bound to the surface of both N- and C-domains (20). In this study we used site-directed mutagenesis, which combined with previous mutagenesis, biochemical studies (5, 6), and the crystal structure (20) of arrestin, allows us to propose a mechanism for arrestin’s response to the phosphorylated, activated receptor.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP, [14C]leucine, and [3H]leucine were purchased from NEN Life Science Products. All restriction enzymes were purchased from New England Biolabs. Sepharose 2B and all other chemicals were from sources described previously (6). Rabbit reticulocyte lysate and SP6 RNA polymerase were prepared as described previously (10). 11-cis-Retinal was generously supplied by Dr. R. K. Crouch.

Site-directed Mutagenesis—Bovine visual arrestin cDNA (11) was generously supplied by Dr. T. Shinohara. The plasmid pARR-VSP was constructed and modified as described earlier (12, 13). This pGEM2-based plasmid encodes bovine wild type arrestin with an “idealized” 5’-untranslated region (10) under control of a SP6 promoter. Construct pARR-SC was used for all further mutagenesis. All mutations were introduced by PCR using an appropriate mutagenizing oligonucleotide 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 all constructs was confirmed by dideoxy sequencing.

In Vitro Transcription, Translation, and Evaluation of Mutants’ Stability—Plasmids were linearized with HindIII before in vitro transcription to produce mRNAs encoding full-length arrestin proteins. In vitro transcription and translation were performed as described previously (6, 10). All arrestin proteins were labeled by incorporation of rhodopsin; Rh, dark nonphosphorylated rhodopsin; P-Rh*, light-activated phosphorylated rhodopsin; P-Rh, dark phosphorylated rhodopsin; PCR, polymerase chain reaction; WT, wild type.

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rhodopsin, P-Rh*, Rh*, or 329G-Rh* (12, 14, 15). Mutagenesis can bypass the need for phosphorylated receptors, with a particular residue’s charge neutralized or reversed by affinity rhodopsin binding state. Therefore, mutant arrestins of the constraints that prevent arrestin from assuming its high charge, disrupting these interactions, thereby releasing some 

ries have identified Arg175 as a phosphorylation-responsive trig-

phosphorylated Rh* and even 329G-Rh* (rhodopsin from which yields proteins with dramatically enhanced binding to non-

ceptor, [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/mmol). The translation of each of the arrestin mutants used in this study produced a single labeled protein band with the expected mobility on SDS-polyacrylamide gel electrophoresis. Two parameters were used for the assessment of muta-

Phosphate Sensor in Arrestin

The relative stability of all mutants used in this study exceeds 0.5.

Rhodopsin Preparations—Urea-treated rod outer segment mem-

branes were prepared, phosphorylated with rhodopsin kinase, and re-

generated with 11-cis-retinal as described (14). The stoichiometry of

RESULTS AND DISCUSSION

Perturbing the Polar Core—Our previous mutagenesis studies have identified Arg72 as a phosphorylation-responsive trigger in visual arrestin (12, 14, 15). Every mutation in the 175 position, with the exception of the most conservative R175K, yields proteins with dramatically enhanced binding to non-phosphorylated Rh* and even 329G-Rh* (rhodopsin from which the COOH terminus along with all rhodopsin kinase phosphorylation sites has been proteolytically removed) (14, 15). These mutations led us to conclude that Arg175 may interact with negatively charged neighboring residues and that this type of intramolecular interaction contributes to the stability of arrestin’s inactive basal state. We hypothesized that the association of the receptor’s phosphate with Arg175 neutralizes its positive charge, disrupting these interactions, thereby releasing some of the constraints that prevent arrestin from assuming its high affinity rhodopsin binding state. Therefore, mutant arrestins with a particular residue’s charge neutralized or reversed by mutagenesis can bypass the need for phosphorylated receptors, i.e. they bind with high affinity to any light-activated form of rhodopsin, P-Rh*, Rh*, or 329G-Rh* (12, 14, 15).

Both crystal structures (9, 20) show that Arg175 in the basal state of visual arrestin has three negatively charged partners: Asp302, Asp306, and Asp303. These residues are the primary components of a network of interactions, which we refer to as the polar core (Fig. 1). We replaced each of these Asp residues with Asn (to test whether the residue serves as a counter-ion or forms a hydrogen bond), with Ala (to disrupt any potential interaction the side chain may be engaged in), and with Arg to preclude the interaction with Arg175 while creating a potential for an alternative ion pairing with the remaining negative charges). All mutants were expressed in the in vitro translation system (6), and their binding to four functional forms of rhodopsin (dark P-Rh, P-Rh*, dark Rh, and Rh*) was compared with that of wild type (WT) arrestin, as well as the most potent Arg175 mutant, R175E, whose receptor binding is least dependent on receptor phosphorylation (Fig. 24). Mutations in position 296 markedly increase binding to Rh* (Asp (WT) = Asn << Ala << Arg). This order of potency is the functional expression of the fact that Asp306 forms a hydrogen-bonded ion pair with Arg175, as seen in the crystal structures (9, 20). Asp303 mutations moderately increase the binding to Rh*, whereas Asp306 mutations have minimal effect. None of these mutations compromise P-Rh* binding. Several of these polar core mutations show an increased binding to an inactive phosphorylated receptor, i.e. the altered protein has partially lost its ability to discriminate against inactive rhodopsin. The stereochemical basis of this effect will require more structural information on the receptor binding form of arrestin.

As shown in Fig. 1, the polar core also includes Arg382 of the C-tail (20). The C-tail constitutes the last 22 ordered residues of the crystal structure and loops back into the center of the molecule, where its interactions appear to help stabilize arrestin’s inactive basal state (20). We changed Arg382 to Ala, Glu, and Asn (Fig. 2C). Importantly, all these mutations increase the binding to Rh*, although the effect of these mutations on Rh* binding is less than half that of R175E (Fig. 2C). The results of all three 382 mutations are comparable. Apparently, the removal of the positive charge in either position Arg175 (15) or Arg382 rather than the exact chemical nature of the substituting residue affects the altered binding profile. The crystal structure of Granzin et al. (9) modeled Lys2 as part of the polar core in place of Arg382. Various mutations of Lys2 (K2R, K2A, and even K2E) have no appreciable effect. Moreover, the deletion of residues 2 through 6 has no effect (Fig. 2B). These data strongly argue against any functional role of Lys2 and, by inference, against its positioning as suggested by Granzin et al. (9), supporting the conclusion that Arg382 occupies this place in the polar core (20). In the Arg382 mutations, the binding to dark P-Rh is also increased, similar to the polar core mutations described above.

To position Arg382 in the polar core, additional interactions of the C-tail with the NH2-terminal domain are necessary. Previous studies demonstrated that mutations of important aromatic residues Phe375, Phe377, and Phe380 increase Rh* binding in a manner similar to Arg382 mutations (13). The crystal structure (20) indicates that these phenylalanines interact with residues 11–13. Deletion of residues 11–16 results in a marked increase of binding to Rh* (Fig. 2B). Thus, any mutation that perturbs the positioning of the C-tail with concomi-
FIG. 2. Perturbations in the polar core. Effects of point mutations of negatively charged partners of Arg<sup>175</sup> (A), mutations in the NH<sub>2</sub> terminus (B), and of Arg<sup>382</sup> (C) on arrestin binding to four functional forms of rhodopsin. The indicated forms of rhodopsin (150 nm) were incubated with the indicated arrestin proteins (2 nm, specific activities 120–200 dpm/fmol) at 37 °C for 5 min in a total volume of 50 µl. Samples were then cooled on ice, and bound and free arrestin were separated by Sepharose 2B chromatography as described under “Experimental Procedures.” Means ± S.D. from two to three experiments each performed in duplicate are shown.

FIG. 3. Reconstruction of the polar core. Effects of the charge reversal mutations of the negatively charged partners of Arg<sup>175</sup> in combination with R175E mutation on arrestin binding selectivity. The experiments were performed as described in the legend to Fig. 2. Means ± S.D. from two experiments each performed in duplicate are shown.

tant destabilization of the polar core compromises arrestin’s selectivity for P-Rh<sup>+</sup>.

Reconstructing the Polar Core—Simultaneous charge reversal of Arg<sup>175</sup> and its negatively charged partner could reestablish the ion pair with an altered structure so that it would no longer respond to the phosphorylated COOH-terminal segment of rhodopsin, thus yielding an arrestin “locked” in its basal state. Alternatively, this double charge reversal could reconstruct a fully functional polar core. We constructed mutants that combine the R175E mutation with charge reversal mutations (Asp to Arg) that change each of its partners (Asp<sup>296</sup>, Asp<sup>303</sup>, Asp<sup>30R</sup>). The combination of R175E with the D296R mutation, each of which alone dramatically increases arrestin binding to Rh<sup>+</sup>, yields an arrestin with a virtually wild type binding profile (Fig. 3). Less potent by themselves, either mutation D30R or D303R counteracts the effect of R175E but to a substantially lesser degree than D296R. In addition, we constructed two double mutations (D296R/D303R as well as D296R/D30R) and tested their effects on arrestin’s selectivity with and without a concurrent R175E mutation. These results are presented in Fig. 3. In summary, the more a given mutation enhances arrestin binding to Rh<sup>+</sup>, the more effective it is in suppressing the effect of the R175E mutation (the order of potency being D296R = D296R + D30R ≈ D30R + D303R ≈ D30R ≈ D303R). This correlation suggests that the enhancement of Rh<sup>+</sup> binding by the disruptive effect of charge reversal and second site suppression of the R175E mutation are related stereoechemically.

These results substantiate the assertion that the basal state of arrestin is restrained by a network of interactions in which the electrostatic forces of the polar core play an important role. While the constraints of compensating charge reversal variants (such as R175E/D296R and R175E/D30R/D303R) in the polar core of arrestin may not be identical in structural detail to those of wild type arrestin, the stabilizing principles are the same, and they respond similarly to the intrusion of the receptor’s negatively charged phosphate. This conclusion is consistent with the fact that the presumed disruptive intrusion of the phosphopeptide is not structurally explicit, that is, arrestin responds to a receptor that is phosphorylated at any one or more of seven positions in the receptor’s COOH-terminal segment in vivo.

Conclusions—It appears that the mechanism whereby Arg<sup>175</sup> functions as a phosphorylation-sensitive trigger is more complex than was originally proposed (12). The x-ray crystal structures (9, 20) provide detailed information on the basal state of arrestin. The major drawback is that the crystal structures give a detailed stereoechemical framework of the molecule only in the basal state prior to assuming the conformation necessary to bind P-Rh<sup>+</sup> (6, 8). Mutagenesis studies, on the other hand, provide a wealth of phenomenological data on the role of various elements of the molecule, but cannot unequivocally indicate whether the changes in arrestin behavior occur because of the destabilization of the basal state, stabilization of the high-affinity rhodopsin binding state, or some other primary effect of the mutation. Combining inferences from stereoechemistry and mutagenesis allows one to overcome some of these limitations and to reconstruct arrestin’s behavior in terms of a consistent model. High-affinity binding to receptor occurs after the phosphorylated COOH-terminal segment of rhodopsin intrudes upon arrestin’s polar core (Fig. 1) and releases the interactions that constrain free arrestin in a binding-incompetent form. This model is analogous to the activation of a proenzyme in which the active site is formed only after a strong stereoechemical constraint is released. Mutations that neutralize the charge on Arg<sup>175</sup> or generate an opposing charge in its primary partner, Asp<sup>296</sup>, mimic the effect of phosphate and allow these arrestin mutants to bypass the need for receptor phosphorylation. Compensating second site charge reversal mutations restore the requirement for receptor phosphorylation.

Receptor binding to arrestin involves both the N- and C-domains (6, 16) as discussed in greater detail by Sigler and
In the basal state, the position of the two domains relative to each other is supported by multiple inter-domain interactions (20). The disruption of such interactions occurs as the consequence of the intrusion into the polar core by the receptor’s phosphorylated COOH-terminal segment. Arrestin’s transition into its high-affinity rhodopsin binding state (6) involves a conformational rearrangement (8). It is tempting to speculate that this event is, in fact, the reorientation of the two domains relative to each other, since significant change in the orientation of the domains would be required to bring all of the regions of arrestin implicated by chemical protection (16) into receptor contact. The need for a substantial domain rearrangement follows from the fact that some of the contact sites are 70 Å apart in arrestin’s basal state (16, 20), but the maximum dimension of the cytosolic surface of Rh* is less than 40 Å (17). Based on the crystal structure of free arrestin (20), the effects of the 3A (F375A, V376A, F377A) mutation (13) and short deletions in the NH2 terminus (Ref. 6, Fig. 2B) tempt us to speculate that a rearrangement of the three-element interaction involving residues 9–14, 103–111, and 373–380 is an integral part of the transition into the rhodopsin binding competent state. Interestingly, residues 100–120 were recently implicated in rhodopsin binding (18). Further structure-function studies are necessary to elucidate the molecular mechanism whereby arrestin recognizes the activated and phosphorylated state of rhodopsin, ensuring the exquisite selectivity of visual arrestin for P-Rh*. The elucidation of the three-dimensional structure of an arrestin/rhodopsin complex would be most informative. Since the mutagenically sensitive residues are almost absolutely conserved among all members of the arrestin family, the presented mechanism for arrestin’s activation is most likely to be a general model for arrestin-mediated desensitization of most G-protein-coupled receptors.

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