Sulfhydryl Reactivity Demonstrates Different Conformational States for Arrestin, Arrestin Activated by a Synthetic Phosphopeptide, and Constitutively Active Arrestin†

J. Hugh McDowell,*,‡ W. Clay Smith,‡,§ Ron L. Miller,‡ Michael P. Popp,‡ Anatol Arendt,‡ Galina Abdulaeva,‡ and Paul A. Hargrave‡,||

Departments of Ophthalmology, Neuroscience, and Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610-0284

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ABSTRACT: The sulfhydryl groups of the three cysteines in bovine arrestin react with DTNB very slowly (over a period of several hours). In the presence of the synthetic phosphopeptide comprising the fully phosphorylated carboxyl-terminal 19 amino acids of bovine rhodopsin, the reactivity of one of the sulfhydryls was enhanced while that of another was greatly reduced. Since this synthetic peptide was shown to activate arrestin with respect to its binding to unphosphorylated, light-activated rhodopsin, the reactivity of the sulfhydryl groups of a constitutively active R175Q arrestin mutant was examined. All three of the sulfhydryl groups of the mutant arrestin R175Q reacted rapidly with DTNB, but not as rapidly as with SDS-denatured arrestin. The arrestin mutant R175Q bound to light-activated, unphosphorylated rhodopsin in ROS disk membranes. The arrestin mutant R175Q also inhibited the light-activated PDE activity with an IC_{50} of 1.3 μM under the experimental conditions that were used. These data indicate that each of these forms of arrestin is a different conformation. The activated conformation of arrestin that binds to phosphorylated rhodopsin in vivo may be yet another conformation. We conclude that arrestin is a flexible molecule that is able to attain several different conformations, all of which are able to attain the activated functional state of arrestin.

The visual transduction cascade is an exquisitely sensitive interface of an organism with its environment. This sensitivity is a result of the transduction system's ability to distinguish the absorption of only one or two quanta of light by the visual pigment (rhodopsin in rod cells). Maintaining this sensitivity also requires a rapid and complete termination of the activation. This is provided by phosphorylation of light-activated rhodopsin followed by binding of arrestin. While phosphorylation of rhodopsin reduces its ability to activate the transduction cascade (*1*, *2*), binding of arrestin is required to rapidly terminate the activation (*2*). For arrestin to bind to rhodopsin, rhodopsin must be both phosphorylated and freshly light-exposed, i.e., in an activated conformation (*3*). Recent work has shown not only that rhodopsin must be in the proper conformation for binding to arrestin but also that arrestin must be in an active conformation (*4*−*6*). Arrestin was found to bind to unphosphorylated, freshly bleached rhodopsin if a synthetic peptide comprising the fully phosphorylated carboxyl-terminal 19 amino acid peptide (7P-peptide)[1] was present (*4*). In fact, the arrestin could bind to the freshly bleached rhodopsin even if rhodopsin's 19-residue carboxyl terminus containing the phosphorylation sites had been proteolytically removed as long as this synthetic fully phosphorylated peptide was present (*4*). Other studies have shown that mutant arrestins in which arginine 175 has been replaced with either glutamine or glutamic acid similarly are able to bind to freshly bleached, unphosphorylated rhodopsin (*5*, *6*), again indicating that a conformational change in arrestin is required for it to bind to rhodopsin. Physiologically, this conformational change is induced by the phosphorylated carboxyl terminus of rhodopsin. In studies in which limited proteolysis was used as a conformational probe, the compounds heparin, phytic acid, and the fully phosphorylated carboxyl-terminal peptide of rhodopsin all similarly promote an initial fast digestion of arrestin by trypsin, compared to that with native arrestin with no additions, followed by an inhibition of further digestion (*4*, *7*). These observations suggest that arrestin exists in more than one conformation induced by the presence of various ligands.

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* To whom correspondence should be addressed: Department of Ophthalmology, Box 100284 JHMHC, University of Florida, Gainesville, FL 32610-0284. Phone: (352) 392-9097. Fax: (352) 392-0573. E-mail: mcdowell@EYE1.EYE.UFL.EDU.

‡ Department of Ophthalmology.

§ Department of Neuroscience.

|| Department of Biochemistry and Molecular Biology.

[1] Abbreviations: 7P-peptide, bovine rhodopsin residues 330−348 with all seven serine and threonine residues phosphorylated; Arr, arrestin; DTNB, 5,5′-dithiobis(2,2′-nitrobenzoic acid); PDE, phosphodiesterase; R, rhodopsin; R*, photoactivated rhodopsin; R175Q, arrestin mutant with the native arginine in position 175 replaced with a glutamine; ROS, rod cell outer segments; RP, phosphorylated rhodopsin; RP*, photoactivated phosphorylated rhodopsin; SDS, sodium dodecyl sulfate.

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The reactivity of the sulfhydryl groups of native arrestin has been previously examined (*8*, *9*). In these studies, the rate of reaction of the three sulfhydryl groups is very slow compared to that of most proteins with free sulfhydryl groups. All three sulfhydryls were available for reaction under nonreducing conditions, indicating that there are no disulfide bonds in arrestin. The rates of the reactions were also dependent on salt concentration, ligands, and denaturants, indicating that the reactivities of the sulfhydryls are sensitive to the conformation of arrestin as well as suggesting that the conformation of arrestin is dependent on these factors. We decided to examine the reactivities of the sulfhydryls of arrestin under conditions known to induce an active conformation of arrestin.

MATERIALS AND METHODS

Preparation of Bovine Retinal Arrestin. Bovine arrestin was prepared from bovine retinas as described previously (*4*). Both native arrestin and arrestin mutant R175Q were produced in *Pichia pastoris* and purified in a manner similar to that used for retinal arrestin from retinas and is discussed later.

Expression of Recombinant and Mutant Forms of Bovine Arrestin. Bovine retinal arrestin cDNA (a kind gift from C. Craft) was cloned into the *Eco*RI restriction site of the *P. pastoris* shuttle vector pPIC-ZA (Invitrogen). The R175Q mutation of arrestin was introduced into the cDNA using oligonucleotide-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene). The primers used for mutagenesis were 5′-CTGTACCTTCTGGATCAGCAAAC and 5′-TTGCTGATCCAGAAGGTACAG. Following mutagenesis, the R175Q mutation was confirmed by dideoxy chain termination sequencing (Sanger et al., 1977; Sequenase, version 2.0, Amersham) of the entire cDNA.

The native arrestin and the R175Q cDNAs were linearized by digestion with *Pme*I (New England Biolabs) and linearized products separated from uncut plasmid by electrophoresis through 1.2% agarose followed by gel extraction of the linearized band (Qiaex II, Qiagen). This linearized DNA was then electroporated into the GS115 strain of *Pichia* (1.5 kV, 200 Ω, and 25 mF) with selection for recombination on YPD (1% yeast extract, 2% proteose peptone, and 2% dextrose) plates containing 100 μg/mL zeocin (Invitrogen). Isolated colonies were cultured in MGY-H (1.34% yeast nitrogen base, 1% glycerol, 0.00004% biotin, and 0.004% histidine), followed by exchange with MM-H (1.34% yeast nitrogen base, 0.5% methanol, 0.00004% biotin, and 0.004% histidine) to induce expression of the proteins which were under the control of the alcohol oxidase promoter. Expression of arrestin and the R175Q protein were assayed by Western blots, using the anti-arrestin monoclonal antibody SCT-128 (an antibody directed against amino acids 300−320 of arrestin). Colonies that gave the best expression were selected for expression at larger volumes.

For large-scale expression, overnight cultures were begun in 5 mL of YPD with zeocin (100 μg/mL, 30 °C). One liter cultures in MGY-H were seeded with 1 mL from the overnight cultures and allowed to grow for 20 h. The medium in these cultures was then exchanged with 250 mL of MM-H. These cultures were allowed to grow for 3 days (30 °C), adding 0.5% methanol (final concentration) to the cultures every 24 h. Cells were collected from the cultures (2000*g* for 5 min) and resuspended in 10 mM HEPES (pH 7.0) with 3 mM EDTA, 1 mM benzamidine, and 0.1 mM phenylmethanesulfonyl fluoride. The yeast cells in this solution were then lysed using a French press operated at 20 000 psi. Cell debris was pelleted (31000*g* for 30 min) and the supernatant filtered (0.45 μm cellulose acetate, Nalgene) and loaded onto DEAE-Sephacyl (2.2 cm × 19 cm) at a rate of 0.85 mL/min. After loading, the column was washed with about 90 mL of 10 mM HEPES, 1 mM EDTA, 1 mM benzamidine, and 15 mM NaCl (pH 7.0). A gradient was applied from 10 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM benzamidine, and 15 mM NaCl to the same buffer with 200 mM NaCl beginning with 125 mL of each buffer and collecting 5 mL fractions. The column was then washed with 125 mL of the 200 mM NaCl buffer again, collecting 5 mL samples. SDS−PAGE was carried out with aliquots from every fifth fraction, and the fractions containing the 48 kDa protein were pooled and loaded onto a heparin−agarose column (20 mL). The column was washed with 30 mL of 10 mM HEPES and 150 mM NaCl (pH 7.0). A gradient was applied from 0 to 12 mM phytic acid in 10 mM HEPES and 150 mM NaCl (pH 7.0), collecting 2.5 mL fractions. The OD_{278} of the fractions was monitored, and the peak fractions were pooled and dialyzed against 2 L of 10 mM HEPES and 15 mM NaCl (pH 7.0), changing the buffer once after 8 h. The dialyzed pool was reloaded onto the stripped [100 mL of 10 mM HEPES and 750 mM NaCl (pH 7.0)], re-equilibrated [100 mL of 10 mM HEPES and 15 mM NaCl (pH 7.0)] heparin−agarose column and was then washed with 25 mL of 10 mM HEPES and 15 mM NaCl (pH 7.0). A gradient was applied using 50 mL each from 10 mM HEPES and 15 mM NaCl (pH 7.0) to 10 mM HEPES and 750 mM NaCl (pH 7.0), collecting ∼2.5 mL fractions. The OD_{278} of the effluent was monitored, and the peak fractions containing the arrestin (>95% by SDS−PAGE) were pooled and stored at −75 °C in 1 mL aliquots. Immediately prior to the experiments, the arrestin samples were subjected to a buffer exchange using PD-10 columns with 10 mM HEPES and 400 mM NaCl (pH 7.5).

DTNB Reaction. A 2.5 mM 5,5′-dithiobis(2,2′-nitrobenzoic acid) (DTNB) stock solution was prepared by dissolving DTNB in 20 mL of 10 mM HEPES and 400 mM NaCl (pH 7.5) and adjusting the pH to 7.5 by carefully adding 10 μL aliquots of 5 N NaOH. Solutions of heparin (Sigma H3993, 5 mg/mL), phytic acid (Sigma P-3168, 5.4 mM), and 7P-peptide (76 μM) were also prepared in reaction buffer and the pHs adjusted. Arrestin and R175Q were changed to reaction buffer [10 mM HEPES and 400 mM NaCl (pH 7.5)] using PD-10 columns (Pharmacia), just before the experiment.

The DTNB reaction was monitored using a Hewlett-Packard model 8452 diode array spectrophotometer. The reaction was carried out at 25 °C with final concentrations of 8.5 μM arrestin and 500 μM DTNB. All solutions were passed through a 0.45 μm filter just prior to use. A DTNB blank was used to zero the instrument. Arrestin and buffer were allowed to reach 25 °C, and then DTNB was added and the $OD_{412} - OD_{600}$ monitored at 20 s intervals.

The kinetic data as moles of sulhydryl modified per mole of arrestin were initially fit via the least-squares method using the program Scientist (MicroMath Scientific Software, Salt

Lake City, UT). Since there is such a large difference in the fast and slow rate constants, the program was unable to fit the data to two rate constants simultaneously. However, the fast reaction was fit using data from the first 10 min of the reaction because there was essentially no contribution by the slow reaction during this time period. The calculated parameters were then used to extrapolate the contribution of the fast reaction for the entire 2 h time course. The extrapolated fast contribution was subtracted from the observed reaction, leaving only the contribution of the slow reaction which could then be fit to a single exponential. The fit for each set of data was started from the same parameters.

ROS Preparation. ROS for the PDE assay were prepared by sucrose density differential centrifugation in ROS buffer which contained 100 mM KCl, 50 mM NaCl, 10 mM $MgAc_2$, and 20 mM MOPS (pH 7.5). Frozen retinas were thawed and then homogenized in ROS buffer supplemented with 2 mM DTT and 1 mM benzamidine. The pellet collected after centrifugation was submitted to the ROS preparation as described previously (*10*). The ROS were collected by centrifugation and then suspended in the buffer used in the phosphodiesterase activity assay [100 mM KCl, 4 mM $MgSO_4$, and 10 mM HEPES (pH 7.95)] to about 3 mg of rhodopsin/mL. Milliliter aliquots were frozen in liquid N_2 and stored at −85 °C wrapped in foil in the dark.

PDE Assay. ROS (1 mL) were thawed; 50 μL of 1.9 mM GTP was added, and the mixture was incubated at 27 °C for 30 min. This procedure reduced the background dark activity in these preparations.

The volume of the reaction was 600 μL, and the conditions for each determination are given in the figure legends. The pH was monitored using a Beckman ϕ40 pH meter equipped with a Corning flat surface electrode. The data were collected and stored at 1 s intervals using a ComputerBoards CIO-DAS08-PGH analog input card and Control-CB data aquisition program on a Gateway 486-33 computer.

Phosphopeptide Synthesis and Purification. The peptide comprising the carboxyl-terminal 19 amino acid residues of bovine rhodopsin was synthesized and purified in its fully phosphorylated form as described previously (*11*). In this peptide, all three serine residues and all four threonine residues are present as phosphorylated residues.

RESULTS AND DISCUSSION

Reactions of the three sulfhydryl groups of native arrestin are quite slow (Figure 1; *8*, *9*), suggesting their limited accessibility to the reagent. The reaction of the sulfhydryl groups of arrestin in the presence of various compounds known to interact with arrestin is shown in Figure 1. Phytic acid, heparin, and 7P-peptide affect arrestin in a similar manner as judged by their effects on the limited proteolysis of arrestin (*4*, *7*); however, these compounds have very different effects on the reactivity of arrestin's sulfhydryl groups (Figure 1). The presence of heparin has little effect on the reactivities of the sulfhydryl groups. Phytic acid has a greater effect, in the direction of the effect of the rhodopsin 7P-peptide. The 7P-peptide has a dramatic effect, greatly enhancing the rate of reaction of 1 mol of sulfhydryl while apparently reducing the reactivity of the other sulfhydryls. In the presence of the unphosphorylated peptide (residues 330−348), there is no effect on the reactivity of the sulfhydryl groups. SDS denaturation of arrestin greatly accelerates the rate of reaction, rendering all three sulfhydryl groups essentially immediately available for reaction with DTNB.

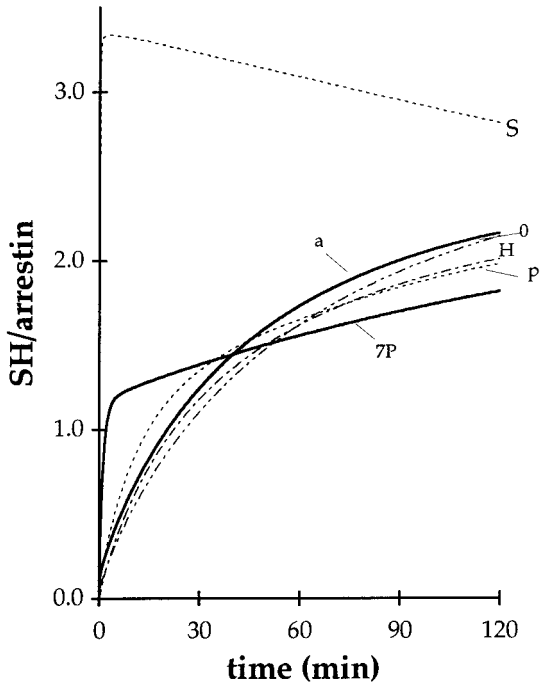

FIGURE 1: Rate of reaction of arrestin's sulfhydryl groups with DTNB in the presence of compounds known to affect the conformation of arrestin. The experimental protocol is described in Materials and Methods: (—) arrestin only (a), (—) arrestin and 7P-peptide (7P), (— - - —) arrestin and unphosphorylated residues 330−348 (0), (— - —) arrestin and heparin (H), (-----) arrestin and phytic acid (p), and (-----) arrestin and SDS (S). The number of moles of sulfhydryl groups modified by DTNB is plotted vs the reaction time in minutes.

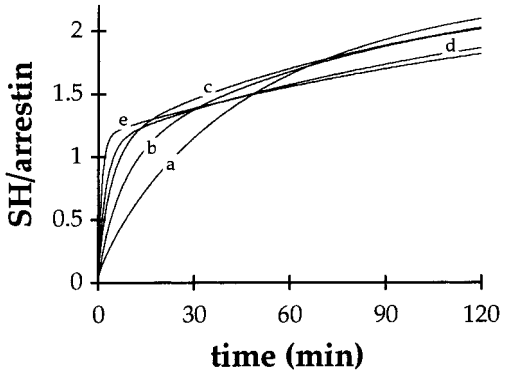

FIGURE 2: Effect of the concentration of 7P-peptide on the reactivity of the sulfhydryl groups of arrestin with DTNB. The concentration of the 7P-peptide was as follows: curve a, 0 μM; curve b, 10 μM; curve c, 19 μM; curve d, 38 μM; and curve e, 76 μM. The reaction was performed as described in Materials and Methods.

The effect of the 7P-peptide appears to be complex, enhancing the rate of reaction of 1 mol of sulfhydryl per mole of arrestin and reducing the reactivity of another. The concentration of the 7P-peptide was varied to resolve the effects of the phosphorylated peptide on the sulfhydryl reactivities (Figure 2). The OD_{412} drifts due to the long reaction time required by the slow reactivity of the sulfhydryl groups; nevertheless, analysis of the kinetic data is instructive, if not very exact. The data were fit to the sum of two

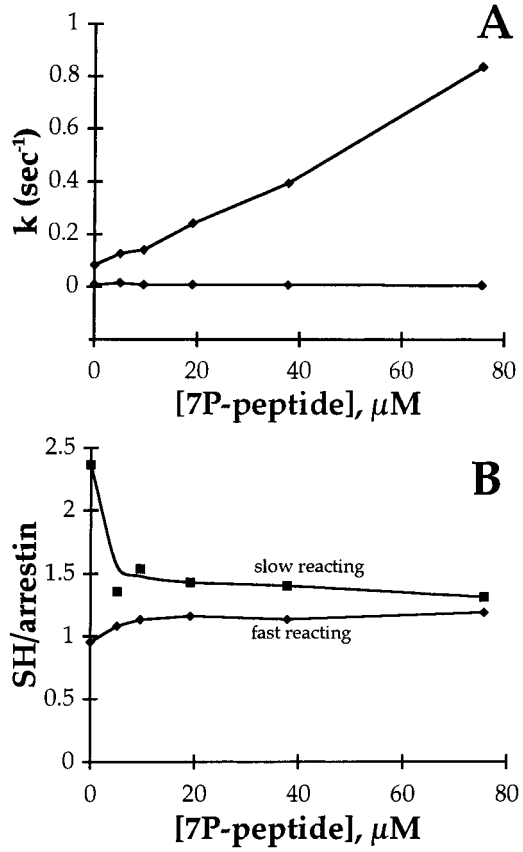

FIGURE 3: Effect of the concentration of 7P-peptide on the kinetic parameters of the reaction of arrestin's sulfhydryl groups with DTNB. (A) The effect of the concentration of 7P-peptide on the pseudo-first-order rate constants for the slow- and fast-reacting sulfhydryl groups of arrestin. (B) The effect of the concentration of 7P-peptide on the number of sulfhydryl groups of arrestin available to react with DTNB.

single exponentials as described in Materials and Methods, and the rate constants and pre-exponential factors are plotted as functions of the 7P-peptide concentration in Figure 3. The fast rate constant is linearly dependent on the concentration of the 7P-peptide over the entire concentration range that was examined (Figure 3A). On the other hand, there is little if any effect on the slow rate constant (Figure 3A). There is no effect of the 7P-peptide on the pre-exponential factor (i.e., the number of moles of sulfhydryls per mole of arrestin) of the fast-reacting SH, which remains about 1. However, the number of moles of slow-reacting sulfhydryls per mole of arrestin is reduced by about 1 (from 2.3 to 1.3) (Figure 3B) even at the lowest concentration that was used, indicating that the 7P-peptide blocks reaction with 1 mole of sulfhydryl groups (per mole of arrestin) even at very low concentrations. Thus, the 7P-peptide has two effects on arrestin: (1) to block 1 mol of sulfhydryl groups (per mole of arrestin) from reacting and (2) to enhance the rate of reaction of DTNB with another mole of sulfhydryl groups (per mole of arrestin).

We examined the effect of the 7P-peptide on light-induced phosphodiesterase activity in rod outer segments containing all of the phototransduction components (*12*). Photoactivated rhodopsin activates the G-protein transducin which in turn increases the activity of the phosphodiesterase (PDE). The photoactivated rhodopsin is then phosphorylated by rhodopsin kinase, allowing arrestin to bind to the photoactivated, phosphorylated rhodopsin and stop the activation process.

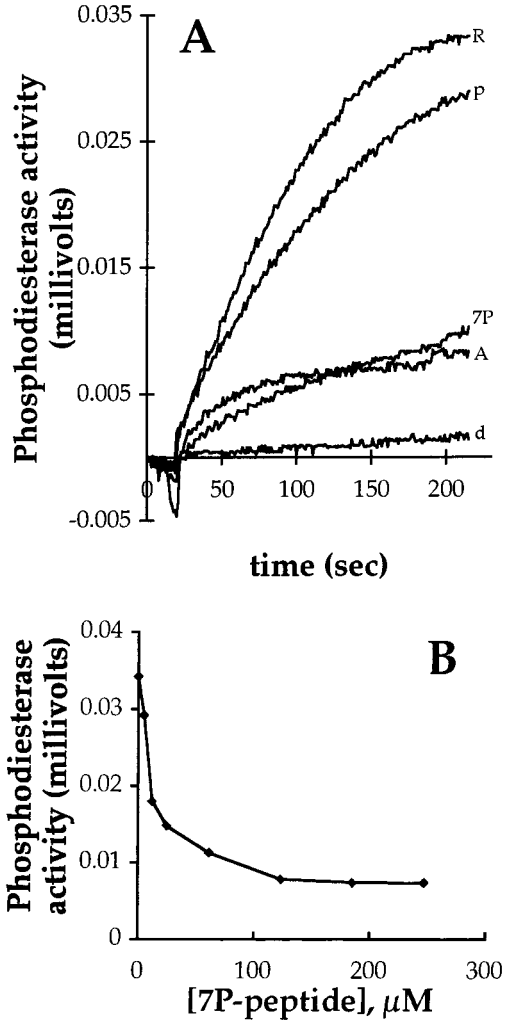

FIGURE 4: Effect of 7P-peptide on the light-induced phosphodiesterase activity of rod outer segments. The final concentrations in the reaction mix were 7.3 μM rhodopsin in ROS, 4.2 μM arrestin, 96 μM GTP, 57 μM GDP, and 2.8 mM cGMP. Data collection was begun at time 0, and cGMP was added at 20 s, initiating the reaction. Panel A shows the phosphodiesterase activity of the control in the light (R) and in the dark (d), the effect of 7P-peptide (62 μM) on the time course of the phosphodiesterase activity (7P), and the effect of 0.9 mM phytic acid (p) and of 33 μM ATP. Panel B shows the effect of varying the concentration of 7P-peptide on the total activity elicited by the flash as measured by averaging the last ten 1 s measures of traces carried out as described for panel A.

In Figure 4A, the light-induced PDE activity is observed by comparing the reaction in light and dark. In the presence of ATP, the PDE activity is markedly reduced. In the presence of 7P-peptide but with no ATP added, the PDE activity is also greatly reduced. This indicates that the 7P-peptide activates the arrestin so that phosphorylation of rhodopsin is not necessary to turn off the PDE activation. The effect of the concentration of 7P-peptide on the light-induced PDE activity was determined (Figure 4B). The half-maximal concentration of 7P-peptide is quite low (<10 μM). It should be noted that the effect required the addition of supplemental arrestin to the preparation as noted in the legend of Figure 4. Little effect on the PDE activity was observed using only the endogenous arrestin in the ROS preparation. It seems likely that since both the blocking of a sulfhydryl group and the PDE effect occur at a low 7P-peptide concentration these two effects are related.

The enhancement of the rate of reaction of the fast-reacting sulfhydryl occurs over a much higher 7P-peptide concentration and would therefore seem to be unrelated to the activation of arrestin. However, the following analysis indicates how this could also be correlated with the activation of arrestin. If arrestin is activated by binding the 7P-peptide, i.e.,

$$a + \text{7P-peptide} \overset{K}{\rightleftharpoons} A-\text{7P-peptide}$$

and the reactions of DTNB with a and A are described by

$$a + \text{DTNB} \xrightarrow{k_1} a\text{-TNB} + \text{TNB}^-$$

$$\text{A-7P-peptide} + \text{DTNB} \xrightarrow{k_2} \underset{\text{TNB}}{\text{A-7P-peptide}} + \text{TNB}^-$$

where a is unactivated arrestin, A is activated arrestin, k_1 is the pseudo-first-order rate constant of the fast-reacting cysteine in the unactivated state, and k_2 is the pseudo-first order rate of the fast-reacting cysteine in the activated state. For this model, it is assumed that only one cysteine residue is converted from a slow- to a fast-reacting state. If the equilibrium constant, K, is very low, at equilibrium there is only a small amount of arrestin in the activated (A) state. This is consistent with our observation that extra arrestin must be present to observe the effect of the 7P-peptide on the PDE activity; i.e., there must be excess arrestin as well as excess 7P-peptide to yield enough activated arrestin to block the activation of transducin. It is also consistent with our thus far unsuccessful attempts to demonstrate direct binding of the 7P-peptide to arrestin (unpublished). Another reasonable assumption is that the forward and reverse rates for the equilibrium are very fast relative to the slow reaction of DTNB with the cysteine residues. Finally, if the cysteines are not directly involved in the activation of arrestin but are only indicators of the conformational state of arrestin, then it seems to be reasonable to assume that reacting the fast-reacting cysteine with DTNB would not affect the equilibrium. If only the fast reactive cysteine is considered, solving for the apparent rate of reaction of this cysteine yields

$$k_{\text{fast,observed}} = k_1 + k_2K[\text{7P-peptide}]$$

Therefore, in this model where the conversion of the slow-reacting cysteine to a faster-reacting cysteine is correlated with the activation of arrestin, a linear dependence of the rate constant on the 7P-peptide concentration over a large concentration range would be observed.

To ascertain whether the reactivity of the sulfhydryl groups is related to the activation of arrestin, the reactivity of the sulfhydryl groups of a constitutively active arrestin was examined. It has been reported that arrestin in which arginine 175 has been replaced by glutamine (R175Q) exhibits light-dependent binding to rhodopsin but, unlike native arrestin, does not require the rhodopsin to be phosphorylated (*5*, *6*). R175Q was produced in yeast and purified as described in Materials and Methods. In Figure 5A, R175Q and native arrestin were tested for their ability to bind to membranes containing rhodopsin, photoactivated rhodopsin, phosphorylated rhodopsin, and photoactivated phosphorylated rhodopsin. Native arrestin (Figure 5A, lanes 1−4) bound very little to unphosphorylated rhodopsin whether photoactivated or not (Figure 5A, lanes 1 and 2). Photoactivation enhanced native arrestin binding to phosphorylated membranes (Figure 5A, lane 4), as expected. The binding of R175Q (Figure 5A, lanes 5−8) to unphosphorylated rhodopsin was enhanced by photoactivation (Figure 5A, compare lanes 5 and 6). This shows that R175Q binds to unphosphorylated rhodopsin in a light-dependent fashion, indicating that the R175Q is "constitutively active" in agreement with the findings of Gurevich et al. (*5*, *6*). R175Q bound to both unbleached and photoactivated phosphorylated rhodopsin (Figure 5A, lanes

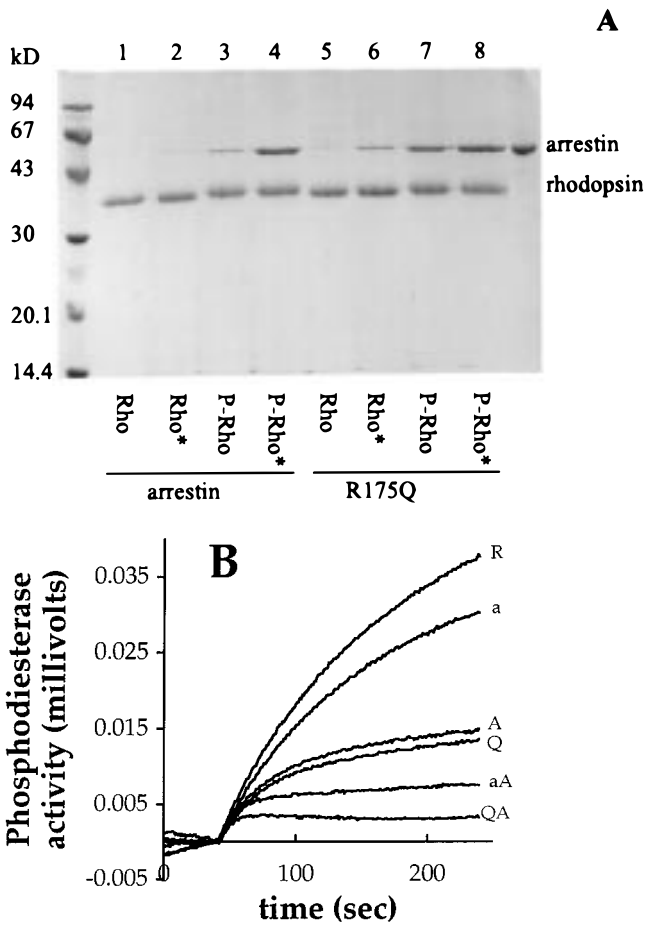

FIGURE 5: Arrestin mutant R175Q is constitutively active. (A) Light-induced binding of arrestin mutant R175Q to bleached, unphosphorylated ROS membranes. Lanes 1−4 show native arrestin binding as a control: lane 1, arrestin and unbleached, unphosphorylated ROS membranes; lane 2, arrestin and bleached, unphosphorylated ROS membranes; lane 3, arrestin and unbleached, phosphorylated ROS membranes; and lane 4, arrestin and bleached, phosphorylated ROS membranes. In lanes 5−8, the binding of arrestin mutant R175Q is shown: lane 5, R175Q and unbleached, unphosphorylated ROS membranes; lane 6, R175Q and bleached, unphosphorylated ROS membranes; lane 7, R175Q and unbleached, phosphorylated ROS membranes; and lane 8, R175Q and bleached, phosphorylated ROS membranes. (B) Light-induced phosphodiesterase activity is inhibited in the presence of R175Q without ATP. The reaction mixture contained 7.5 μM rhodopsin in ROS, 64 μM GTP, and various combinations of arrestin, R175Q, and ATP. At time zero, 4.5 mM cGMP was added, and at $t = 40$ s, the sample was illuminated for $^1/_8$ s. The traces are for ROS alone (R), ROS and 3.68 μM arrestin (a), ROS and 0.33 mM ATP (A), ROS, 0.33 mM ATP, and 3.68 μM arrestin (aA), ROS and 4.19 μM R175Q (Q), and ROS, 4.19 μM R175Q, and 0.33 mM ATP (QA).

Table 1: Activities of the Conformational States of Arrestin

activity	native	heparin-induced	7P-peptide-induced	constitutively active	P-Rho*-induced
sulfhydryl reactivity	three slow-reacting	three slow-reacting	one slow, one fast, and one blocked	three fast	ND[a]
PDE activity	little effect	little effect	inhibits	inhibits	inhibits
proteolysis	slow, continuous	initially fast, then protected	initially fast, then protected	ND[a]	initially fast, then protected
binds to R*	no	no	yes	yes	−

[a] ND, not determined.

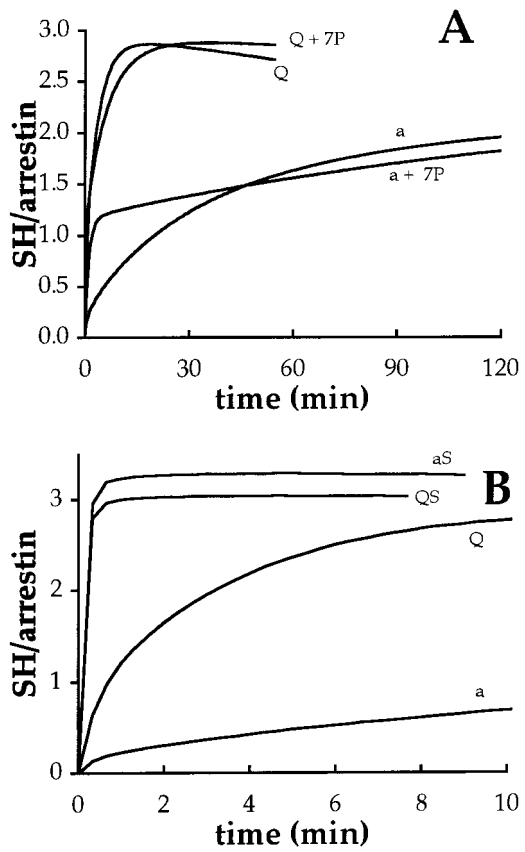

FIGURE 6: Reactivity of the sulfhydryl groups of arrestin mutant R175Q. (A) Time course of the reactivity of the SH groups of arrestin (a) and R175Q (Q) as well as arrestin in the presence of 76 μM 7P-peptide (a + 7P) and R175Q in the presence of 76 μM 7P-peptide (Q + 7P). (B) Time course of the reactivity of the SH groups of arrestin (a) and R175Q (Q) as well as that of arrestin (aS) and R175Q (QS) in the presence of 1% SDS.

7 and 8), although gel scanning indicated that about twice as much R175Q was bound to the bleached, phosphorylated rhodopsin compared to the unbleached.

GrayKeller et al. (*6*) have shown that a similar mutant, R175E, was effective in inhibiting the light-induced phosphodiesterase activity. To test whether the mutant arrestin R175Q is physiologically active, the R175Q was tested in the PDE assay (Figure 5B). Indeed, R175Q does inhibit PDE activity in the absence of ATP, similar to that of arrestin and ATP (Figure 5B). Under these conditions, the concentration of R175Q needed to achieve half of its maximal effect was 1.3 μM.

The reactivity of the sulfhydryl groups of constitutively active R175Q arrestin was then examined (Figure 6). All of the sulfhydryls of the constitutively active R175Q react with DTNB and at a much faster rate than those of native arrestin (Figure 6A), though not nearly as fast as in the presence of SDS (Figure 6B). Native bovine arrestin grown in yeast does not exhibit an enhanced rate of SH reaction with DTNB, indicating that the enhanced rate with R175Q is not an artifact of the production in yeast. With native arrestin, the 7P-peptide enhances the rate of the reaction of 1 mol of sulfhydryl (per mole of arrestin) and blocks the reaction of another (Figure 1), but with R175Q, the 7P-peptide appeared to slow but not block the reaction of DTNB with at least one of the sulfhydryls of R175Q (Figure 6A). A major difference in sulfhydryl reactivity between native arrestin and R175Q suggests a significant conformational difference between these two molecules even though they differ by only a single amino acid.

CONCLUSIONS

Bovine arrestin appears to be able to adopt a variety of conformational states that were detected by different techniques as summarized in Table 1. Native arrestin exhibits a susceptibility to proteolytic digestion by trypsin that is different than that of arrestin in the presence of several negatively charged compounds (heparin, phytic acid, and 7P-peptide) (*4*, *13*). These compounds must also promote somewhat different conformational states of arrestin since only the 7P-peptide induces arrestin to bind to light-activated unphosphorylated rhodopsin (*4*) and because they have different effects on the reactivity of the sulfhydryl groups of arrestin. The constitutively active arrestin mutant R175Q exhibits yet another conformational state that, like arrestin in the presence of 7P-peptide, binds to unphosphorylated R*, but in contrast to arrestin in the presence of 7P-peptide, all three of the sulfhydryl groups easily react with DTNB. Arrestin bound to P-Rho* is difficult to assess using these techniques, but it may exist in a different conformation than the constitutively active form. Gurevich (*14*) has recently proposed a model for the interaction of arrestin and rhodopsin. In this model, there are two tiers of constraining intramolecular interactions in arrestin that must be relieved for arrestin to attain its highest rhodopsin affinity form. The first constraint involves the trigger associated with arginine 175 which is disrupted by phosphorylated R* or presumably the 7P-peptide from rhodopsin's C-terminus, and the second involves the arrestin carboxyl-terminal regions of residues 369−378 and 379−404. This model therefore also assumes multiple conformations of arrestin. There are clearly different states of arrestin that are not associated with its activation, e.g., the state induced by heparin which does not bind rhodopsin, but is demonstrably different from the native state. There are also clearly different "activated" states of arrestin such as the R175Q mutant and those induced by the 7P-peptide. Even on crystallization, Granzin et al. (*15*) found two conformations in their crystals of native, presumably unactivated arrestin.

It is not clear what function is served by the apparent flexibility or multiple conformations of arrestin. Arrestin must be activated before it can bind to activated rhodopsin, preventing further activation of transducin. In vivo, this activation is performed by binding to the phosphorylated C-terminal region of rhodopsin. However, binding to activated, unphosphorylated rhodopsin can occur if arrestin is activated by the synthetic 7P-peptide, and this binding occurs even if the C-terminal region of rhodopsin is removed (*4*). One can speculate that arrestin's flexibility allows it to more easily bind to the other regions of rhodopsin after interacting with and being activated by the phosphorylated C-terminus.

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