Rapid affinity purification of retinal arrestin (48 kDa protein) via its light-dependent binding to phosphorylated rhodopsin

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Arrestin (also named '48 kDa protein' or 'S-antigen') is a soluble protein involved in controlling light-dependent cGMP phosphodiesterase activity in retinal rods, and is also known for its ability to induce autoimmune uveitis of the eye. We report a rapid and simple purification method based on the property of arrestin to bind specifically and reversibly to illuminated and phosphorylated rhodopsin [(1984) FEBS Lett. 176, 473–478]. This method does not require column chromatography and yields about 2–4 mg purified arrestin from 15 bovine retinas. Pure arrestin can be resolved by isoelectric focusing into at least 10 distinct bands, all of which stain with a monoclonal antibody specific for S-antigen.

Arrestin *48 kDa protein* *Retinal S-antigen* *Rhodopsin phosphorylation* *Purification* *Isoelectric heterogeneity*

1. INTRODUCTION

Arrestin (previously called '48 kDa protein') is one of the most abundant soluble proteins of the vertebrate photoreceptor cell [1] and is presently being investigated in many laboratories for different reasons. Since its discovery in 1978, as one among three proteins which bind to the ROS disk membrane in a light-dependent manner [2], it has recently attracted interest for two reasons. Firstly, it has been shown to play an important role in the light-induced enzyme cascade of vision: it binds to photoisomerized and phosphorylated rhodopsin [3] and thereby quenches rhodopsin's capacity to activate PDEase [4]. This function has led to its name 'arrestin' [5]. Secondly, arrestin has been shown to be identical with 'retinal S-antigen' [6], a retina protein known to cause experimental autoimmune uveitis when injected into animals. Some methods have been published, describing the purification of retinal S-antigen [7–10], all of which involve several time-consuming column chromatographic steps. We report a rapid and simple method which takes advantage of the ability of arrestin to associate reversibly with bleached and phosphorylated rhodopsin.

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Abbreviations: ROS, rod outer segment; PDEase, cGMP phosphodiesterase; IEF, isoelectric focusing; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; FPLC, fast protein liquid chromatography

2. MATERIALS AND METHODS

ROS were prepared from fresh bovine retinas as described [11].

2.1. *Retina extract*

Retina extract was prepared in dim red light as follows. 150 retinas that had been frozen in liquid N_2 were thawed in 200 ml of 70 mM sodium phosphate buffer (pH 7.2)/2 mM $MgCl_2$/0.1 mM EDTA. They were shaken vigorously by hand for

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1 min and then gently stirred for 30 min on ice. After centrifugation for 30 min at 20000 rpm in a Beckman JA 20 rotor, the supernatant was collected and centrifuged again. The final supernatant was stored at −80°C in aliquots of 20 ml until use.

2.2. *Arrestin purification*

An ROS pellet containing ~15 mg rhodopsin was homogenized in 15 ml of 70 mM sodium phosphate (pH 7.2)/2 mM $MgCl_2$/0.1 mM EDTA/3 mM ATP/1 mM GTP and illuminated at 30°C with white light (150 W). After 20 min, 20 ml retina extract, which had been separately warmed to 30°C, was added, and the mixture was illuminated for another 10 min. The suspension was centrifuged for 20 min at 20000 rpm. The supernatant was discarded and the pellet washed twice by resuspending it each time with 30 ml of 10 mM Hepes (pH 7.2) at 4°C and centrifuging for 40 min at 20000 rpm. In the first washing, 0.1 mM GTP was additionally present. The final pellet was suspended in 5 ml of 2 mM Hepes (pH 7.2)/500 mM KCl and incubated at 4°C overnight. The suspension was then centrifuged at 20000 rpm for 20 min. The supernatant containing the purified arrestin was centrifuged again to remove any residual membrane particles and was frozen in small aliquots and stored at −80°C until use. (If a salt-free preparation is desired, the final pellet can be extracted with 5 ml of 300 mM $(NH_4)HCO_3$ instead of 500 mM KCl. The buffer can then be removed by lyophilization without loss of activity of the purified arrestin.)

2.3. *Isoelectric focusing*

IEF was carried out on vertical slab gels (135 × 170 × 1 mm), containing 5% polyacrylamide, 4% ampholines (LKB) (pH 5–7), 1% ampholines (pH 3.5–10), and normally 0.5% octylglucoside. Electrode solutions were 20 mM NaOH and 50 mM H_3PO_4. Focusing power was usually 2500 V·h.

For immunoblotting the proteins were transferred to nitrocellulose sheets [12] and stained using the anti-S-antigen monoclonal antibody S6H8 (kind gift of J.P. Faure, see [13]) and the biotin-streptavidin system (Amersham), with 1-chloro-4-naphthol as a substrate.

2.4. *Phosphodiesterase activity*

Phosphodiesterase activity was monitored through the pH changes associated with cGMP hydrolysis [4,14]. Phosphorylated ROS membranes (P-disks) for PDEase assay were prepared as in [3].

3. RESULTS AND DISCUSSION

The affinity purification procedure described here makes use of the specific and reversible binding of arrestin to bleached and phosphorylated rhodopsin [3]. During the illumination period in the presence of ATP, rhodopsin becomes phosphorylated and therefore binds arrestin; other proteins are removed by centrifugation, and the purified arrestin is then eluted from the membranes simply by an overnight incubation in the dark in high ionic strength buffer.

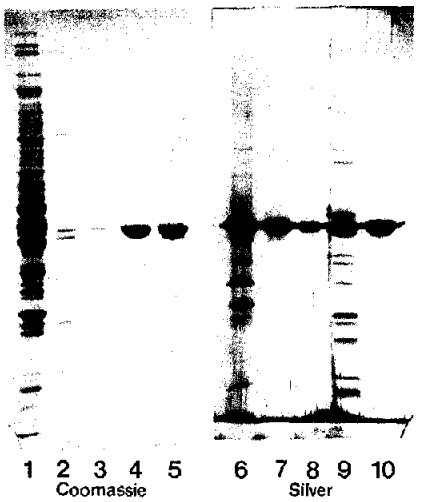

Fig.1. Polypeptide composition [15] of arrestin as prepared using our method (lanes 4,6,7,8) or that of Dorey et al. [8] (lanes 5,9,10). Lanes 1–5 were stained with Coomassie blue and lanes 6–10 by silver staining [16]. Lanes 4–7, 9 and 10 each contained 5 μg protein [17], lane 8 contained 2 μg. Lane 1, supernatant (5 μl) obtained after mixing retina extract with bleached ROS; lanes 2,3, 20 μl and 50 μl, respectively, of the two successive washing supernatants (see section 2); lanes 4,6, crude arrestin, prepared by our method; lane 8, after further purification by an additional wash (see text); lanes 5,9, crude arrestin, prepared according to Dorey et al. [8]. Aliquots of crude arrestin obtained either by our method or by that of Dorey et al. [8] were further purified by FPLC (Pharmacia Mono Q column, see [18]); the resulting preparations are shown on lanes 7 and 10, respectively.

Fig.1 shows that the resulting arrestin preparation is at least as pure as a typical preparation purified by a conventional column chromatographic method [8]. The two crude preparations contain a different set of impurities (cf. lanes 6 and 9, fig.1). The yield is normally 2–4 mg arrestin for 15 retinas. This preparation normally contains some rhodopsin kinase activity which can be removed by a subsequent FPLC chromatographic step. FPLC purification of our crude arrestin yields a preparation which is essentially pure by the criterion of silver staining on SDS-PAGE (5 μg protein, see lane 7, fig.1).

The advantage of our 'crude' purification procedure is its simplicity and rapidity as compared with conventional procedures involving successive column chromatographic steps [7–10]. Our procedure takes only about 3 h on 1 day and 1 h on the following morning, and requires no more than a few centrifugation steps. It yields a product at least as pure as the column chromatographic methods (see fig.1) and can easily be scaled up by a factor of 10 or more.

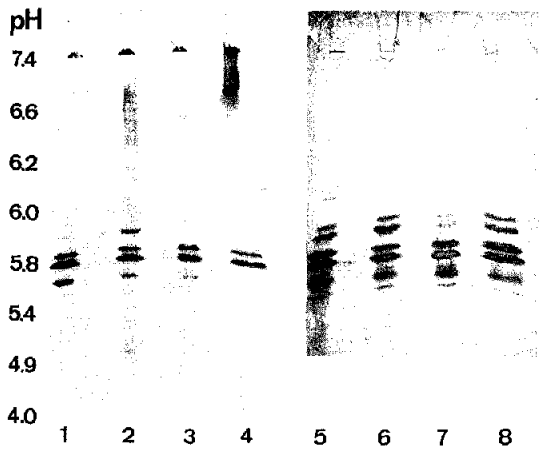

Fig.3. Isoelectric focusing pattern of different arrestin preparations (10 μg protein each). Lanes: 1–4, staining with Coomassie blue; 5–8, corresponding immunoblot. Lanes: 1,2, arrestin preparation according to Dorey et al. [8], in lane 1 further purified by FPLC; 3,4, preparation according to our method, in lane 3 further purified by FPLC. The 4 preparations shown here are the same as those shown in fig.1, lanes 10, 9, 7 and 6, respectively. More bands are seen on the immunoblot than on the Coomassie blue-stained gel because of the greater sensitivity of the antibody staining reaction.

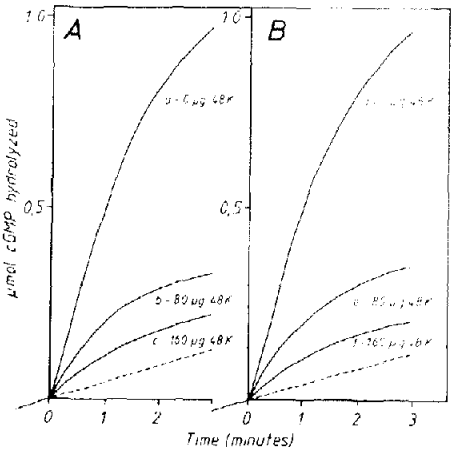

Fig.2. Inhibition of light-induced PDEase activity by arrestin purified by our method (A) or according to Dorey et al. [8] (B). PDEase activity was measured with purified transducin and PDEase reassociated to phosphorylated and regenerated rod outer segment membranes as in [4]. Each sample contained 5 nmol rhodopsin, 0.04 nmol PDEase and 0.6 nmol transducin in a final volume of 1.4 ml. Initial concentration of cGMP was 1.5 mM, and of GTP 0.1 mM in 10 mM Hepes (pH 7.9)/120 mM KCl (2 mM $MgCl_2$. The reaction was started by a flash bleaching 10^{-4} of the rhodopsin. Amounts of arrestin (μg) added to the various samples are indicated on the curves.

A nearly pure arrestin preparation can be achieved even in the absence of FPLC, if an additional centrifugation step is included (fig.1, lane 8). Washing the pellet gently and briefly (see section 2) with 3–5 ml of 2 mM Hepes/500 mM KCl, before its overnight incubation in the same buffer, removes most of the impurities present in the crude preparation (fig.1, lane 6) with relatively small losses (~30%) of arrestin.

Fig.2 compares the activity [4] of our arrestin preparation with that of a preparation by Dorey et al. [8]. Both preparations are equally active in suppressing light activation of PDEase in the presence of phosphorylated rhodopsin [4].

IEF resolves arrestin into a number of subspecies (fig.3) as mentioned earlier [9,17]. Even FPLC-purified arrestin, which is essentially homogeneous on SDS-PAGE, is resolved into 2–4 major bands and 5–10 minor bands with isoelectric points between pH 5.5 and 6.1. All of these subspecies are stained by an antibody specific [13]

for S-antigen (= arrestin; see fig.3, lanes 5–8). Qualitatively similar IEF patterns are observed with arrestin purified by our method or that of Dorey et al. [8], either crude or FPLC-purified (fig.3, lanes 5–8). Specificity of the antibody was verified by blotting an SDS gel containing all of the soluble proteins of the retina (similar to lane 1 in fig.1): only arrestin was recognized by the antibody. The IEF pattern was the same in the presence (fig.3) or absence (not shown) of octylglucoside, and was the same at 4 or 20°C. Partial preparative separation of the subspecies could be achieved by careful fractionation on the Mono Q column, as well as by differential elution from the disk membranes (not shown). This indicates that the observed multiplicity is not an IEF artefact. Addition of a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine, leupeptin, aprotinine, EGTA) during purification did not change the IEF pattern.

The observed multiplicity of IEF subspecies may be partially due to phosphorylation of arrestin (Weyand, I., unpublished), and perhaps to additional factors which are at present unknown. Such multiplicity raises the question of whether the different subspecies may play differential roles in regulating PDEase activity [4], and/or in causing the autoimmune disease [6–8].

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