In this study, we address the mechanism of visual arrestin release from light-activated rhodopsin using fluorescently labeled arrestin mutants. We find that two mutants, I72C and S251C, when labeled with the small, solvent-sensitive fluorophore monobromobimane, exhibit spectral changes only upon binding light-activated, phosphorylated rhodopsin. Our analysis indicates that these changes are probably due to a burying of the probes at these sites in the rhodopsin-arrestin or phospholipid-arrestin interface. Using a fluorescence approach based on this observation, we demonstrate that arrestin and retinal release are linked and are described by similar activation energies. However, at physiological temperatures, we find that arrestin slows the rate of retinal release ~ 2-fold and abolishes the pH dependence of retinal release. Using fluorescence, EPR, and biochemical approaches, we also find intriguing evidence that arrestin binds to a post-Meta II photodecay product, possibly Meta III. We speculate that arrestin regulates levels of free retinal in the rod cell to help limit the formation of damaging oxidative retinal adducts. Such adducts may contribute to diseases like atrophic age-related macular degeneration (AMD). Thus, arrestin may serve to both attenuate rhodopsin signaling and protect the cell from excessive retinal levels under bright light conditions.

The visual photoreceptor rhodopsin is perhaps the best model system for understanding the mechanisms used in G-protein-coupled receptor (GPCR) signaling, as detailed information exists on the structures and dynamic interactions of the protein constituents (1). Visual activation begins with absorption of light by the 11-cis-retinal chromophore in rhodopsin. The photoactivated form of rhodopsin, Rho* or “Meta II,” interacts with and activates the G-protein transducin, which exchanges nucleotide and then diffuses to interact with downstream effectors. Signaling by Rho* is terminated by slow thermal decay and the release of retinal. Alternatively, signaling can be quickly terminated by a process that begins with phosphorylation of rhodopsin’s C-terminal tail through the action of rhodopsin kinase (2, 3). The phosphorylated Rho* is then bound by arrestin, which stops signaling by physically occluding the G-protein binding site (4, 5).

In the present study, we address how these two inactivation mechanisms are related and, specifically, what governs arrestin release from rhodopsin. Arrestin is known to bind to phosphorylated Meta II, a form in which the photolyzed chromophore all-trans-retinal is still attached to the receptor by a deprotonated Schiff base. Arrestin does not bind phosphorylated opsin, but all-trans retinal added exogenously can stimulate arrestin binding to phosphorylated opsin (6, 7). Early studies showed indirectly that retinal release and arrestin release are probably interrelated events (7, 8). However, how these processes are linked or whether arrestin binds other photointermediates of rhodopsin (such as the storage form Meta III) is still unknown.

In this paper, we demonstrate that arrestin binding and release can be directly observed in real time by monitoring spectral changes in fluorescently labeled arrestin mutants. Our studies employed two different cysteine mutants of arrestin, I72C and S251C, two sites that lie within the experimentally proposed rhodopsin-binding surface on arrestin (9, 62–64). When these mutants are labeled with the fluorescent probe monobromobimane and incubated with phosphorylated rhodopsin, they exhibit changes in fluorescence upon rhodopsin photoactivation. Importantly, these changes do not occur in the presence of nonphosphorylated rhodopsin, and subsequent analysis suggests that the spectral changes are a direct result of interaction with activated, phosphorylated rhodopsin. In the present study, we demonstrate how these spectral changes can be exploited to obtain information on the rate of arrestin release. Our results provide direct evidence that arrestin and retinal release are indeed linked events and that arrestin slows the rate of retinal release from Rho*-P by ~2-fold, possibly through a kinetic trap mechanism. In addition, we find that arrestin binding eliminates the pH dependence of retinal release. Surprisingly, we find that a subfraction of arrestin consistently remains bound to phosphorylated rhodopsin long after the decay of the active Meta II species. This latter result provides compelling evidence that arrestin binds Meta III or some other photodecay product.

**EXPERIMENTAL PROCEDURES**

**Materials**—Frozen bovine retinas were obtained from Lawson and Lawson, Inc. (Lincoln, NE). GBX red light filters were purchased from...
Dynamics of Arrestin-Rhodopsin Interactions

Eastman Kodak Co., and [γ-32P]ATP was from PerkinElmer Life Sciences. Nitrocellulose filters (0.45 μm) and Biamax centrifugal concentrators (10-kDa cut-off) were from Millipore Corp. (Bedford, MA). Mono-bromobimane was obtained from Molecular Probes, Inc. (Eugene, OR), and [1-oxyl-2,2,5,5-tetramethyl-p- pyrroline-3-methyl]methanethiosulfonate spin label was from Toronto Research Chemicals (North York, Canada). 11-cis-Retinal was a generous gift from Rosalie Crouch (Medical University of South Carolina and NRI, National Institutes of Health). Cuvettes were purchased from Uvonics (Plainview, NY), and round capillaries for EPR measurements were from VitroCom, Inc. (Monmouth Lakes, NJ). Band pass filters and long pass filters were acquired from Oriel (Stratford, CT). Acrylamide/bisacrylamide solution (37.5:1) and microcolumns were purchased from Bio-Rad. Spectroscopic grade buffers were from USB Corp. All other chemicals and reagents were obtained from Sigma.

Preparation of Rod Outer Segments (ROS)—ROS were isolated from bovine retinas as described previously (10). All sucrose solutions were made in ROS buffer (70 mM potassium phosphate, 1 mM magnesium acetate, pH 6.8), and all procedures were done at 4 °C under red lights. Rhodopsin concentration was assessed by difference spectra in the presence of hydroxyamine (ε500 = 40,800 liters cm⁻¹ mol⁻¹). Stocks were snap-frozen and stored at −80 °C.

Highly phosphorylated ROS (ROS-P) were prepared by suspending ROS (0.5 mg final rhodopsin concentration) in ROS buffer, with 20 μM GTP and 3 mM ATP (10-ml final volume). Phosphorylation of rhodopsin by the native rhodopsin kinase was initiated by illumination with a 15-watt bulb from a Kodak safelight (without filter) placed ~20 cm away, and sedimentation of the membranes was prevented by gently rocking the sample. After 2 h, the reaction was stopped by a 4-fold dilution with ROS buffer (plus 50 mM hydroxyamine and 2% bovine serum albumin). Phosphorylated opsin membranes were then collected by centrifugation (40,000 × g, 50 min). Levels of phosphorylation were quantified with the use of [γ-32P]ATP (10–100 cpm/μmol) as a tracer. Aliquots were removed from the tracer reaction, spotted onto nitrocellulose filters, washed, and subjected to scintillation counting. Our assay indicated that ROS-P with ~6.4 ± 1.5 phosphates/rhodopsin was created (11).

Phosphorylated opsin membranes were washed by resuspending the pellet using a tissue homogenizer, followed by centrifugation. Membranes were washed twice with low ionic strength buffer (5 mM PIPES, 1 mM EDTA, pH 7.0) to remove peripheral proteins (12), followed by three additional washes with ROS buffer. The final washed opsin membranes were regenerated overnight by the addition of a 2-fold excess of 11-cis-retinal (4 °C). The regenerated samples were washed once with ROS buffer containing 2% bovine serum albumin in and 50 mM hydroxyamine, twice with 2% bovine serum albumin in ROS buffer, and three times with ROS buffer alone. Nonphosphorylated ROS membranes were prepared identically, except that no ATP was added during the phosphorylation procedure. The turbidity of ROS samples was reduced by continuous sonication using a Branson 1210 bath sonicator (4 °C, 3 times with ROS buffer alone. Nonphosphorylated ROS membranes were then collected by centrifugation (40,000 × g, 50 min). Levels of phosphorylation were quantified with the use of [γ-32P]ATP (10–100 cpm/μmol) as a tracer. Aliquots were removed from the tracer reaction, spotted onto nitrocellulose filters, washed, and subjected to scintillation counting. Our assay indicated that ROS-P with ~6.4 ± 1.5 phosphates/rhodopsin was created (11).

Synthesis of Synthetic Phosphopeptide 7FP—The 19-amino acid-long peptide analogous to the fully phosphorylated C-terminal tail of rhodopsin was synthesized and purified as described previously (13).

Construction, Expression, and Purification of Arrestin—Recombinant bovine visual arrestin with an N-terminal His tag was expressed and purified from Pichia pastoris as described previously (14). Mutant constructs I72C and S251C were created utilizing PCR, and the constructs were confirmed by DNA sequencing on both strands.

Labeling of Arrestin—Arrestin samples were buffer-exchanged and concentrated (~50 μM) in labeling buffer (10 mM MES, 150 mM NaCl, pH 6.5) by ultrafiltration (Millipore Biomax). Monobromobimane was added from a stock in Me2SO in 10-fold molar excess to arrestin (final Me2SO concentration below 1%). After an incubation of 3 h at room temperature with gentle agitation, samples were centrifuged briefly at 100,000 × g to remove aggregates. Labeled arrestin was bound by multiple passages over His-select resin (Sigma) equilibrated with buffer (10 mM HEPES, 250 mM NaCl, pH 7.4), followed by extensive washing with buffer. Arrestin was eluted with 500 mM imidazole, and the imidazole was removed by size exclusion chromatography (Superdex 75; Sigma; 10 mM HEPES, 150 mM NaCl, pH 7.4). The labeling efficiency was calculated using ε592 = 26,360 liters cm⁻¹ mol⁻¹ for arrestin and ε529 = 5,000 liters cm⁻¹ mol⁻¹ for bimane (15, 16). The absorbance at 392 nm was subtracted from the 280-nm value to compensate for bimane’s absorbance at 280 nm. Using this method, labeling efficiencies of ~83 and 88% were determined for arrestin mutants I72C and S251C, respectively. Since both of these mutants contain the three native cysteines of arrestin (Cys66, Cys128, and Cys143), a sample of wild-type (WT) arrestin was labeled using the same conditions as a control. The WT cysteines labeled at less than ~2% efficiency.

To assess possible free label contamination, the fluorescence of 2 μM labeled arrestin was compared with an identical sample, which had been precipitated with trichloroacetic acid (10%). For both I72B and S251B, free label contamination was well below 1%.

Functional Pull-down Assay—A simple centrifugation assay was used to assess arrestin functionality. Briefly, sonicated ROS containing 12 μM rhodopsin or phosphorylated rhodopsin and 3 μM arrestin were mixed in 10 mM HEPES, 150 mM NaCl, pH 7.4, in the dark at room temperature (20 μl). Reactions were either kept in the dark or bleached for 5 min using a 150-watt fiber optic light source (λ = 495 nm), followed by 10-fold dilution with ice-cold buffer and centrifugation at 100,000 × g for 10 min at 4 °C. The pellets were solubilized in loading buffer and subjected to SDS-PAGE (10%). Proteins were visualized by Coomassie staining, and densitometry was performed using AlphaEase FC software.

To assess arrestin affinity for post-Meta II rhodopsin, ROS-P in 10 mM HEPES, 150 mM NaCl, pH 7.0, was photobleached at room temperature for 90 s and then transferred to a 35 °C water bath. The sample was kept in the dark after the initial photobleach. After 12 min, arrestin I72B was added (1 μM) and incubated at room temperature for an additional 3 min. The samples were diluted, centrifuged, and subjected to SDS-PAGE as described above. The fluorescently labeled arrestin was visualized with a gel-doc apparatus (Alpha-Innotech FluoroChem 5500). The gel was excited from above with a short wave UV source, and the fluorescent bands were detected through a cut-off filter (535 ±
FIG. 2. Labeled arrestin mutants are functional. Arrestin affinity for dark rhodopsin (ROS), light-activated rhodopsin (ROS*), dark phosphorylated rhodopsin (ROS-P), and light-activated phosphorylated rhodopsin (ROS*P) was assayed by centrifugal “pull-down” assay (see “Experimental Procedures”). The data indicate the labeled mutants retain WT-like specificity and affinity for ROS*P. Upper panel, lanes 1–4, arrestin I72C. Lanes 5–8, arrestin I72C labeled with monobromobimane (I72B). Lane 9, for comparison, WT arrestin binding to ROS*P is shown. Lower panel, lanes 1–4, arrestin S251C. Lanes 5–8, S251B. Lane 9, WT control. Molecular weight markers in kDa (MW) are indicated.

50 nm) by a CCD camera (5-min exposure). Densitometry was performed using AlphaEase FC software.

Steady-state Fluorescence Assays—All fluorescence measurements were made using a Photon Technologies QM-1 steady-state fluorescence spectrophotometer with a single excitation source and two emission detectors (T format). Temperature was controlled and monitored using a water-jacketed cuvette holder connected to a circulating water bath (YVR Scientific) and a digital thermometer, which was submerged into a water-filled well in the sample chamber. Typically, 100-μL samples containing 1 μM labeled arrestin in a 2-mm black-jacketed cuvette were excited at 380 nm, and the emitted fluorescence was measured from 400 to 600 nm using 2 nm increments. Each data point was integrated for 0.25 s, and the average of two scans yielded the final spectrum. Excitation bandpass was kept at 0.25 nm to avoid bleaching of rhodopsin samples (emission bandpass at 15 nm). Spectra were smoothed and normalized using the PTI software program Felix, and the fluorescence spectra of buffer or rhodopsin alone were subtracted where appropriate. Rhodopsin was generally present at a 4-fold excess to arrestin, which was found to be sufficient for complete arrestin binding. The samples were bleached using a 150-watt fiber optic light source (>495 nm) for 40 s.

Fluorescence Lifetime Measurements—A PTI Laserstrobe fluorescence lifetime instrument was used to measure I72B and S251B under different conditions. Typically, 500 nM arrestin in a 4-mm black-jacketed cuvette (200 μL) was measured at 20 °C using 381-nm excitation pulses (full-width half-maximum, ~1.5 ns) with a 298–435-nm band pass filter on the excitation beam. The emission was monitored through two >470-nm long pass filters, and neutral density filters were used to modulate the intensity. Each data point collected represented two averages of five laser shots, and typically 150 points were collected over the lifetime decay curve. The instrument response function, which must be deconvoluted from lifetime decay data, was determined from the scatter from a solution of Ludox through a 400-nm broadband interference filter. Data points were acquired randomly to minimize the impact of laser misfires on the decay curve, and data were analyzed using the commercial PTI software TimeMaster. The “goodness of fit” was evaluated by plotting the residuals and the χ² value (0.7 < χ² < 1.2 was considered acceptable) (17, 18).

Fluorescent lifetimes of these labeled arrestins were also measured in the presence of the phosphopeptide 7PP (100 μM) or ROS-P (2 μM) in the dark state, after photoactivation, and after the addition of 50 mM hydroxyamine). The lifetime data acquisition scheme described above was found to bleach <2% of rhodopsin, and the total time elapsed from the start of photoactivation to the conclusion of the measurement was 4 min, or ~0.5 Meta II decay half-lives at 20 °C. ROS-P was found to introduce a significant amount of scatter and a fluorescent component with an extremely short lifetime (<1 ns) into the samples. To correct for these components, they were simply subtracted from the arrestin lifetime by measuring the lifetime of ROS-P alone with matched concentration under the same conditions.

Fluorescence Quenching Analysis—To measure the quenching effects of I on free versus rhodopsin-bound arrestin, a stock of 4 μM ROS-P and 1 μM labeled arrestin was divided among five tubes (0, 10, 20, 30, or 50 mM KI). KCl was added to keep the ionic strength consistent, and 0.1 mM sodium thiosulfate was present to suppress I₂ formation. The steady-state fluorescence of each sample was measured in the dark state and immediately after photoactivation, and the average of two independent experiments was used to calculate quenching constants. KSV is derived from the Stern-Volmer equation F/F₀ = 1 + KSV [Q], where F₀ and F represent the fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the concentration of quencher. KSV values were also determined for free arrestin and arrestin in the presence of the phosphopeptide 7PP by titration with KI. The fluorescence was corrected for dilution, and an independent titration with KCl was performed to assess any potential ionic effects on the fluorescence. For time-based quenching studies of arrestin I72B, the
Monoexponential and double exponential fluorescence lifetime analysis of bimane-labeled arrestin mutants I72C and S251C

Lifetimes were measured as described under “Experimental Procedures.” Samples contained 500 nM I72B or S251B alone in buffer, in the presence of 100 μM phosphopeptide 7PP, or in the presence of 2 μM ROS-P (dark, after photoactivation (+hv), and after the addition of 50 mM hydroxylamine). Two or three sets of lifetime data from independent experiments are shown for each sample. τ1 and τ2 are the lifetimes in nanoseconds, and α1 and α2 are the fractional amplitudes of each lifetime τ1 and τ2, respectively (the sum of the pre-exponential factors α is normalized to 1).

| Sample         | α1  | τ1  | α2  | τ2  | χ² | (γ)² | τ⁺  |
|----------------|-----|-----|-----|-----|----|------|-----|
|                | ns  | ns  | ns  | ns  |    |      |     |
| I72B           | 1.0 | 11.3| 1.0 | 1.0 | 1.1| 1.1  | 1.1|
| I72B + 7PP     | 1.0 | 11.2| 1.0 | 1.0 | 0.8|      |     |
| I72B + ROS-P (dark) | 1.0 | 10.9| 1.0 | 1.0 | 1.0|      |     |
| I72B + ROS-P (+hv) | 1.0 | 10.4| 1.0 | 1.0 | 0.9|      |     |
| I72B + ROS-P (+NH₂OH) | 1.0 | 10.4| 1.0 | 1.0 | 1.0|      |     |
| S251B          | 0.27| 7.8 | 0.73| 1.6 | 0.9| 2.9  | 5.3|
| S251B + 7PP    | 0.27| 6.8 | 0.73| 1.1 | 1.0|      |     |
| S251B + ROS-P (dark) | 0.34| 6.0 | 0.73| 1.4 | 0.9|      |     |
| S251B + ROS-P (+hv) | 0.34| 6.0 | 0.73| 1.4 | 0.9|      |     |
| S251B + ROS-P (+NH₂OH) | 0.34| 6.0 | 0.73| 1.4 | 0.9|      |     |

Summary of lifetime and quenching values for bimane-labeled arrestin mutants I72C and S251C

| Sample         | τ⁺  | k⁻¹ | k⁺  |
|----------------|-----|-----|-----|
|                | ns  |      |     |
| I72B           | 11.1 ± 0.1| 267 ± 5.2| 2.4 |
| I72B + 7PP     | 10.6 ± 0.1| 261 ± 4.5| 2.5 |
| I72B + ROS-P (dark) | 10.5 ± 0.1| 256 ± 1.6| 2.4 |
| I72B + ROS-P (+hv) | 9.5 ± 0.2| 9.4 ± 2.3| 1.0 |
| S251B          | 5.3 ± 0.2| 12.9 ± 1.4| 2.4 |
| S251B + 7PP    | 5.0 ± 0.1| 10.0 ± 0.7| 2.0 |
| S251B + ROS-P (dark) | 4.8 ± 0.1| 12.3 ± 2.0| 2.5 |
| S251B + ROS-P (+hv) | 6.0 ± 0.1| 5.5 ± 0.3| 0.9 |

The intensity-weighted average lifetime (Table I).

The bimolecular quenching constant (k⁻¹), which represents collisions per second between the fluorophore and the quencher, was derived using the relationship $K_{q} = k_{q} \times \tau_{q}$ (where $\tau_{q}$ represents the intensity-weighted average fluorescence lifetime in the absence of quencher) (28, 60, 61). Note that $k_{q}$ is dependent on diffusion rates, and thus the reported $k_{q}$ values may reflect not only a change in solvent accessibility but also a change in arrestin’s rate of diffusion that occurs when it binds the ROS*-P vesicle.
Determined as described above (35°C).

**EPR**—Arrestin I72C and S251C were prepared for EPR in an identical manner as described for monobromobimane labeling, except that a 5-fold molar excess of nitroxide spin label was used during labeling. The EPR spectrum of a sample of WT arrestin, labeled using identical conditions, showed less than 10% incorporation of spin label at the native cysteines compared with I72C. For EPR measurements, a 6-μl volume of 50 μM spin-labeled arrestin and 200 μM ROS-P were measured in the dark at room temperature (19–21 °C) and at various time points following photoactivation (45 s using a 150-watt >485-nm fiber optic light source). To assess whether the observed spectral changes were due to changes in protein rotational rates, EPR spectra were also measured in the presence of 20% Ficoll 400 (21). Ficoll concentrations higher than 20% could not be used, since they caused arrestin precipitation. EPR measurements were made using a Varian E-104 instrument fitted with a loop-gap resonator and the EWWIN 5.22 data acquisition package (Scientific Software Services, Plymouth, MI). Measurements were carried out at ~9.3-GHz microwave frequency, using 2-milliwatt incident microwave power, a modulation amplitude of ~2 gauss, and a 100-gauss sweep (29 s/scan). Multiple scans were taken and averaged where appropriate (see the legend to Fig. 7 for details).

**RESULTS**

Numerous studies suggest the two concave surfaces of arrestin are involved in binding light-activated phosphorylated rhodopsin (5, 9, 22). In the present study, we introduced two cysteine residues between these surfaces and labeled them with the cysteine-specific fluorescent probe monobromobimane (Fig. 1). We and others have extensively characterized bimane and found that it can reliably report on protein dynamics and structure due to its small size, its sensitivity to polarity, and its ability to be quenched by nearby tryptophan and tyrosine residues (15, 23–26). Below, we report our studies on the bimane-labeled arrestin mutants I72C and S251C.

**Fluorescently Labeled Arrestin Mutants Are Functional**—The relative functionality of the mutants with and without the bimane label was first assessed using a centrifugal “pull-down” assay (Fig. 2). Mutant I72C shows proper binding specificity to ROS*-P but binds ROS*P with less affinity (43% of WT). Interestingly, bimane labeling of I72C (I72B) appears to restore the binding ability of this mutant to ~99% of WT levels. Both unlabeled arrestin S251C and labeled S251B bind to similar levels as WT (89 and 101%, respectively).

**Spectral Properties of Labeled Arrestin Mutants**—Both I72B and S251B fluoresce with a λmax of ~470 nm, and their spectra do not change in the presence of an excess of dark ROS-P. However, upon photoactivation, the emission spectrum of arrestin I72B blue-shifts to a λmax of 456 nm and increases in total integrated intensity by ~12% (Fig. 3A). The fluorescence of arrestin S251B increases ~100% upon photoactivation but displays no shift in its λmax (Fig. 3D). Importantly, these changes are not observed using nonphosphorylated ROS (Fig. 3, B and E) and are abolished by 50 mM hydroxyamine (data not shown), presumably because hydroxyamine catalyzes the decay of activated rhodopsin by cleaving the retinal Schiff base.

**Spectral Changes Require Interaction with Rhodopsin**—We next assessed whether the observed spectral changes were due to conformational changes within arrestin itself rather than interactions with ROS*-P. To do this, we tested the effect of phosphorylated peptide 7PP, which represents the fully phosphorylated form of rhodopsin’s C-terminal tail and has been shown to transactivate arrestin to bind nonphosphorylated ROS* (13). The peptide 7PP causes an ~20% decrease in intensity for mutant I72B (Fig. 3C) and induces no change in arrestin S251B (Fig. 3F). We obtained similar results as for 7PP using heparin and phytic acid, two polyamionic compounds that have been reported to bind arrestin and induce activating conformational changes (data not shown) (20, 27).

**Fluorescence Lifetime Analysis**—To further elucidate the cause of these spectral changes, we measured the fluorescent decay lifetimes of I72B and S251B under different conditions (Table I). The fluorescence lifetime (τ) of I72B is 11.1 ns, and this value is shortened slightly by the phosphopeptide 7PP (10.6 ns). In the presence of ROS-P, the τ shortens by ~1 ns after photoactivation but reverts to ~10.5 ns after the addition of hydroxyamine. These values are all similar to free bimane (9.1 ± 0.1 ns; data not shown), suggesting no major perturbation of the probe at this site. In contrast, the lifetime of S251B is multiexponential, with components of ~7.4 and 1.4 ns, resulting in an amplitude-weighted average fluorescence lifetime...
Dynamics of Arrestin-Rhodopsin Interactions

Fluorescence Quenching Analysis—Fluorescence quenching studies were carried out using the soluble quencher Iγ, to determine whether binding to ROS*-P shields the probes on I72B and S251B from the solvent. The Stern-Volmer constants (KSV) and bimolecular quenching constants (kq) determined under different conditions are reported in Table II. The data indicate that the probe at I72 is more solvent-accessible on free arrestin (higher KSV and kq) than when arrestin is bound to ROS*-P (lower KSV and kq). Consistent with this observation, we note that the fluorescence intensity increase that occurs upon I72B binding ROS*-P is directly proportional to temperature (12% increase at 20 °C versus 50% increase at 35 °C; data not shown). We believe this effect is due to differences in solvent accessibility for the following reasons. At warmer temperatures, the probe on unbound I72B experiences more collisions with the solvent than at cooler temperatures and is thus de-exited more. Upon binding ROS*-P, the probe is protected from collisions, resulting in a higher relative change in fluorescence at warmer temperatures. These results also complement the blue shift in the I72B emission spectra upon ROS*-P binding (Fig. 3A), which indicates a movement of the bimane probe to a more hydrophobic environment (15, 29).

The lower KSV for S251B is probably due to its shorter lifetime compared with I72B. When the quenching data are analyzed using the lifetime values to obtain the kq, or the true number of collisions/s of quencher and probe, the probe at Ser251 is seen to be as accessible as at Ile72. Importantly, the kq for S251B is also significantly reduced in the presence of ROS*-P, implying that the probe at this site is also shielded from the solvent when bound to rhodopsin.

Kinetics of Arrestin Binding and Release—The fluorescence changes described above can be used to directly monitor arrestin binding to ROS*-P. As shown in Fig. 4A, the fluorescence of I72B increases and then plateaus after photoactivation. A second bleach produces no further increase, indicating that all of the arrestin is bound to ROS*-P after the initial bleach. The data are well fit to a monoexponential, yielding a binding rate constant of k = 27.6 ± 1.6 × 10⁻³ s⁻¹ or a t1/2 value of 25.1 s at (τ) of 2.6 ns. Note that (τ) is proportional to steady-state intensity (18, 28), and thus the short (τ) of S251B is consistent with its quenched fluorescence compared with I72B and indicates some sort of dynamic quenching mechanism. Binding of S251B to ROS*-P causes a significant increase in its (τ), which correlates to an increased steady-state intensity (Fig. 3D).

![Fig. 5. Temperature, pH, and arrestin dependence of retinal release from phosphorylated rhodopsin.](image-url)

Arrestin affects the rate and pH dependence of retinal release from ROS*-P. A, using the fluorescence approach described under “Experimental Procedures,” retinal and arrestin release from ROS*-P can be monitored simultaneously. In this example, retinal release (λem = 395 nm, λex = 330 nm, black trace) and arrestin release (λem = 380 nm, λex = 456 nm, gray trace) from a sample of 4 μM I72B and 2 μM ROS-P was monitored at 25 °C. Rates were determined by fitting the buffer-subtracted raw data to a single exponential. The residuals demonstrate the goodness of fit. B, arrestin slows retinal release from ROS*-P. Retinal (atR) release from ROS*-P in the absence of arrestin (closed circles), retinal release from ROS*-P in the presence of an excess of arrestin I72B (open circles), and arrestin release from ROS*-P (triangles) were measured at different temperatures (15–40 °C). The average of two independent experiments is shown for each point. C, Arrhenius analysis indicates that arrestin slows the rate of retinal release without dramatically affecting the energetics. The slopes of the plots indicate similar activation energies for retinal release from ROS*-P without arrestin (closed circles, Ea = 21.6 ± 0.9 kcal/mol), with arrestin (open circles, Ea = 19.4 ± 0.9 kcal/mol) and the release of arrestin from ROS*-P (triangles, Ea = 18.0 ± 0.7 kcal/mol) at pH 7.5. D, Arrestin abolishes the pH dependence of retinal release. The data show the effect of pH on retinal release from ROS*-P without arrestin (closed circles), retinal release from ROS*-P with an excess of arrestin I72B (open circles), and arrestin release from ROS*-P (triangles) at 35 °C. Each point represents data from two independent experiments.
creases with increasing pH (Fig. 5). Retinal release from ROS*-P in the absence of arrestin is slow without arrestin.

Arrhenius plots are kinetically shifted below that of retinal release. As expected, we find the rate of retinal release from ROS*-P increases with temperature (Fig. 5A), as described previously (19, 30). However, although we find that arrestin and retinal release occur at the same rate, it appears that arrestin slows retinal release ~2-fold at physiological temperatures (Fig. 5B). This slowing of retinal release is also observed with unlabeled arrestin (data not shown), indicating that these results are not simply due to the use of the bimane-labeled arrestin. It should be noted that, although arrestin is present in excess in these samples, not all rhodopsin proteins might be bound by arrestin after illumination. A portion may be inaccessible to arrestin, due to inside-out vesicles or heterogeneous phosphorylation. Thus, the experiment shown in Fig. 5A may reflect two different rates of retinal release, from unbound rhodopsin and from arrestin-bound rhodopsin, and this may account for the slight deviation of the residuals for the calculated curve.

Analysis of Arrhenius plots using these rates reveals that retinal release from ROS*-P occurs with an \( E_a \) of 21.6 kcal/mol (Fig. 5C), in good agreement with previous findings (19, 30). The \( E_a \) values for arrestin release and retinal release in the presence of arrestin are similar (\( E_a = 18.0 \) and 19.4 kcal/mol, respectively). However, it is important to note that these Arrhenius plots are kinetically shifted below that of retinal release without arrestin.

We also find that at 35 °C and above pH 6.5, the rate of retinal release from ROS*-P in the absence of arrestin increases with increasing pH (Fig. 5D). Strikingly, in the presence of arrestin, the rates of retinal and arrestin release are pH-independent. These results are intriguing, since they closely mirror those seen by Heck et al. (30) using a peptide analogue derived from the C terminus of the \( \alpha \)-subunit of transducin (G\(_\alpha\)-HAA) and suggest a coupling between events at the cytoplasmic face and Schiff base hydrolysis.

**Dynamics of Arrestin Release from ROS*-P**—Rather than decay to opsin and free retinal, a significant population of rhodopsin can also decay to a photoproduct called Meta III at physiological temperature and pH (30, 31). Although the exact nature of Meta III is still under active investigation, it is clear that Meta III differs from Meta II in its absorbance (\( \lambda_{\text{max}} = 470 \) nm), its structure, and its ability to activate transducin. Below, we describe our surprising finding that suggests that long after Meta II decay, arrestin remains bound to Meta III or some post-Meta II photoproduct.

**Fig. 6. A fraction of arrestin remains bound to rhodopsin after Meta II decay.** Fluorescence and centrifugation studies both indicate that some arrestin remains bound to ROS-P after Meta II decay. A, after the initial increase after photoactivation (arrow), the fluorescence of arrestin I72B in the presence of ROS-P decreases as the arrestin dissociates, but it does not return to dark state levels and plateaus at ~20% of the starting state intensity. The addition of 10 mM hydroxylamine returns the fluorescence to dark state levels. Inset, a centrifugal pull-down assay also reveals that arrestin I72B remains bound to ROS-P long after Meta II decay. In this experiment, arrestin I72B and ROS-P were mixed in the dark and centrifuged at 90 s (left lane) or 1600 s after photobleach (middle lane). As a control, 10 mM hydroxylamine was added prior to centrifugation (right lane). The membrane pellets were subjected to SDS-PAGE, and the bimane-labeled arrestin bands were visualized by fluorescence and quantified by densitometry. A significant amount of arrestin remains bound at 1600 s, corresponding to ~25% of the 90-s sample (subtracted for nonspecific pull-down seen in far right lane). Both experiments used 1 \( \mu \)M I72B and 4 \( \mu \)M ROS-P (pH 7, 35 °C). B, fluorescence quenching analysis also indicates that a fraction of arrestin remains bound long after Meta II decay. Experiments similar to that shown in A were carried out with increasing concentrations of KI (0, 10, 20, 30, 50, 70, and 100 mM). Raw data (smoothed for the increase in noise due to the transformation) is plotted in three dimensions: time, KI concentration, and fractional change in fluorescence (where \( F \) represents fluorescence and \( F_0 \) is the fluorescence without quencher). Data from two independent experiments were used to derive Stern-Volmer constants of I72B in the presence of dark ROS-P (\( K_{SV} = 22.6 \pm 0.4 \text{ M}^{-1} \)) (a), after photoactivation (\( K_{SV} = 5.9 \pm 0.6 \text{ M}^{-1} \)) (b), and at the plateau (\( K_{SV} = 14.5 \pm 1.5 \text{ M}^{-1} \)) (c). The fits of \( F/F_0 \) versus [KI] are indicated as *straight lines* in the graph.
As shown in Fig. 6A, after the initial increase that occurs upon I72B binding to ROS*-P, the fluorescence decreases at a rate that matches Meta II decay and retinal release, yet consistently plateaus at some value ~20% higher than the initial dark state intensity. To make the fluorescence fully return to the starting value, we find it necessary to add hydroxylamine, which cleaves the Schiff base and converts all rhodopsin photointermediates to opsin. This effect is highly reproducible. To ensure that this “plateau effect” is not simply a spectral artifact, we carried out centrifugal pull-down analysis, and we find the same result (Fig. 6A, inset). A majority of arrestin I72B is pulled down 90 s after photoactivation with Meta II ROS*-P, and ~25% of this arrestin is still pulled-down with ROS*-P more than 1600 s after photoactivation (35 °C). Note that this effect cannot be due to Meta II rhodopsin, since Meta II decays with a t1/2 of ~90 s at 35 °C, and thus after 1600 s, less than 0.4 × 10^-4% of the original pool of Meta II remains. Again, as noted for the fluorescence assay described above, we find that it is necessary to add hydroxylamine to abolish this binding.

We explored this phenomenon further by measuring how accessible the probe at Ile72 is to the quencher I in the presence of (a) dark ROS-P, (b) immediately after photobleach, and (c) at 15 half-lives after Meta II decay (35 °C) (Fig. 6B). The probe at Ile72 is relatively accessible in the dark (Ksv of 22.6 m^-1), but upon arrestin binding to ROS*-P, the probe becomes more buried (Ksv = 5.9 m^-1). Interestingly, even after 15 Meta II decay half-lives, the accessibility of the probes has not returned to that of the original dark state (Ksv = 14.5 m^-1), indicating that some population of the probes on I72B are still buried relative to the original dark state.

Fluorescent Changes Are Not Simply Due to Spectral Artifacts—Fluorescence experiments involving rhodopsin must take into account possible complications caused by the spectral properties of rhodopsin. We are confident that the fluorescence changes described above are not simply due to trivial optical artifacts for the following reasons. (a) Under the conditions used, the optical density of the samples was measured to be less than 0.1; thus, the data should be free of inner filter effects. (b) Similar results were obtained when these mutants were labeled with the fluorophore PyMPO, whose excitation (415 nm) is much more than 0.1; thus, the data should be free of inner filter effects. (c) The EPR spectrum of nitroxide spin-labeled I72C (I72-SL) also changes upon arrestin binding ROS*-P. The spectral changes indicate that the spin label at Ile72 becomes significantly less mobile (Fig. 7A), further suggesting that the probe at Ile72 becomes buried in the arrestin-ROS*-P interface. This immobilization of the probe at Ile72 could not be mimicked by 20% Ficoll 400; thus, the spectral changes are not simply due to a decreased rotational mobility of the protein upon binding to the ROS membranes (Fig. 7B) (21). Furthermore, even after 12 Meta II decay half-lives (90 min, 20 °C), the EPR spectrum of I72-SL differs significantly from the starting state dark spectrum (Fig. 7A), again suggesting that arrestin binds to some post-Meta II photodecay product. Similar results were obtained with spin-labeled S251C (data not shown). EPR measures absorption of microwave radiation and is thus not affected by rhodopsin’s spectral properties (32, 39).

Post-Meta II Decay ROS-P Binds Arrestin—To ensure that the results described above were not simply due to the slowing effect of arrestin on Meta II decay, we tested arrestin’s affinity for ROS*-P that had first decayed in the absence of arrestin. As shown in Fig. 8, we find that a significant amount of arrestin is pulled down when added to post-Meta II ROS-P, and the amount of arrestin pulled-down is roughly proportional to the amount of post-Meta II ROS-P. Note that these samples had decayed through eight half-lives and thus contained less than 0.4% of the original Meta II. As a control, we again find that hydroxylamine abolishes arrestin binding to post-Meta II ROS*-P. These results clearly indicate that arrestin can interact specifically with some photodecay product of rhodopsin, possibly Meta III.

DISCUSSION

Arrestin attenuates rhodopsin signaling by binding to ROS*-P and blocking the G-protein binding site. In the present work, we have employed bimane-labeled arrestin mutants to address how arrestin and rhodopsin interact and how arrestin is released from rhodopsin after binding. Our results are discussed below.

Labeled Arrestin Mutants Are Functional—The two bimane-labeled arrestin mutants used in this study, I72B and S251B, have similar binding specificities as WT when assessed by a centrifugal pull-down assay (Fig. 2). Interestingly, the decreased binding affinity of unlabeled I72C is restored upon attaching the probe bimane to this site, suggesting that some hydrophobic and/or steric bulk at this site may be important for affinity.

Possible Reasons for Fluorescence Changes Observed upon Binding ROS*-P—The fluorescence of the bimane-labeled arrestin mutants changes upon binding to ROS*-P, and control experiments demonstrate that these changes require phosphorylated rhodopsin and cannot be mimicked by the phosphopeptide 7PP. What causes the fluorescent changes? For I72B, the blue shift in fluorescence, the fluorescence quenching analysis, and the EPR data all suggest that the probe becomes buried in...
Fig. 8. Binding of arrestin to ROS-P after Meta II decay. ROS-P binds arrestin, even after Meta II has fully decayed. A, ROS-P membranes (10, 20, 30, and 40 μM) were photobleached at room temperature and allowed to decay to 35 °C for 12 min. At this time, arrestin I72B (1 μM) was added and allowed to incubate at room temperature for an additional 3 min. The samples were analyzed by a centrifugal pull-down assay, as described under “Experimental Procedures.” As a control, hydroxylamine (10 mM) was added prior to centrifugation in half of the samples and is seen to abolish the arrestin binding. The far left lane shows the amount of arrestin pulled down 90 s after photobleach at room temperature. B, fluorescence analysis was used to derive normalized, background-subtracted values of bound arrestin from two independent experiments, as shown in A. The arrestin that was pulled down in hydroxylamine-containing samples represents the amount of nonspecific pull-down.

A rhodopsin-arrestin or phospholipid-arrestin interface. Similarly for S251B, the fluorescence quenching analysis and EPR data also suggest that the probe becomes buried upon binding ROS*-P. In contrast to I72B, no blue shift in fluorescence is observed for S251B, but instead a dramatic increase in fluorescence intensity is observed. What causes this large increase? We believe that the most likely explanation is a conformational change in arrestin itself. Structural analysis using coordinates provided by Hirsch et al. reveals that two tyrosine residues, 67 and 250, lie very close to Ser251 (see Fig. 1) (34). Since tyrosine residues can quench bimane fluorescence, albeit much less effectively than tryptophan (35), the probe at Ser251 is probably susceptible to quenching from at least one of these nearby tyrosine residues, which results in perturbed fluorescence. Upon binding to ROS*-P, this quenching is partially relieved, which suggests that at least one of the quenching tyrosines may move away from the probe at Ser251 upon binding. A possible candidate is Tyr67, since it is unlikely that neighboring Tyr250 can move very far from Ser251. However, since Tyr67 is located on the opposite lobe of arrestin, our results may support the proposed interlobal movement in arrestin upon its activation and binding to ROS*-P (65).

Fluorescence Changes Can Be Used to Monitor Arrestin Binding and Release—The fluorescently labeled mutants provide a novel way to directly monitor arrestin binding and release. Using these mutants, we find that the rate of arrestin binding to ROS*-P at 8 °C to be 27.6 ± 1.6 × 10^{-8} s^{-1}, or a t_{1/2} of 25.1 s. Note that this value is substantially lower than that obtained by Schleicher et al. (36), using the extra-Meta II assay (−0.5 s^{-1} or a t_{1/2} of 1.4 s). We are not sure of the cause of this discrepancy, although it may simply be due to differences in the measuring techniques and reagents.

We used this fluorescence approach to directly and simultaneously monitor both arrestin and retinal release. Our studies indicate that whereas these two events do occur at the same rate, arrestin slows retinal release by ~2-fold at physiological temperature and pH. Arrhenius analysis shows that when arrestin and ROS-P are present together, retinal and arrestin release have E_a of ~20 kcal/mol but are kinetically shifted (slower) compared with retinal release without arrestin. This intriguing finding suggests that arrestin acts to slow retinal release, perhaps through a “kinetic trap” mechanism (Fig. 9), by apparently stabilizing rhodopsin’s structure so that the retinal remains in the binding pocket longer but not affecting the energetics of retinal release. Arrestin binding also abolishes the pH dependence of retinal release, perhaps by affecting certain protonation events, which normally speed the rate of retinal release with increasing pH. A similar effect is observed for the transducin peptide analogue G_{αs}-HAA (30), suggesting that the two may bind at the same site or affect the rhodopsin structure in similar ways.

Previous work has also suggested a slowing effect of arrestin on retinal release, using the enzyme rhodopsin dehydrogenase (RDH), which reduces all-trans-retinal to all-trans-retinol (7, 8). Hofmann et al. (7) found that arrestin slowed the rate of RDH activity by ~40% (30 °C) compared with control reactions in which no arrestin was present. Similarly, we find that arrestin slows the rate of retinal release by ~42% at 30 °C using our direct fluorescent method.

**Arrestin Binds to a Post-Meta II Decay Product**—We were surprised to find that a population of arrestin remains bound to ROS-P, even after all Meta II has decayed. Evidence for this post-Meta II decay binding includes the following. (a) Neither the steady-state fluorescence of I72B nor the EPR spectrum of I72-LS return to dark state levels even after Meta II decay (Figs. 6 and 7); (b) arrestin is “pulled down” with post-Meta II ROS-P in centrifugal pull-down assays (Fig. 8); and (c) fluorescence quenching studies show that long after Meta II decay, accessibility of the probe at Ile72 does not return to dark state values (Fig. 6). Because these effects are all reversed by hydroxylamine, which converts all rhodopsin photointermediates into opsin and free retinal oxime, we hypothesize that arrestin may be binding to Meta III, the long lived storage form of rhodopsin (Fig. 9). Although it would seem counterintuitive for arrestin to bind a nonsignaling form of rhodopsin, Meta III has recently been shown to have some activating ability toward transducin (37). Thus, arrestin may bind Meta III to attenuate its activity and prevent inappropriate signaling.

As far as we are aware, the work presented here is the first demonstration that arrestin binds a post-Meta II decay photoproduct of rhodopsin. Although it has been shown that arrestin will bind phosphorylated opsin membranes to which a large excess of all-trans-retinal has been added exogenously (7), such a nonphysiological situation ignores the potentially important role of secondary retinal binding sites and ligand channeling (30, 37, 38).

One puzzling question raised by our results is how arrestin binding to Meta III (or some other post-Meta II photoprodut) would affect the measurement of “extra-Meta II.” Arrestin stabilizes Meta II at the expense of Meta I, and measurement of this extra-Meta II has been the basis for many quantitative studies of arrestin binding (36, 39, 67). We do not believe

\[ a + b \]
arrestin’s interaction with post-Meta II photoproducts would impact measurement of extra-Meta II, since it is unlikely that any significant amount of late photoproducts exists under the conditions used to measure extra-Meta II (low temperatures and within the first minute after photoactivation).

Furthermore, since Meta III has recently been shown to evolve from Meta I (40, 41), it is reasonable to speculate that the arrestin-bound post-Meta II complex may convert Meta III to a Meta II-like species (7), as has been suggested for transducin (37). We stress that the work we present here has not established the nature of the post-Meta II decay photoproduct to which arrestin binds, and this question will require future studies.

Possible Implications of These Findings on the Visual Cycle—In the visual retinoid cycle, all-trans-retinal released from opsin is reduced by RDH to all-trans-retinol and then transported to the retinal epithelium, enzymatically converted into 11-cis-retinal, and recycled to regenerate rhodopsin in the rod outer segment (4). Our results imply that arrestin influences the first step of this cycle, by slowing the rate at which free all-trans-retinal is released and by affecting the release of retinal from Meta III or other photodecay products. Why might arrestin affect retinal release in these ways? One possibility is that full-length arrestin serves a protective role in the rod cell, by capping bleached rhodopsin and retarding the release of free retinal into the cell. Considering that the rate of RDH activity is slow compared with other visual processes, RDH may become limiting under high bleaching conditions, and levels of free all-trans-retinal may become quite high (42). High levels of free retinal have been shown to be cytotoxic by forming adducts with phosphatidyethanolamine (A2E), which can become highly reactive epoxides that damage DNA and proteins, and thus may contribute to macular degeneration (43). Thus, arrestin may allow the cell sufficient time to deal with the excess retinal and prevent wasteful regeneration of rhodopsin under high bleaching conditions. Consistent with this hypothesis, it has been proposed that full-length arrestin serves to quench rhodopsin activity only under bright light conditions, whereas the short splice variant p44 carries out most of the rhodopsin inactivation within the low light operation range of the rod cell (44). Under dim light conditions, full-length arrestin is sequestered in the inner segment of the rod cell and is only transported to the outer segment under high bleaching conditions (45–47).

Caveats for the Present Work—Although the above scenario suggests that arrestin plays a key protective role in the retinoid cycle, in vivo work by Palczewski, Saari, and others suggests that the situation may not be so simple, since they found no difference in retinoid levels in mouse eyes between WT and arrestin knock-out mice (48). One possible explanation for this apparent discrepancy is that their experiments measured retinoid kinetics after a flash of light followed by recovery in the dark. This approach did not address the situation of constant illumination, where RDH activity would become limiting and arrestin’s role might be more pronounced. Another difference is that our in vitro method required the use of prephosphorylated rhodopsin in washed native membranes and fluorescently labeled recombinant arrestin. Although we strove to carry out experiments under near physiological conditions (temperature, pH, ionic strength), we did not examine the role of other constituents of the visual cycle, which may also affect apparent rates. For example, Hofmann et al. (7) have shown that RDH activity can speed arrestin release, presumably by reducing all-trans-retinal and removing it from the binding pocket.

Possible Implications of These Results on General Mechanisms of GPCR Desensitization—A hallmark of β-arrestin-mediated GPCR attenuation is receptor internalization, which removes the receptor from the cell surface and desensitizes the cell (49). If β-arrestin slows the rate of ligand release fromGPCRs (66), as arrestin does for retinal, it might allow the cellular machinery more time to coordinate and induce internalization. Alternatively, perhaps different ligands for a single receptor affect β-arrestin binding in different ways, and this is why some ligands induce arrestin-mediated GPCR internalization and others do not (50–52).

Summary of Future Directions—The novel method we describe here sets the stage for directly answering many questions regarding the dynamics of arrestin binding and release. For example, do the dynamics of full-length arrestin binding and release differ from the splice variant p44 (53)? What are the effects of exogenously added retinal on arrestin binding and release? Finally, our assay may also be extended to test arres-
tin’s interaction with interesting rhodopsin mutants, such as those that are constitutively active (54) or show extended amounts of Meta III formation. Arrestin-rhodopsin aggregates formed due to certain rhodopsin or arrestin mutations can be a cause of retinitis pigmentosa (55). The site-directed spectroscopic techniques described in this study will allow further investigation of these and other interesting questions.

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