Dynamics of Arrestin-Rhodopsin Interactions

LOOP MOVEMENT IS INVOLVED IN ARRESTIN ACTIVATION AND RECEPTOR BINDING*

Received for publication, March 13, 2007, and in revised form, June 28, 2007 Published, JBC Papers in Press, July 2, 2007, DOI 10.1074/jbc.M702155200

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In this study we investigate conformational changes in Loop V-VI of visual arrestin during binding to light-activated, phosphorylated rhodopsin (Rho*-P) using a combination of site-specific cysteine mutagenesis and intramolecular fluorescence quenching. Introduction of cysteines at positions in the N-domain at residues predicted to be in close proximity to Ile-72 in Loop V-VI of arrestin (i.e. Glu-148 and Lys-298) appear to form an intramolecular disulfide bond with I72C, significantly diminishing the binding of arrestin to Rho*-P. Using a fluorescence approach, we show that the steady-state emission from a monobromobimane fluorophore in Loop V-VI is quenched by tryptophan residues placed at 148 or 298. This quenching is relieved upon binding of arrestin to Rho*-P. These results suggest that arrestin Loop V-VI moves during binding to Rho*-P and that conformational flexibility of this loop is essential for arrestin to adopt a high affinity binding state.

Rhodopsin activity is controlled by a multistep process whereby activated rhodopsin is phosphorylated on its C terminus by rhodopsin kinase, leading to the binding and quenching of rhodopsin activity by visual arrestin. Visual arrestin belongs to the arrestin superfamily, which includes rod and cone arrestins as well the β-arrestins (1). The β-arrestins, which are involved in the inactivation of agonist-occupied G-protein-coupled receptors (for review, see Ref. 2), have an additional role not seen in visual arrestins whereby they direct receptor endocytosis by acting as a clathrin or AP-2 adaptor; e.g. Refs. 3 and 4. Visual arrestin is an important model system not only for understanding the visual response but also for understanding the broad-reaching mechanism for controlling cellular signal transduction cascades mediated by G protein-coupled receptors.

The crystal structures of rod, cone, and β-1 arrestin are remarkably similar. All show a structure consisting of two cup-shaped domains formed from a seven-stranded β sandwich (5–7). These structures, of the unbound state, have provided an important framework on which to base predictions for the binding interaction between arrestin and light-activated phosphorylated rhodopsin (Rho*-P).4 Interestingly, in the crystal structures of visual arrestin, Loop V-VI (residues 68–78 in bovine visual arrestin (7)) is seen in two primary conformers, one in which Loop V-VI is extended away from the main bulk of the protein (Fig. 1, α conformer) and one with the loop folded into the N-terminal domain of the protein (β conformer). Several groups have identified that Loop V-VI has an important role in receptor binding. Experimental evidence shows (i) this loop is a vital component of arrestin that directs receptor preference (8), (ii) insertion of a peptide into this loop can either directly block or be used to competitively block binding to Rho*-P (9, 10), (iii) a fluorescent label at residue 72 in this loop apparently buries itself into the rhodopsin interface upon binding (10, 11), and (iv) spin labels in this loop have reduced mobility upon binding to Rho*-P (11, 12).

Although these prior studies indicate the importance of Loop V-VI and show it lies at the interface of the arrestin-rhodopsin complex, they do not indicate if the loop itself changes structure during binding to Rho*-P, and if so, then what might be the importance of these conformational changes in the binding of arrestin to Rho*-P. In this study we use a combination of experimental techniques to show that Loop V-VI moves relative to residues in the β-strands of the N-terminal domain and that this conformational flexibility is necessary for arrestin to bind to its receptor.

EXPERIMENTAL PROCEDURES

Materials—Frozen bovine retinas were purchased from Lawson and Lawson, Inc. (Lincoln, NE), and 11-cis-retinal was a generous gift from Rosalie Crouch (Medical University of South Carolina and National Eye Institute, National Institutes of Health). Monobromobimane was purchased from Molecular Probes (Invitrogen), and Bioman centrifugal concentrators (10 kDa cut-off) were obtained from Millipore (Bedford, MA). Cuvettes were purchased from Uvonics (Plainview, NY), and band-pass filters and long-pass filters were obtained from Oriel (Stratford, CT). Acrylamide/bisacrylamide solution (37:5:1) and microcolumns were purchased from Bio-Rad. Spectroscopic-grade buffers were from U. S. Biochemical Corp. (Cleveland, OH). All other chemicals and reagents were purchased from Sigma.

* This work was supported in part by grants from the Karl Kirchgeesser Foundation, National Institutes of Health Grants EY06225 and EY08571 (to W. C. S.) and DA018169 and EY015436 (to D. L. F.), and a National Defense Science and Engineering Graduate Fellowship (to M. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: Rho-P, phosphorylated rhodopsin; Rho*-P, light-activated rhodopsin; λmax, wavelength of maximum absorption or emission; λex, wavelength of excitation; λem, wavelength of emission; DTNB, 5,5′-dithiobis(2,2′-nitrobenzoic acid); TPCP, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DTT, dithiothreitol.
Preparation of Rod Outer Segment and Recombinant Arrestin Mutants—Rod outer segment membranes were prepared for pulldown assays and trypsinolysis studies as described (13). Rod outer segment membranes used for fluorimetry studies were prepared as described (14) and later modified (11). Recombinant arrestins were prepared and heterologously expressed in Pichia pastoris as described in McDowell et al. (15). His-tagged arrestins were purified by nickel-agarose and heparin-agarose affinity chromatography as described (10). All arrestin mutants were created in a background that lacked tryptophans (W194F) and contained an N-terminal His(6) repeat inserted after the initiating methionine and contained C63A/C143A to minimize cysteines. Cys-128 was retained because mutagenesis of this cysteine lowered the extinction coefficient for arrestin at 280 nm (18, 19). The extinction coefficient for arrestin at 280 nm (ε280) is 26,360 liter M⁻¹ cm⁻¹ (17), and for tryptophan-less arrestin mutants, a modified extinction coefficient was used (ε280 = 20,760 liters M⁻¹ cm⁻¹) (18, 19).

Functional Pulldown Assay—The functionality of expressed and modified arrestins was tested using a pulldown assay with rhodopsin in disc membranes. For the experiment shown in Fig. 3, 1 µM arrestin was mixed with 4 µM phosphorylated rhodopsin in disc membranes (Rho-P) in 10 mM HEPES/100 mM NaCl (pH 7) with or without 1 mM DTT according to the experimental conditions. After light activation of the rhodopsin for 2 min at room temperature, the mixture was centrifuged (32,000 × g) to form a pellet of the rhodopsin-containing membranes along with any bound arrestin. This pellet was washed one time and then resuspended in Laemmli sample buffer (20) for separation by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. For some reactions in which the arrestin was prepared without DTT, the supernatant was retained, and 1 mM DTT (final concentration) was subsequently added. This arrestin was then mixed with a fresh aliquot of 4 µM Rho-P and binding assessed as described above. Gels were quantified by scanning densitometry (Scion Image for Windows, Scion Corp.) of the arrestin band, normalized to wild-type arrestin, and averaged over three replicates. In each case background density was determined from a portion of the gel that contained no protein and was subtracted from each band density. For SDS-PAGE analysis of arrestin variants under non-reducing conditions, arrestin was prepared in Laemmli sample buffer without DTT.

For the experiment shown in Fig. 5, 3 µM bimane-labeled arrestin was added to 12 µM Rho-P in 10 mM HEPES, 150 mM NaCl (pH 7.4). Reactions were either kept in the dark or photoactivated (≥495 nm) for 2 min at room temperature followed by a 10-fold dilution with buffer (4 ºC) and centrifugation (100,000 × g for 10 min). The supernatant was removed, and pellets were solubilized in loading buffer and subjected to SDS-PAGE (10%). The fluorescence of the bands was visualized using a gel-doc apparatus (Alpha-Innotech FluorChem 5500), and gels were subsequently stained with Coomassie Brilliant Blue.

Measurement of Sulphydryl Groups—The reaction of 5,5′-dithio-bis(2,2′-nitrobenzoic acid) (DTNB) with a free sulphydryl group results in an absorptive species, which can be used to quantify reactive sulphydryl groups in arrestin (15). Briefly, a 2.5 mM DTNB stock solution was prepared in 10 mM HEPES and 750 mM NaCl (pH 7.5). For the DTNB reaction, 8.5 µM arrestin was mixed with 500 µM DTNB, and 0.75% sodium dodecyl sulfate was present to denature the protein and expose any buried sulphydryl groups (30 ºC). The DTNB reaction product (A412) was monitored in a quartz cuvette using a Hewlett-Packard model 8452 diode array spectrophotometer. The number of reactive sulphydryls was determined by using the extinction coefficient of the indicator product (13,600 liters M⁻¹ cm⁻¹ at 412 nm). For quantitation of sulphydryls under non-reducing conditions, arrestin was first dialyzed overnight in the above HEPES buffer, changing the buffer twice to ensure that all DTT was removed. For reducing conditions, a separate aliquot of arrestin was treated with 2 mM DTT overnight and then rapidly buffer-exchanged using a PD-10 column (GE Healthcare) immediately before the addition of DTNB.

Limited Trypsinolysis—Limited digestion of arrestin with TPCK-treated trypsin (Sigma) was performed as originally introduced (21) and later modified (9). Briefly, all arrestins were dialyzed against 50 mM Tris (pH 8) without DTT. Arrestin (3.7 µM) was mixed with 9.6 µM phosphorylated rhodopsin in disc membranes to which 1 unit of TPCK-treated trypsin was added at room temperature. Reaction mixtures were kept in the dark or exposed to light for 2 min, after which aliquots were removed at timed intervals, quenched by the addition of Laemmli sample buffer, and separated by 12% SDS-PAGE.

Fluorescence Spectroscopy—Fluorescence steady-state experiments were conducted as described previously (11) using a Photon Technologies (PTI) spectrophotometer with a DeltaRam excitation source. For experiments using rhodopsin, excitation slits were set <0.25-nm band pass (to avoid bleaching of the rhodopsin), and emission slits were set at 15-nm band pass. Samples were excited at 380 nm, and emission was measured from 400 to 600 nm using 2-nm increments (0.25-s integration per point). Determination of accurate emission λmax values was done as described previously (22). Briefly, excitation slits were set at 1-nm band pass, and emission slits were set at 10-nm. A sample of 2 µM bimane-labeled arrestin (20 ºC) was excited at 381 nm, and emission was collected from 400 to 600 nm using 1-nm increments (1-s integration per point).

Quantum Yield Measurements—The quantum yield (φ) of each labeled arrestin mutant was determined as described previously (22). Briefly, the optical density at 360 nm (OD) and integrated fluorescence intensity (F) (λem 360 nm; λex 370–700 nm) of each arrestin were compared with that of a standard with a known quantum yield (0.55), quinine sulfate in 1 N H2SO4, using the relation,

\[
\phi_S = \phi_A \times \frac{F_A \text{OD}_s}{F_S \text{OD}_A} \quad (\text{Eq. 1})
\]

where the subscripts A and S stand for the arrestin sample and the standard, respectively.
Movement of Loop V-VI in Arrestin

Steady-state Fluorescence Anisotropy Measurements—The steady-state fluorescence anisotropy, which is a measure of the rotational diffusion of a fluorophore (19), was measured on a PTI QM-1 steady-state fluorescence spectrophotometer. Polarizers were fitted over the excitation source and the emission detector and used to vary the polarization of the light. Anisotropy \( r \) is defined as,

\[
r = \frac{I_\| - G I_\perp}{I_\| + 2GI_\perp}
\]

where \( I_\| \) and \( I_\perp \) are the intensities of the fluorescence emission parallel and perpendicular to the plane of the excitation light, respectively. The G-factor, a term used to correct for the different sensitivities of the emission detector for vertically and horizontally polarized light, was determined before measuring the anisotropy. In a typical experiment the fluorescence emission of 1 \( \mu \text{M} \) bimane-labeled arrestin (200 \( \mu \text{l} \) sample volume, 4-mm black-jacketed cuvette, 14 °C) was measured at 475 nm (380 nm excitation) for 15 s (1-s integration per point). The average of the 15 points was used for \( I_\| \) and \( I_\perp \), and measurements were done in triplicate. Excitation slits were set at 1 nm, and emission slits were set at 10 nm. These settings caused <1% rhodopsin bleaching in samples containing Rho-P in disc membranes, as measured by absorbance spectroscopy. Background fluorescence of buffer or Rho-P was subtracted where appropriate before the calculation of anisotropy.

Fluorescence Lifetime Analysis—The fluorescence decay lifetime of each bimane-labeled arrestin mutant was determined using a PTI Laserstrobe instrument (11). Briefly, 1 \( \mu \text{M} \) arrestin (with or without a 4-fold excess of Rho-P in disc membranes) in 200 \( \mu \text{l} \) (4-mm black-jacketed cuvette) was measured at 20 °C using 381-nm excitation pulses (full width half-maximum ~1.5 ns). A 298–435-nm band pass filter was placed over the excitation beam, and the intensity was modulated using neutral density filters. Emission was monitored through three long pass filters (>399 nm, >418 nm, >450 nm) and a monochromator set at 470 nm (slits at 15-nm band pass). Lifetime data were collected identically as described previously (11), and data were analyzed using the commercial FeliX32 Analysis software (PTI). Each lifetime data set was fit to a double-exponential decay curve, and the amplitude-weighted average fluorescence lifetimes were reported.

RESULTS

Rationale of Mutant Design—The arrestin crystal structures show that Loop V-VI can adopt multiple conformations (see Fig. 1) (5–7). To test if movement of this loop might be important in the binding of arrestin to rhodopsin, we focused on the relative movement of Ile-72. This residue in Loop V-VI is in close proximity (<9 Å) to Glu-148 and Lys-298 when the loop is in the \( \beta \) conformation and further away (>20 Å) in the \( \alpha \) conformation (Fig. 1). We used two methodologies to measure the distance between Ile-72 and residues Glu-148 and Lys-298; that is, cysteine cross-linking and intramolecular fluorescence quenching.

Di-cysteine Mutants Form Intramolecular Disulfides—We first tested whether cysteines introduced at sites 148 and 298 were in sufficient proximity to a cysteine at residue 72 to form a disulfide bond, which would be predicted to tether Loop V-VI to the N-domain of arrestin. These di-cysteine mutants of arrestin were expressed and purified to >95% homogeneity. They were subsequently dialyzed at pH 7.5 without DTT (to promote the formation of a disulfide bond) or with 1 mM DTT (to reduce any potential disulfide bonds). We then measured sulfhydryl reactivity of I72C/E148C and I72C/K298C to assess the number of free sulfhydryl groups present in oxidizing and reducing conditions (Fig. 2A). This analysis showed that both I72C/E148C and I72C/K298C had only one reactive sulfhydryl group before treatment with DTT (presumably from the native Cys-128). After treatment with DTT, the number of reactive sulfhydryl groups increased to nearly three for I72C/K298C and to ~2.5 for I72C/E148C. This increase of approximately two reactive groups is consistent with the reduction of a disulfide bond.

Because it is possible that these cysteine mutants of arrestin could form dimers (i.e. intermolecular disulfide bonds), we also performed SDS-PAGE gel analysis of each of the di-cysteine mutants under non-reducing and reducing conditions to assess the multimeric state of the arrestin (Fig. 2B). These included I72C/E148C and I72C/K298C, in which the cysteines are predicted to be in close proximity, and I72C/K276C, in which the cysteines are not close and, therefore, should not form an intramolecular disulfide bond (Fig. 1). This analysis showed
that for all of the cysteine-containing variants of arrestin, more than 90% of the total arrestin existed as a monomer, with the remainder forming multimeric complexes. Interestingly, the two di-cysteine mutants that are predicted to form an internal disulfide bond (I72C/E148C and I72C/K298C) show even less propensity to form multimeric complexes. Under reducing conditions, all of the arrestin variants migrated as a single band.

Diulfide Formation Inhibits Arrestin Binding to Rho*P—

The functionality of the arrestin di-cysteine mutants was tested using a centrifugal pulldown assay with phosphorylated rhodopsin in native rod outer segment disc membranes. Fig. 3A shows an example of the pulldown assay using the I72C/K298C mutant. This arrestin bound well to Rho*-P when DTT was present (lane 6) but had very poor binding to Rho*-P when DTT was absent (lane 8). The lack of binding of I72C/K298C in the absence of DTT could be restored to its original level by the subsequent addition of 1 mM DTT (lane 10).

Fig. 3B shows a summary of the binding of each of the various arrestin mutants to Rho*-P in the presence or absence of 1 mM DTT. Note that both arrestins with cysteine replacements predicted to be in close proximity to I72C (i.e. E148C and K298C) showed a dramatic increase in binding in the presence of DTT. Also note that for I72C/K276C, in which the cysteines are not in close proximity, binding to Rho*-P was not influenced by DTT. Similarly, DTT also had a very small effect (<15%) on mutants with a tryptophan at sites 148 and 298 (these tryptophan mutants were used in our fluorescence experiments described below). Although all of these arrestin variants retained their selectivity for Rho*-P, these binding results show that the substitutions used in this study decrease the steady-state affinity of the mutants compared with wild-type arrestin to varying degrees. This effect suggests that these residues (Ile-72, Glu-148, and Lys-298) may either participate directly in binding of arrestin to Rho*-P or that mutating these residues may affect the arrestin structure, indirectly altering the binding site.

The results from the pulldown assay indicate that tethering Loop V-VI blocks arrestin binding to Rho*-P. As an additional measure of arrestin functionality, we performed limited trypsinolysis. Arrestin binding to Rho*-P is characterized by an increase in the susceptibility of the C terminus of arrestin to trypsin cleavage. This technique has been used previously to show the integrity of mutated arrestin proteins and to...
assess changes in arrestin conformation upon binding to rho-dopsin (9, 21, 23).

Fig. 4 shows examples of the proteolysis that occurs for arrestin treated with limiting amounts of trypsin for 10 min (note that all arrestins were prepared under non-reducing conditions in the absence of DTT). In the absence of receptor binding (Rho-P, −), wild-type arrestin was slowly proteolysed by trypsin. However, upon light activation and receptor binding (Rho*-P, +), arrestin was rapidly cleaved by trypsin as indicated by the appearance of a 44-kDa band below the full-length arrestin. In contrast, I72C/E148C and I72C/K298C both had very slow trypsinolysis, even in the presence of Rho*-P, implying that they were unable to bind Rho*-P. For the control I72C/K276C, proteolysis was virtually identical to wild-type arrestin, showing essentially no proteolysis with Rho-P but rapid cleavage in the presence of Rho*-P. A similar proteolysis pattern was observed for each of the control arrestins in which I72C was paired with a tryptophan at residue 148 or 298. Note that mutant I72C/K298W showed increased trypsinolysis in the presence of dark Rho-P compared with WT. This increased sensitivity to trypsin may be due to some instability of this mutant. However, similar to WT, trypsinolysis of I72C/K298W increased in the presence of Rho*-P.

**Fluorescently Labeled Arrestin Mutants**—The results described above suggest that movement of Loop V-VI is required for arrestin to bind to Rho*-P. However, these results could also be interpreted to mean that creating an internal disulfide bond between I72C and residues in the N-domain simply blocks a conformational change that is essential in a different and potentially remote portion of the arrestin polypeptide or sterically occludes the binding pocket. To address these alternatives, we fluorescently labeled Loop V-VI at site 72 and placed quenching tryptophan residues at sites 148 and 298 to determine whether Loop V-VI moves relative to other portions of the arrestin protein during binding to Rho*-P.

The bimane-labeled arrestin mutant I72C (I72B) has been well characterized (11, 16) and is an ideal position to measure the relative movement of Loop V-VI using Trp-bimane quenching. Comparison of the α and β conformers of the crystal structure show that the distance between Ile-72 and residues Glu-148 and Lys-298 is >20 Å in the α-conformer, whereas it is <9 Å in the β conformer (Fig. 1). Because Trp-bimane quenching only occurs when the two groups are within near-contact distance (<15 Å) (24), these residues are ideal for this methodology.

To assess any potential effects of mutating Glu-148 and Lys-298 on arrestin function, we also placed Phe at these sites as a control. Phe has a bulky side chain like Trp but does not quench bimane fluorescence (24).

We first assessed whether these bimane-labeled arrestin mutants were functional by a centrifugal pulldown assay (Fig. 5). The data indicate that all mutants showed selectivity for Rho*-P and all bound at nearly the same level as wild-type arrestin, except for I72B/K298W, which pulled down at ~70% that of the levels of wild-type, and I72B/K298F, which pulled down at ~50% that of the level of wild type.

**Trp Residues in the N-domain Quench I72B Fluorescence**—We next characterized the fluorescence properties of the arrestin mutants (Table 1). Note that I72B/K298W and I72B/E148F had significantly diminished quantum yields compared with I72B and the controls I72B/K298F and I72B/E148F. Interestingly, the quantum yield of I72B/E148F was actually 37% higher than that of I72B, which may be because the E148F mutation increases the local hydrophobicity around the probe.

The amplitude-weighted average fluorescence lifetime (τ) of arrestin I72B was ~10 ns (Table 1). Both Trp-containing mutants had similar (τ) values. The fact that I72B/K298W and I72B/E148F had significantly reduced quantum yields but similar (τ) values as the Trp-less controls, implies that the bimane probe was being quenched through a static mechanism, indicating that the bimane and the Trp residue in these mutants were quite close (5–10 Å) (24). This static quenching was also reflected in the red-shifted absorbance λ_{max} of
these three mutants (Table 1), which suggests a ground-state complex between the Trp and the bimane. Note that the lifetime of I72B/E148F was significantly longer (14.5 ns) than I72B, which explains why its quantum yield was higher. This increased lifetime, quantum yield, and blue-shifted emission are all consistent with the bimane fluorophore being in a more hydrophobic environment through interacting with the Phe at 148.

**Bimane Quenching Is Relieved by Rho*-P Binding**—The steady-state fluorescence spectra of the I72B mutants in the presence of rhodopsin in disc membranes are shown in Fig. 6. In the presence of unphosphorylated rhodopsin, none of the mutants exhibited significant fluorescence changes upon light activation (data not shown). In the presence of Rho-P, however, the fluorescence of each mutant increased and blue-shifted in intensity upon light activation (λ<sub>max</sub> values and relative increases in intensity are summarized in Table 2). Note that for both I72B/E148W and I72B/K298W, the steady-state fluorescence in the dark was significantly reduced compared with I72B (compare Fig. 6, A to B and D). Upon light activation, the intensities of these mutants increased to a level similar to that of I72B. These results suggest that binding to Rho*-P relieves the Trp-bimane quenching in these mutants. Arrestin I72B/K298F exhibited a diminished fluorescence change compared with I72B (Fig. 6E), probably because it bound less well to Rho*-P (as suggested by pulldown analysis, Fig. 5). Arrestin I72B/E148F exhibited only a ~30% increase in intensity compared with ~60% for I72B, because its initial, dark-state fluorescence intensity was higher (Fig. 6C).

For each arrestin mutant we also measured the steady-state anisotropy, which gives information regarding the relative rotational freedom of the probe. For nearly every arrestin mutant, the anisotropy of the probe at site 72 is higher in the presence of dark Rho-P (Table 2) than in its absence (Table 1). This result suggests that arrestin interacts with dark Rho-P, which has been previously observed (12). Upon light activation, the anisotropy of each mutant increases, which is probably due to a burying of the probe at 72 in the arrestin/Rho*-P interface. The addition of hydroxylamine returns the anisotropies to lower values.

Finally, the fluorescence lifetime values of the I72B mutants do not change significantly upon Rho*-P binding (Table 2),

![Fluorescence changes of bimane-labeled arrestin mutants suggest movement of Loop V-VI upon binding Rho*-P.](image)

**TABLE 2**

Fluorescence characteristics of bimane-labeled arrestin mutants in the presence of membrane-bound phosphorylated rhodopsin

| Mutant       | + Rho-P | Emission λ<sub>max</sub><sup>a</sup> nm | Change in integrated intensity<sup>b</sup> | Anisotropy<sup>c</sup> | τ<sup>d</sup><sub>0</sub> ns |
|--------------|---------|----------------------------------------|------------------------------------------|------------------------|--------------------------|
| I72B         | Dark    | 471                                    | +58 ± 5                                  | 0.099 ± 0.007          | 10.2 ± 1.0               |
|              | + Light | 454                                    |                                          | 0.165 ± 0.003          | 9.4 ± 0.5                |
|              | + NH₂OH | ND                                     |                                          | 0.081 ± 0.005          | 10.9 ± 0.2               |
| I72B/E148W   | Dark    | 473                                    | +187 ± 13                                | 0.082 ± 0.010          | 8.1 ± 0.2                |
|              | + Light | 454                                    |                                          | 0.152 ± 0.004          | 9.1 ± 0.1                |
|              | + NH₂OH | ND                                     |                                          | 0.075 ± 0.002          | 8.7 ± 0.5                |
| I72B/E148F   | Dark    | 466                                    | +29 ± 3                                  | 0.113 ± 0.004          | 14.0 ± 0.1               |
|              | + Light | 454                                    |                                          | 0.166 ± 0.008          | 10.0 ± 0.7               |
|              | + NH₂OH | ND                                     |                                          | 0.085 ± 0.006          | 13.1 ± 0.7               |
| I72B/K298W   | Dark    | 469                                    | +226 ± 14                                | 0.146 ± 0.005          | 8.2 ± 0.4                |
|              | + Light | 455                                    |                                          | 0.045 ± 0.016          | 9.7 ± 0.5                |
|              | + NH₂OH | ND                                     |                                          | 0.070 ± 0.003          | 9.3 ± 1.2                |
| I72B/K298F   | Dark    | 471                                    | +41 ± 4                                  | 0.112 ± 0.007          | 10.2 ± 0.2               |
|              | + Light | 460                                    |                                          | 0.045 ± 0.000          | 10.1 ± 0.4               |
|              | + NH₂OH | ND                                     |                                          | 0.070 ± 0.002          | 8.5 ± 0.1                |
| I72W/K298B   | Dark    | 472                                    | +91 ± 8                                  | 0.096 ± 0.005          | 9.5 ± 0.1                |
|              | + Light | 473                                    |                                          | 0.086 ± 0.001          | 8.6 ± 0.3                |
| I72F/K298B   | Dark    | 472                                    | −3 ± 1                                   | 0.101 ± 0.014          | 11.0 ± 0.5               |
|              | + Light | 472                                    |                                          | 0.098 ± 0.004          | 9.5 ± 0.1                |
|              | + NH₂OH | ND                                     |                                          | 0.095 ± 0.005          | 11.2 ± 0.2               |

<sup>a</sup>The average of two independent experiments (380 nm excitation; 20 °C). The S.E., which is not reported, was <2 nm for each value.

<sup>b</sup>Average from two independent experiments ± S.E.

<sup>c</sup>The steady-state anisotropy of the bimane-probe. Results are the mean ± S.E. from two measurements (14 °C).

<sup>d</sup>\( \tau = \alpha_1 T_1 + \alpha_2 T_2 \), the amplitude-weighted average fluorescence lifetime. Results are the mean ± S.E. from two independent fluorescence lifetime measurements and two-component analyses (20 °C). The \( \chi^2 \) value for each fit was between 0.8 and 1.2.
Movement of Loop V-VI in Arrestin

FIGURE 7. Fluorescence changes of the reversal mutants also suggest movement of Loop V-VI upon Rho*-P binding. The fluorescence of 1 μM arrestinI72W/K298B (A) or I72F/K298B (B) was measured in the presence of a 4-fold excess of Rho*-P in disc membranes before (solid traces) and after (dashed traces) light-activation. Conditions were the same as described in Fig. 6. Notice that the fluorescence probe on I72F/K298B does not change upon binding Rho*-P. Thus the changes seen in A are most consistent with movement of I72W away from K298; the steady-state spectra represent smoothed and buffer-subtracted raw data.

except for I72B/E148F, which goes from ~14 ns in the dark to 10 ns upon light activation.

Quenching Is Also Observed with a Reversal Mutant—The results discussed above are consistent with a scenario where the bimane probe at 72 moves away from tryptophan residues at 148 and 298 when arrestin binds Rho*-P. Unfortunately, the data are complicated by the fact that the bimane probe at 72 exhibits an increase in intensity that is independent of any potential Trp-quenching (Fig. 6A) (11, 16). As an additional control, we switched the fluorophore and quencher sites on arrestin (i.e. I72W/K298B). This fluorescent mutant was functional (Fig. 5, far right), and the bimane at 298 appeared to be quenched by the Trp at 72 (Fig. 7A and Table 1). Importantly, this quenching was relieved when arrestin bound Rho*-P. Because the control I72F/K298B did not show any change in fluorescence upon binding Rho*-P (Fig. 7B), the increase in fluorescence of I72W/K298B was due solely to the movement of site 72 away from site 298. Note that, in contrast to site 72, when the bimane probe is located at site 298 there was neither a change in fluorescence intensity nor anisotropy when arrestin bound Rho*-P (Table 2).

DISCUSSION

In the crystal structures of visual arrestin, two principal conformers are identified (α and β) that differ primarily in loop structures, especially Loop V-VI (Fig. 1). In the α conformer, Loop V-VI is extended away from the N-terminal domain, with a distance of more than 20 Å between Ile-72 in Loop V-VI and Glu-148 or Lys-298 in β-strands of the N-domain, (distance between β carbons). In the β-conformer, Loop V-VI is folded toward the N-domain such that there is only ~9 Å between residue 72 and 148 or 298. In this study we assessed the relative placement of Loop V-VI in arrestin in solution and how this placement changes when arrestin binds light-activated, phosphorylated rhodopsin using cysteine substitution and cross-linking and intramolecular fluorescence quenching. Our results and their implications are discussed below.

Loop V-VI Makes Close Contact with the N-Domain in Inactive Arrestin—Using site-specific cysteine substitutions, we showed that a cysteine introduced at residue 72 in Loop V-VI can efficiently cross-link to cysteines substituted at either Glu-148 or Lys-298 (Fig. 2). Because a typical disulfide bond is on the order of ~6 Å, these results imply that in the unbound state, Loop V-VI must be able to come close (<10 Å) to residues 148 and 298 in the β-strands of the N-domain. Likewise, we observed significant intramolecular fluorescence quenching (nearly 50%) of a bimane probe at residue 72 when tryptophan residues were placed at sites 148 and 298 (Fig. 6 and Table 1). Moreover, this quenching occurred through a static mechanism, implying that the probe and quencher were in very close contact (5–10 Å). The fluorescence quenching was also observed when the placement of the fluorophore and tryptophan were reversed (Fig. 7). Our previous studies using the Trp-bimane quenching pair indicate that the Trp residue must be within near-contact distance (5–10 Å) of the bimane probe to observe these types of quenching effects. These results collectively show that in solution, Loop V-VI of arrestin is folded into the N-domain or at least exists in a conformational equilibrium in which Loop V-VI can make close contact at times with the β-strands of the N-domain. Thus, in its basal state at least a subpopulation of arrestin may resemble the β conformer in the vicinity of Loop V-VI.

Although our results suggest that the residue Ile-72 on Loop V-VI can come into close contact with the N-domain, our previous studies (11) and those of Hubbell and co-workers (12) have reported that a spin-label at site 72 is highly mobile in the basal state of arrestin. These EPR results suggest that significant backbone fluctuations occur at this site. Likewise, we observe that a bimane probe at this site has a low anisotropy, again indicating probe mobility. Together, these results are best explained by a model in which the loop exists in a conformational equilibrium with a more mobile (possibly extended) state. Alternatively, it may be that the rotameric state of residue 72 allows it an unusually high degree of freedom.

Loop V-VI Is in an Extended State When Arrestin Binds Rho*-P—Our results further show that when arrestin binds Rho*-P, Loop V-VI is stabilized in a more extended conformation in which site 72 is not in close contact with the N-domain. The significant intramolecular quenching of the bimane on Loop V-VI (residue 72) by tryptophans in the N-domain (residues 148 and 298) was relieved upon arrestin binding to Rho*-P, restoring the bimane emission to that of I72B in the absence of a tryptophan quencher (Fig. 6). Likewise, when the bimane was located on the N-domain and the quencher on Loop V-VI, the fluorescence increased nearly 100% upon binding to Rho*-P (Fig. 7). Together, these results indicate that when arrestin binds Rho*-P, Ile-72 on Loop V-VI is out of the “quenching range” (>15 Å) of the N-domain (24). This release of quenching is consistent with movement of the loop from a folded (or partially folded) conformer to an extended conformation, such as would occur if the β conformation of arrestin converts to the α conformation upon receptor binding.

Importantly, our results also show that this conformational flexibility is necessary for arrestin binding to Rho*-P. When a disulfide bond was formed between a cysteine at position 72 and a cysteine at either 148 or 298, then binding of arrestin to Rho*-P was severely diminished. These oxidized arrestin mutants were not “pulled-down” with Rho*-P disc membranes (Fig. 3), and the trypsin sensitivity of these arrestins did not
change in the presence of Rho*-P (Fig. 4) under non-reducing conditions. However, binding of these same cysteine-substituted arrestins to Rho*-P was completely retained under reducing conditions (1 mM DTT) or could be restored if DTT was subsequently added to the arrestin in which disulfide bonds had previously formed. Although it is possible that the loss of binding seen under non-reducing conditions could be the result of the formation of a multimeric complex of arrestin molecules, we do not think it to be likely since the binding of the I72C and I72C/K276C variants of arrestin did not show any sensitivity to the reducing conditions (Fig. 3B), and only a small proportion of multimeric complexes could be detected by non-reducing SDS-PAGE (Fig. 2B). Furthermore, we can also rule out the formation of nonspecific, denatured aggregates of arrestin in the absence of DTT since the reintroduction of DTT restored the binding of I72C/E148C and I72C/K298C to Rho*-P.

Mechanism of Arrestin Activation and Binding to Rho*-P in the Vicinity of Loop V-VI—Our data suggest that Loop V-VI undergoes a conformational change upon arrestin binding to Rho*-P in which the loop is stabilized in an extended conformation away from contact with the N-domain. Why might this movement be necessary for binding? Two possibilities are easily envisioned. First, the extended conformation of Loop V-VI may be an important element that directly interacts with rhodopsin. There are several lines of evidence that support direct interaction of the loop with rhodopsin. Loop V-VI is highly conserved among all members of the arrestin superfamily (6), suggesting that particular side chains may have key interactions with residues on the receptor. The importance of hydrophobic residues, particularly at position 72, is shown by decreased binding when Ile-72 is substituted with cysteine but which can be restored by attachment of a hydrophobic fluorophore (11). The fluorophore at site 72 shows a blue shift and increase in fluorescence when arrestin binds Rho*-P, which suggests that Loop V-VI is buried in a protein-protein interface. Likewise, a spin-label probe at this site becomes highly immobilized when arrestin binds Rho*-P (11, 12). If Loop V-VI directly interacts with Rho*-P, it is possible this interaction is similar to the role of the C terminus of the α-subunit of transducin, which inserts into the hydrophobic cleft opened by the movement of helix F of rhodopsin (25, 26). Alternatively, if a single arrestin binds a dimer of rhodopsin (27), perhaps Loop V-VI inserts between the two rhodopsin molecules (our experiments were all performed with rhodopsin in the native disc membranes, where rhodopsin oligomerization likely occurs (27–31)).

A second mechanism by which the movement of Loop V-VI could be involved in the binding to Rho*-P is as a steric regulator of receptor binding elements in the N-domain. In this concept, originally proposed by Granzin et al. (5), the β-conformer represents the “closed” conformation, where critical receptor-binding elements are occluded, and the α-conformer represents the “open” state in which rhodopsin binding residues are exposed. Our results do not favor one mechanism over the other, but it is important to note that they are not mutually exclusive.

Although our study focused on Loop V-VI, many past studies have collectively suggested that nearly the entire concave surfaces of both the N- and C-domains are involved in receptor binding (8–10, 12, 32, 33). Thus, Loop V-VI is certainly important, but is clearly not the only element involved. Furthermore, Loop V-VI movement may be coincident with other proposed conformational changes in arrestin (34–36). For example, the C-terminal tail of arrestin is clearly important for regulating arrestin function since truncation of the C terminus and various C-terminal mutations result in constitutively active arrestin (34, 37–40). It is possible the C terminus may “hold” arrestin in an inactive conformation, and its mobilization may have to occur to enable arrestin to bind Rho*-P. Such movement has in fact been measured using spin probes in the C terminus of arrestin (12). An intriguing possibility is that the C terminus of arrestin may interact directly with Loop V-VI, and when the C terminus is displaced by interaction with Rho*-P, Loop V-VI is free to extend so that arrestin binding can occur. Unfortunately, the C terminus is not resolved in the current crystal structures beyond residue 386 in the β conformation such that the proximity and orientation of the C-terminal 18 amino acids relative to Loop V-VI is not known.

In summary, our studies show that Loop V-VI is an important element that mobilizes during arrestin binding to activated receptor and that this movement is essential for high affinity binding. Future investigations should consider the potential for additional loop movements and also the dynamics of Loop V-VI interaction with the arrestin C terminus and with rhodopsin.

REFERENCES

1. Craft, C. M., and Whitmore, D. H. (1995) FEMS Lett. 362, 247–255
2. Lefkowitz, R. J., and Whalen, E. J. (2004) Curr. Opin. Cell Biol. 16, 162–168
3. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) J. Biol. Chem. 275, 23120–23126
4. Kirchhausen, T. (1999) Annu. Rev. Cell Dev. Biol. 15, 705–732
5. Granzin, J., Wilden, U., Choe, H.-W., Labahn, J., Krafft, B., and Buldt, G. (1998) Nature 391, 918–921
6. Han, M., Gurevich, V. V., Vishnivskiy, S. A., Sigler, P. B., and Schubert, C. (2001) Structure 9, 869–880
7. Hirsch, J. A., Schubert, C., Gurevich, V. V., and Sigler, P. B. (1999) Cell 97, 257–269
8. Vishnivskiy, S. A., Hosey, M. M., Benovic, J. L., and Gurevich, V. V. (2004) J. Biol. Chem. 279, 1262–1268
9. Dinculescu, A., McDowell, J. H., Amici, S. A., Dugger, D. R., Richards, N., Hargrave, P. A., and Smith, W. C. (2002) J. Biol. Chem. 277, 11703–11708
10. Smith, W. C., Dinculescu, A., Peterson, J. J., and McDowell, J. H. (2004) Mol. Vis. 10, 392–398
11. Sommer, M. E., Smith, W. C., and Farrens, D. L. (2005) J. Biol. Chem. 280, 6681–6671
12. Hansson, S. M., Francis, D. J., Vishnivskiy, S. A., Kolobova, E. A., Hubbell, W. L., Klug, C. S., and Gurevich, V. V. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4900–4905
13. McDowell, J. H. (1993) Methods Neurosci. 15, 123–130
14. Papermaster, D. S. (1982) Methods Enzymol. 81, 48–52
15. McDowell, J. H., Smith, W. C., Miller, R. L., Poppe, M. P., Arendt, A., Abdulaeva, G., and Hargrave, P. A. (1999) Biochemistry 38, 6119–6125
16. Sommer, M. E., Smith, W. C., and Farrens, D. L. (2006) J. Biol. Chem. 281, 9407–9417
17. Schubert, C., Hirsch, J. A., Gurevich, V. V., Engelman, D. M., Sigler, P. B., and Fleming, K. G. (1999) J. Biol. Chem. 274, 21186–21190
18. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
19. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, Kluwer Academic, New York
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Palczewski, K., Pulvermüller, A., Buczylko, J., and Hofmann, K. P. (1991) J. Biol. Chem. 266, 18649–18654
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22. Mansoor, S. E., McHaourab, H. S., and Farrens, D. L. (1999) Biochemistry 38, 16383–16393
23. Ascano, M., Smith, W. C., Gregurick, S. K., and Robinson, P. R. (2006) Mol. Vis. 12, 1516–1525
24. Mansoor, S. E., McHaourab, H. S., and Farrens, D. L. (2002) Biochemistry 41, 2475–2484
25. Altenbach, C., Yang, K., Farrens, D. L., Farahbakhsh, Z. T., Khorana, H. G., and Hubbell, W. L. (1996) Biochemistry 35, 12470–12478
26. Janz, J. M., and Farrens, D. L. (2004) J. Biol. Chem. 279, 29767–29773
27. Liang, Y., Fotiadis, D., Filipek, S., Saperstein, D. A., Palczewski, K., and Engel, A. (2003) J. Biol. Chem. 278, 21655–21662
28. Fotiadis, D., Liang, Y., Filipek, S., Saperstein, D. A., Engel, A., and Palczewski, K. (2003) Nature 421, 127–128
29. Jastrzebska, B., Maeda, T., Zhu, L., Fotiadis, D., Filipek, S., Engel, A., Stenkamp, R. E., and Palczewski, K. (2004) J. Biol. Chem. 279, 54663–54675
30. Mansoor, S. E., Palczewski, K., and Farrens, D. L. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 3060–3065
31. Medina, R., Perdomo, D., and Bubis, J. (2004) J. Biol. Chem. 279, 39565–39573
32. Hanson, S. M., and Gurevich, V. V. (2006) J. Biol. Chem. 281, 3458–3462
33. Pulvermuller, A., Schroder, K., Fischer, T., and Hofmann, K. P. (2000) J. Biol. Chem. 275, 37679–37685
34. Gurevich, V. V., and Benovic, J. L. (1993) J. Biol. Chem. 268, 11628–11638
35. Vishnivetskiy, S. A., Hirsch, J. A., Velez, M. G., Gurevich, Y. V., and Gurevich, V. V. (2002) J. Biol. Chem. 277, 43961–43967
36. Vishnivetskiy, S. A., Schubert, C., Climaco, G. C., Gurevich, Y. V., Velez, M. G., and Gurevich, V. V. (2000) J. Biol. Chem. 275, 41049–41057
37. Gurevich, V. V. (1998) J. Biol. Chem. 273, 15501–15506
38. Palczewski, K., Buczylko, J., Ohguro, H., Annan, R. S., Carr, S. A., Crabb, J. W., Kaplan, M. W., Johnson, R. S., and Walsh, K. A. (1994) Protein Sci. 3, 314–324
39. Pulvermuller, A., Maretzi, D., Rudnicka-Nawrot, M., Smith, W. C., Palczewski, K., and Hofmann, K. P. (1997) Biochemistry 36, 9253–9260
40. Schroder, K., Pulvermuller, A., and Hofmann, K. P. (2002) J. Biol. Chem. 277, 43987–43996