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Similarities between G-proteins in visual cells of *Sepia* and cattle

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In contrast to antisera against native transducin a polyclonal antiserum raised against heat-denatured bovine transducin crossreacts with the G-protein from *Sepia* visual cells. This antiserum recognizes a 44 kDa (G_α) and a 36 kDa (G_β) protein band from *Sepia* photosensory membrane preparation. Furthermore we purified the antibody-binding G-protein from *Sepia* by binding it to light-activated rhodopsin of *Sepia* and GTP-induced extraction, similar to the purification of bovine transducin. This G-protein is probably involved in the phototransduction process. The purified *Sepia* G-protein did bind to vertebrate photosensoric membrane upon illumination, but was not eluted by GTP-containing buffer solution. After extensive bleaching, the G-protein became soluble.

Visual transduction; G-protein; Heat denaturation; Antibody; Crossreaction; (*Sepia officinalis*)

1. INTRODUCTION

A family of GTP-binding regulatory proteins (G-proteins) transduce signals from membrane receptors in the cells. Activated membrane receptors stimulate the α-subunit of G-proteins to bind GTP. In visual cells of invertebrates, photoactivated rhodopsin [1] catalyzes the exchange of GTP for bound GDP on a G-protein [2,3]. The active, GTP-bound form (G_α-GTP) activates a phospholipase C. This active PLC catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3) [4–7]. Either calcium [8], cGMP [9,10] or some agent which is still unknown is the terminal intracellular messenger acting to open the light-modulated ion channel.

In outer segments of Octopus photoreceptors a 41 kDa is ADP-ribosylated by pertussis toxin [11,12]. Furthermore, an antiserum raised against the β- and γ-subunit of bovine transducin crossreacts with a 36 kDa protein of Octopus retina [11,12].

Experiments in our and G. Schultz's (Berlin) laboratories showed that the outer segments of the visual cells of *Sepia* contain a 44 kDa and 36 kDa protein which strongly crossreact with two different antibodies raised against synthetic peptides (15 AA and 16 AA) of identical amino acid sequence of vertebrate T_α- and T_β-subunits and other GTP-binding proteins, whereas antisera raised against purified bovine transducin recognized neither the 36 kDa (G_β) nor the 44 kDa (G_α) protein of *Sepia* G-protein [13–15]. The experiments reported here were made in the hope of solving this controversy.

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Abbreviations: PLC, phospholipase C; GTP, guanosine trisphosphate; GDP, guanosine diphosphate; G-protein, GTP-binding protein; T_α, $T_{\beta\gamma}$, subunits of transducin (G-protein of vertebrate photoreceptor); G_α, G_β, subunits of G-protein; cGMP, cyclic guanosine monophosphate; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine *N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl) aminomethane

2. MATERIALS AND METHODS

2.1. *Preparation of photosensoric membrane from* Sepia

Dark-adapted eye cups were obtained according to the method described by Saibil and Michel-Villaz [16]. The outer

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segments were isolated according to the method described by Conen [13]. Deviating from this method we isolated the rhodopsin-containing membrane by using a different sucrose step gradient (1 ml, 20% w/w; 2 ml, 30% w/w; 2 ml, 40% w/w; and 3 ml, 50% w/w sucrose).

2.2. *Purification of G-protein*

The G-protein from *Sepia* was purified according to the method described by Kühn [17] adapted to *Sepia* preparation. It was purified specifically by binding it to light-activated rhodopsin and elution by GTP-containing buffer solution. The important modifications are as follows. Buffer solutions: (i) physiological buffer solution, 400 mM KCl, 25 mM $MgCl_2$, 50 mM NaCl, 5 mM EGTA, 1 mM DTT, 50 mM Hepes, 0.1 mM PMSF, pH 7.5; (ii) hypotonic buffer solution, 15 mM Tris-HCl, 0.4 mM EGTA, 1 mM PMSF, pH 7.4. Light conditions: illumination, 1.6×10^{17} photons $\times$ $cm^{-1} \times s^{-1}$ (Osram 4563). The steps after this illumination were carried out under normal laboratory illumination. GTP extraction and following incubation (30 min, 0°C) were done in complete darkness. All other steps were done under dim red light.

2.3. *Purification of transducin*

Transducin was kindly given us by U. Wilden. It had been purified from bovine retinas as described by Kühn [17].

2.4. *Heat denaturation of bovine transducin*

160 μg transducin were slowly thawed on ice. After addition of Freund's adjuvant the probe was shaken and boiled in a water bath for 5 min. Subsequently the probe was alternately shaken and sonicated (5 s) three times (sonifier B12 cell disruptor Bronson).

2.5. *Production of antibodies against heat-denatured transducin in rabbit (female New Zealand White)*

The rabbit was immunized interdermally with 160 μg heat-denatured transducin conjugate in Freund's complete adjuvant followed by booster injections (every 10 days, 4 times) of the same amount of antigen in incomplete adjuvant. For test bleeds only a small quantity of blood (1–3 ml) was taken from the ear-vein. After the blood had coagulated at room temperature the serum was separated from the cells by sedimentation. The antibody titer was determined according to the ELISA technique [18].

2.6. *Immunoblotting of proteins*

Immunoblotting was performed according to Towbin et al. [19].

For detection of bound antibodies we used immunogold-silver staining (firm Janssen, Belgium).

3. RESULTS AND DISCUSSION

3.1. *Purification of the G-protein from* Sepia

We purified the G-protein from *Sepia* by repeated light-induced binding to light-activated rhodopsin and GTP-induced dissociation. After illumination of the membrane preparation no subunits of the G-protein were washed off the membranes (fig.1). Addition of GTP to the membrane preparation in hypotonic buffer caused a release of the 44 kDa and the 36 kDa protein together with other proteins. We then mixed the eluted G-protein with the same pellet which had been previously freed of G-protein and illuminated it again: the rebound G-protein could again be extracted by another GTP addition. We repeated this cycle three times. After each cycle the G-protein subunits were less contaminated by other proteins (fig.2), which indicates that these proteins are regulated by guanine nucleotides.

3.2. *Crossreaction of the antiserum raised against heat-denatured transducin*

The crossreactivity of the immunoreactive proteins of *Sepia* to the subunits of vertebrate transducin was examined by use of an antiserum raised against heat-denatured purified transducin. The specificity of the antiserum was tested by the immunoblot technique (fig.3). Fig.3A shows the SDS-PAGE of *Sepia* photosensoric membranes and purified G-protein. Fig.3B shows an immunoblot made from the identical SDS gel using

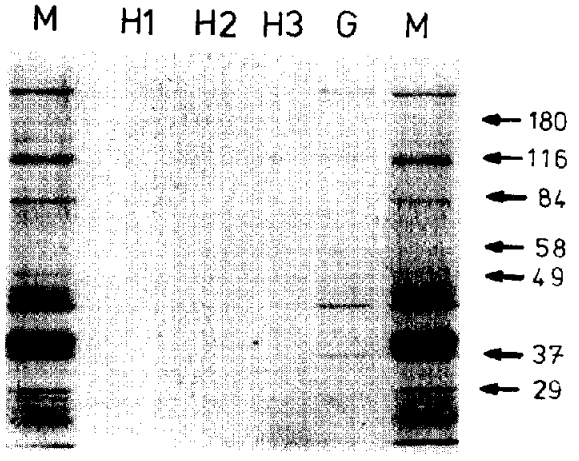


Fig.1. Silver stained SDS-disc-PAGE (10%) of GTP extracted proteins of photosensoric membrane preparation of *Sepia*. M, 5 μl photosensoric membrane preparation from *Sepia*. H1, H2, H3, 5 μl supernatant of hypotonic washes after illumination, before extraction by GTP. G, 5 μl supernatant of hypotonic wash with 250 μM GTP. Arrows indicate the molecular masses. Line G shows that a 44 kDa and a 36 kDa protein were solubilized from light-adapted membrane preparation by GTP-containing buffer.

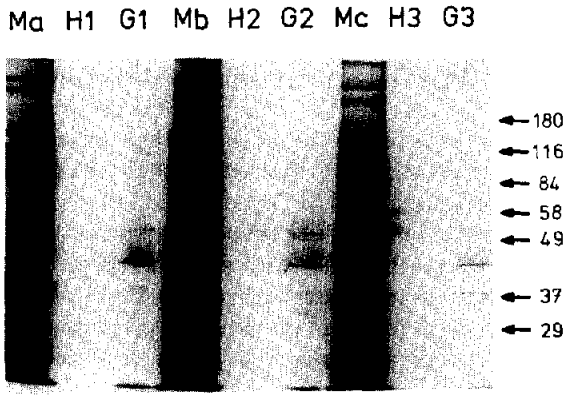


Fig.2. Repeated light-induced binding to photosensoric *Sepia* membrane and dissociation from membrane by GTP results in purification of G-protein from *Sepia*. Ma, Mb, Mc, 3 μl photosensoric membrane from *Sepia*. H1, H2, H3, 10 μl supernatant of hypotonic wash after illumination, before extraction with GTP. G1, G2, G3, 10 μl supernatant of hypotonic wash with 250 μM GTP. Arrows indicate the molecular masses. Gel shows the increasing purification of the G-protein.

the anti-heat-denatured transducin antiserum. This antiserum recognized the α- and β-subunits of purified transducin and the tested invertebrate G-protein subunits: G_α (44 kDa) and G_β (36 kDa). This crossreaction indicates (in contrast to the inefficiency of the antiserum against native transducin [13–15]) that certain immunogenic sites in bovine transducin could be made accessible by heat denaturation. These are sufficiently similar to sites in the G-protein subunits of *Sepia* photoreceptors.

3.3. *Crossreaction of the* Sepia *G-protein with mammal rhodopsin*

Fig.4A shows a test for binding of purified G-protein from *Sepia* to cattle rod outer segment suspension (ROS) in a light- and nucleotide-dependent manner. The G-protein bound to this vertebrate membrane upon illumination but was not solubilized by GTP-containing buffer solution. Extensive bleaching of ROS membrane before the binding of the invertebrate G-protein resulted in its removal with the first washing. Extensive bleaching after binding of the G-protein and the addition of GTP was equally followed by immediate release (see fig.4B,C).

This experiment shows, in addition to the described immunological crossreaction, an unusually strong 'physiological' crossreaction between *Sepia* G-protein and a vertebrate membrane preparation.

It is possible that the interaction of *Sepia* G-

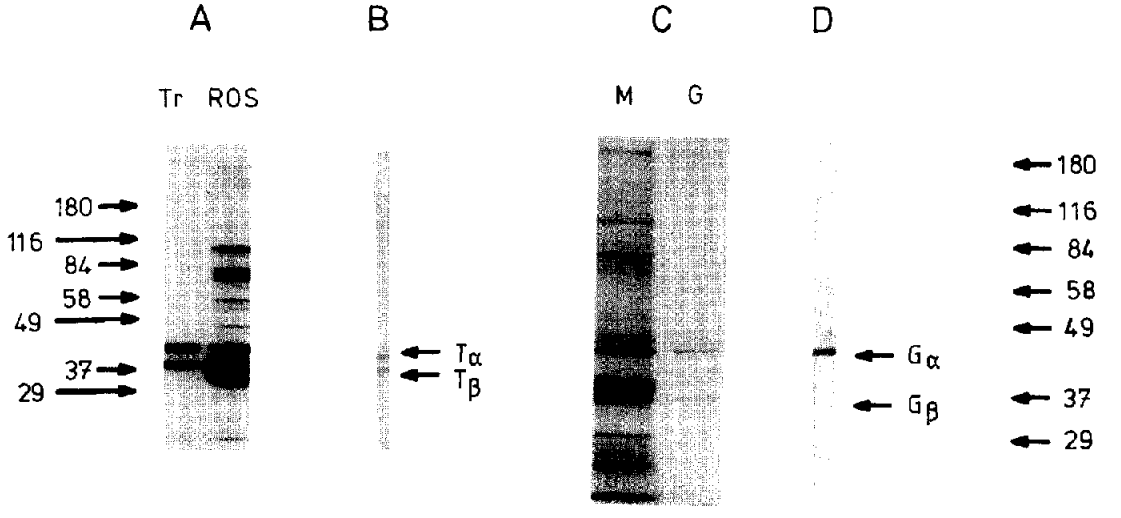
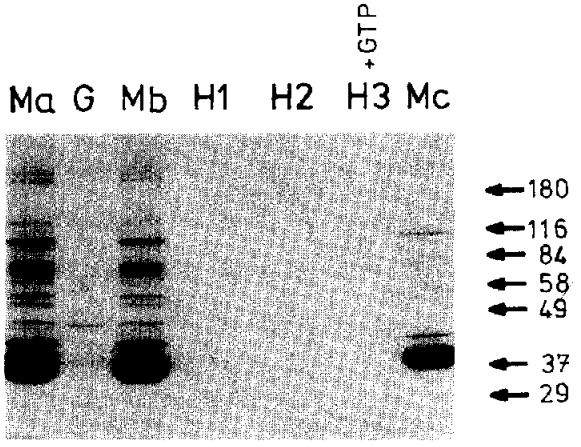
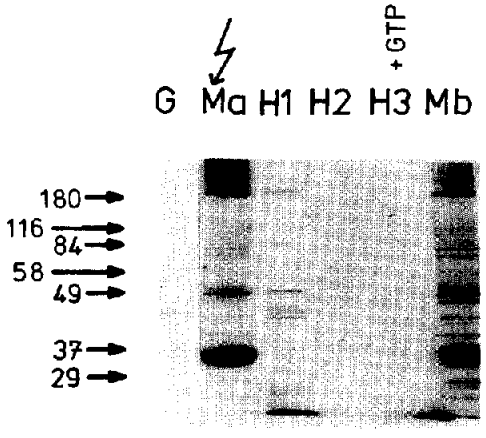


Fig.3. Crossreaction of purified *Sepia* G-protein with antisera (from rabbit) against heat-denatured transducin from cattle. (A) Tr, 3 μl purified transducin from cattle; ROS, 3 μl ROS membrane preparation from cattle. (B) Crossreactivity of protein A-purified antiserum raised against heat-denatured transducin with purified transducin from cattle. (C) M, 3 μl photosensoric membrane from *Sepia*; G, 10 μl supernatant of hypotonic wash with 250 μM GTP. (D) Crossreactivity of protein A-purified antiserum raised against heat-denatured transducin with purified G-protein from *Sepia*.





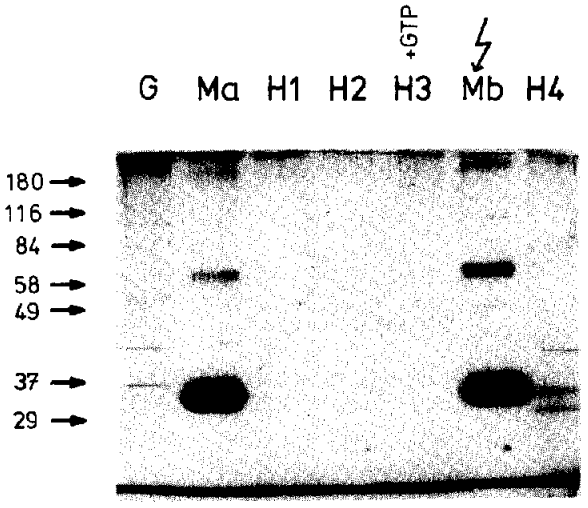


Fig.4. (A) Test to bind G-protein from *Sepia* to bovine photosensoric membrane in a light- and nucleotide-dependent manner (silver stained SDS-disc-PAGE, 10%). Ma, Mb, Mc, 3 μg photosensoric membrane (ROS) from cattle; G, 10 μl purified G-protein from *Sepia*; H1, H2, H3, 10 μl supernatant after hypotonic washes; + GTP, GTP addition to the last hypotonic wash. Arrows indicate the molecular masses. (B) Test to bind G-protein from *Sepia* to bovine photosensoric membrane as in A. After hypotonic wash with 250 μM GTP the pellet was bleached (ϟ) (silver stained SDS-disc-PAGE, 10%). Ma, Mb, 3 μg photosensoric membrane (ROS) from cattle; G, 10 μl purified G-protein from *Sepia*; H1, H2, H3, 10 μl supernatant after hypotonic washes; + GTP, GTP addition to the last hypotonic wash; H4, supernatant after bleaching. Arrows indicate the molecular masses. (C) Test to bind G-protein from *Sepia* to illuminated (ϟ) bovine photosensoric membrane in a light- and nucleotide-dependent manner (silver stained SDS-disc-PAGE, 10%). Ma, Mb, 3 μg photosensoric membrane (ROS) from cattle; G, 10 μl purified G-protein from *Sepia*; H1, H2, H3, 10 μl supernatant after hypotonic washes; + GTP, GTP addition to the last hypotonic wash. Arrows indicate the molecular masses.

Table 1

Sepia G-protein

	G_α 44 kDa	G_β 36 kDa
I. ADP-ribosylation		
CTX	+	
PTX	+	
II. Antisera		
Anti-α-common	+	
Anti-β-peptide		+
Anti-bovine transducin$_{native}$	–	–
Anti-bovine transducin$_{boiled}$	+	+
III. Functional crossreaction		
a. *Sepia* rhodopsin		
light-induced binding	+	+
GTP elution	+	+
b. Bovine rhodopsin		
light-induced binding	+	+
GTP elution	–	–
GTP and bleaching to opsin elution	+	(+)

protein and bovine rhodopsin is not adequate to allow a GDP-GTP exchange.

Table 1 summarizes our results with G-protein of *Sepia* photoreceptor outer segments.

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