Edited by
Paul A. Hargrave

Methods in Neurosciences
Introduction

The binding of arrestin (also referred to as the 48K protein or S-antigen) to freshly bleached phosphorylated rhodopsin occurs rapidly (<200 msec) and quenches the activation of G protein (transducin) (1-4). We have shown that arrestin also acts in the phototransduction process by blocking rapid dephosphorylation of phosphorylated and photolyzed rhodopsin until activated rhodopsin decays (5). The dissociation of arrestin from the photoreceptor protein occurs when photolyzed rhodopsin is completely deactivated by the removal of the photoisomerized chromophore, all-trans-retinal (6).

The amino acid sequence of arrestin was determined first for bovine arrestin (7), and the sequence has now been determined for an additional five species. Arrestin contains a relatively high proportion of nonpolar amino acids (55%) that are assembled in a β-sheet conformation (7, 8). However, very little is known about the biochemical/biophysical and structural properties of the protein.

Arrestin is also of interest because of its ability to cause an autoimmune disease, uveoretinitis, that resembles uveitis in humans (9). Immunization of experimental animals with arrestin results in inflammation of the uvea and retina followed by destruction of retinal photoreceptor cells (reviewed in Ref. 10).

Arrestin-like proteins have been found recently in nonphotosensitive cells such as myocardium, liver, kidney, lung, and cerebellum. These proteins may be involved in the transduction of chemical signals (11), analogous to the function of arrestin in the transduction of light signals in the visual system. For example, β-arrestin has been shown to interact with the β-adrenergic receptor (12, 13) in a manner that is analogous to the interaction of arrestin with rhodopsin.

A variety of methods have been developed for the purification of arrestin. Several are based on conventional chromatographic procedures (9, 14-16) that are time consuming and often produce a mixture of intact arrestin and its proteolytic fragments. The method of Wilden et al. (3) utilizes the binding of arrestin to illuminated phosphorylated rhodopsin. This procedure requires the use of dark-adapted retina, gives low yield, and produces arrestin contaminated by vesicles of rod outer segment (ROS) membranes. We have developed an economical, highly reproducible, and simple method that overcomes
the difficulties mentioned above (17). The method is based on the fact that arrestin binds the ligands heparin and phytic acid (inositol hexakisphosphate, InP$_6$). Thus, after arrestin is first bound to heparin–Sepharose, it is selectively eluted from the resin by phytic acid.

Methods

**Purification of Bovine Arrestin by Affinity Chromatography on Heparin–Sepharose**

All procedures are performed at 5°C. This procedure can be applied to bleached retinas.

**Extract Preparation**

Fifty frozen dark-adapted bovine retinas (obtained from Lawson, Inc., Lincoln, NE, and stored at −70°C) are thawed in a water bath at 20–25°C. The retinas are homogenized in a glass–glass homogenizer under a red safelight with 50 ml of ice cold 10 mM HEPES buffer, pH 7.5, containing 1 mM benzamidine and 20 μg/ml leupeptin (reagents can be purchased from Fisher, Pittsburgh, PA, or Sigma, St. Louis, MO). Soluble proteins are separated from retinal debris by centrifugation for 25 min at 43,700 g. The supernatant is collected and the pellet discarded.

**DEAE-cellulose Chromatography**

The opalescent and reddish extract (51 ml) is applied at a rate of 25 ml/hr to a 1.6 × 17 cm column of DEAE-cellulose (Whatman, Clifton, NJ, DE-52) that has been equilibrated with 10 mM HEPES buffer, pH 7.5. The column is washed with 10 mM HEPES buffer, pH 7.5, containing 15 mM NaCl, at a flow rate of 25 ml/hr until the absorbance at 280 nm drops below 0.1 and a major hemoglobin band is eluted from the column (or moves toward the end of the column; ~150 ml). The washing can be performed overnight, if convenient. Arrestin is eluted with a gradient of NaCl (240 ml total, from 0 to 150 mM NaCl containing 10 mM HEPES buffer, pH 7.5, and 0.5 mM benzamidine). The flow rate is 15 ml/hr, and 3-ml fractions are collected. Arrestin is eluted as a broad peak at about 75 mM NaCl as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Fig. 1).

**Heparin–Sepharose Affinity Chromatography**

Combined fractions containing arrestin from the DEAE-cellulose column (fractions 26–42, total 48 ml) are applied at a flow rate of 20 ml/hr to a 1 ×
Fig. 1  DEAE-cellulose chromatography of arrestin (see text for methods). (Top) Coomassie blue-stained gel of 4 µl of the indicated fractions. (Bottom) Immunoblot...
7.5 cm column of heparin-Sepharose that has been equilibrated with 10 mM HEPES buffer, pH 7.5, and 100 mM NaCl. The column is washed with 10 mM HEPES buffer, pH 7.5, containing 150 mM NaCl at a flow rate of 20 ml/hr until absorbance at 280 nm drops below 0.01 (~60 ml). Arrestin is eluted at a flow rate of 12 ml/hr by a linear gradient of InP₆ (phytic acid, Sigma, P-1916) in 10 mM HEPES buffer, pH 7.5, and 150 mM NaCl (70 ml total, from 0 to 8 mM InP₆ in the washing buffer), and 1.5-ml fractions are collected. Arrestin is eluted as a sharp peak at about 2.5 mM InP₆ as determined by the absorbance at 280 nm (InP₆ does not absorb at this wavelength), SDS-PAGE, and immunoblotting (Fig. 2). Arrestin at this stage is more than 95% pure, but the preparation contains InP₆, which is a competitive inhibitor of the interaction between arrestin and phosphorylated rhodopsin. Thus, careful removal of InP₆ is necessary. Dialysis will remove the bulk of InP₆, but complete removal can only be achieved by rechromatography on heparin-Sepharose with NaCl as the eluant.

Heparin-Sepharose Ion-Exchange Chromatography

To purify arrestin completely and to eliminate InP₆, the protein is rechromatographed on heparin-Sepharose. Fractions containing arrestin that were eluted from heparin-Sepharose by InP₆ (fractions 7-12, 9 ml), are dialyzed against 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl (dialyze against 1 liter buffer for 24 hr). Next, the dialyzate containing arrestin is applied at a flow rate of 13 ml/hr to a 1 x 4 cm column of heparin-Sepharose that has been equilibrated with 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl. The column is washed with HEPES buffer containing 100 mM NaCl (10 ml) at a flow rate of 13 ml/hr. Arrestin is eluted with 400 mM NaCl in the HEPES buffer as determined by absorption at 280 nm and SDS-PAGE (Fig. 3). From 50 bovine retinas, the yield is about 4 mg of homogeneous arrestin.

Assessment of Purity

The purity of arrestin should be assessed using SDS-PAGE (18). Additionally, an immunoblot analysis should be performed to evaluate the structural integrity of arrestin, since the N terminus of arrestin is susceptible to degradation (19). The extent of degradation of the N terminus of the protein can be tested of the fractions from DEAE-cellulose chromatography. The staining was performed employing monoclonal antipeptide antibody C10C10 (generous gift from Dr. L. Donoso). Molecular weight standards were from Pharmacia (Piscataway, NJ). Lane L was loaded with 4 µl of extract.
FIG. 2 Heparin-Sepharose/phytic acid chromatography of arrestin. (Top) The absorption profile was obtained from a heparin-Sepharose column (see text for details). (Bottom left) Coomassie blue-stained gel of 4 µl of the indicated fractions. (Bottom right) Immunoblot of the fractions from heparin-Sepharose. Staining was performed employing monoclonal antipeptide antibody C10C10. Lane L was loaded with 4 µl of the pooled fractions obtained from the DEAE-cellulose chromatography step.
Properties of Purified Arrestin

Stability

Arrestin prepared by this method is stable for several weeks at 0°C. It is important that the buffer used for arrestin storage includes 0.02% NaN₃. We have noticed that arrestin aggregates and precipitates when exposed to very...
Inhibitors of Arrestin Interaction with Photolyzed and Phosphorylated Rhodopsin

We have found that heparin and dextran sulfate compete with photoactivated and phosphorylated rhodopsin to bind arrestin (20). Phytic acid and other highly phosphorylated inositols inhibit this interaction (17). The dissociation constants for arrestin and inositol phosphates are in the low micromolar range. Several other polyanions are poor inhibitors. Neither ATP, GTP, nor Ca$^{2+}$ are ligands for arrestin, despite recent reports (discussed in Ref. 21).

Composition and Posttranslational Modification

It has been reported that bovine arrestin may contain lipids and carbohydrate moieties, covalently attached to the protein (for review, see Ref. 22). It was also reported that arrestin can be modified substoichiometrically by phosphorylation, presumably by protein kinase C (23). Mass spectroscopic analysis reveals that arrestin purified by this method does not have posttranslational modifications, other than acetylation (Fig. 4). It has been shown that all three of the cysteines in arrestin are available as free sulfhydryl groups (8, 24) rather than in disulfide linkage as previously suggested (7).

Molecular Weight

Based on the amino acid composition of arrestin obtained from the sequence of the protein, the molecular weight of arrestin is 45,275 (404 amino acids). The mass spectroscopic analysis of the native protein revealed that arrestin is acetylated on its N-terminal methionine, and that the molecular weight is 45,317.57 ± 4.41 (Fig. 4).

Extinction Coefficient and Ultraviolet and Fluorescence Spectroscopy

The UV absorption maximum for arrestin is at 278 nm. An accurate extinction coefficient was determined by amino acid analysis. These results gave an absorbance for a 0.1% solution at 278 nm of 0.638. On a per amide basis, the molar extinction coefficients are $\epsilon(278) = 71.5$, and $\epsilon(190) = 10,560$ (8). The integrity of arrestin can be conveniently monitored by employing...
Fig. 4 Mass spectrum of arrestin. The predicted mass for the protein obtained from its sequence (7) is 45,275 daltons. If 42 daltons is added for an acetyl group, the molecular mass is then 45,317 daltons. Twenty different peaks (m/z) corresponding to the same mass (m = 45,317 daltons) with charges varying from +56 to +37 were identified. This verifies the molecular size of arrestin and suggests that the protein, the N terminal of which is blocked, is acetylated on its N terminus. The analysis was done by Drs. K. Walsh and L. Ericsson at the University of Washington.

fluorescence spectroscopy as described by Kotake et al. (25) (Fig. 5), since the emission maximum of denatured arrestin is shifted from 306 nm for the intact protein to 340 nm.

Binding Assay for Arrestin to Photolyzed and Phosphorylated Rhodopsin

A convenient assay has been developed to monitor arrestin binding to photolyzed and phosphorylated rhodopsin (17). Under red light, phosphorylated rhodopsin, which has been regenerated with 11-cis-retinal (30 μM; 2 to 5
Fig. 5 UV and fluorescence spectra of arrestin. (Top) UV spectrum of arrestin (0.76 mg/ml) in 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl. (Bottom) Fluorescence spectrum (excitation at 280 nm) of arrestin (1 μM) in 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl at 25°C (dashed line), along with those of the same sample partially denatured by incubating for 10 min at 70°C (dotted line) and buffer alone (solid line).

phosphates per molecule of rhodopsin) in 10 mM HEPES buffer, pH 7.5, containing 100 mM NaCl and 1 mM MgCl₂, is mixed with purified arrestin (5 μM) in a total volume of 60 μl. The sample is illuminated under a 180-W lamp for 5 min from a distance of 10 cm at 30°C. Under these conditions 45–50% of rhodopsin is bleached. The sample is centrifuged in an Airfuge (Beckman, Fullerton, CA) at 180,000 g for 2 min. The supernatant is stored for SDS-PAGE and/or protein content analysis. The pellet is washed with 180 μl of the HEPES buffer, centrifuged under the same conditions, and dissolved in 55 μl of 1% SDS containing 0.1% 2-mercaptoethanol for SDS-PAGE analysis.
Acknowledgments

We thank Drs. K. Walsh and L. Ericsson (University of Washington) for mass spectroscopy analysis of arrestin, Dr. L. Donoso (Jefferson University) for a monoclonal antibody against arrestin (C10C10), and Preston Van Hooser for excellent technical assistance. This research was supported by U.S. Public Health Service Gant EY 09339 and by a grant from the Human Frontiers in Science Program. KP is the recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship.

References