Ca\(^{2+}\) Binding Capacity of Cytoplasmic Proteins from Rod Photoreceptors Is Mainly Due to Arrestin*

Berthold Huppertz, Ingo Weyand, and Paul J. Bauer†

From the Institut für Biologische Informationsverarbeitung, Forschungszentrum Jülich GmbH, 5170 Jülich, Federal Republic of Germany

---

After absorption of a photon by the visual pigment, the photoexcited rhodopsin activates transducin, a GTP-binding protein, which in turn activates a cGMP-phosphodiesterase (PDE) (1, 2). The light-induced activation of the PDE via transducin leads to a decrease in the intracellular cGMP level that entails the closure of cGMP-gated cation channels in the plasma membrane (3, 4). This closure shuts down the Na\(^+\) and Ca\(^{2+}\) influx which persists during darkness (5). As a consequence, the intracellular free Ca\(^{2+}\) concentration decreases due to the light-independent Na\(^+\)/Ca\(^{2+}\) exchange activity in the plasma membrane (4). It was recently proposed that intracellular free Ca\(^{2+}\) is involved in the sensitivity control of the photoreceptor (6–8).

Arrestin (also called S-antigen or 48-kDa protein) binds to photoexcited and phosphorylated rhodopsin and, thereby, blocks competitively the activation of transducin. Using Ca\(^{2+}\) titration in the presence of the indicator arsenazo III and \(^{45}\)Ca\(^{2+}\) autoradiography, we show that arrestin is a Ca\(^{2+}\)-binding protein. The Ca\(^{2+}\) binding capacity of arrestin-containing protein extracts from bovine rod outer segments is about twice as high as that of arrestin-depleted extracts. The difference in the Ca\(^{2+}\) binding of arrestin-containing and arrestin-depleted protein extracts was attributed to arrestin. Both, these differences in protein extracts and the measurements of purified arrestin yield dissociation constants for the Ca\(^{2+}\) binding of arrestin between 2 and 4 \(\mu\)M. The titration curves are consistent with a molar ratio of one Ca\(^{2+}\) binding site per arrestin. No Ca\(^{2+}\) binding in the micromolar range was found in extracts containing mainly transducin and cGMP-phosphodiesterase. Since arrestin is one of the most abundant proteins in rod photoreceptors occurring presumably up to millimolar concentrations in rod outer segments, we suggest that aside from its function to prevent the activation of transducin, arrestin acts probably as an intracellular Ca\(^{2+}\) buffer.

* This work was supported in part by Deutsche Forschungsgemeinschaft Grant Ku 337/3-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

† To whom correspondence should be addressed.

‡ The abbreviations used are: PDE, cGMP-phosphodiesterase; ROS, rod cell outer segments; FPLC, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

---

more, the presence of a significant Ca\(^{2+}\) buffering capacity in ROS has been inferred from electrophysiological data (5). We report here that at least one-half of the observed Ca\(^{2+}\) binding capacity with micromolar affinity of cytoplasmic ROS proteins is due to arrestin, an abundant protein in rod cells, which is involved in the deactivation of the cGMP-PDE enzyme cascade (11, 12).

EXPERIMENTAL PROCEDURES

Preparation of Rod Outer Segments—ROS were prepared in dim red light from fresh and dark-adapted bovine eyes as described (13). All solutions contained 10 mM Hepes/KOH, pH 7.4, 300 mM KCl, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 0.5 mM dithiothreitol, and normally 1 mM EDTA (buffer A).

ROS from illuminated eyes were prepared as follows: 90–100 bovine eyes were illuminated for 30 min with white light (3 Osram lamps, 150 W) in H\(_2\)O thermostated to 30 °C. The retinas were removed under room light and shaken for 90 s in buffer A containing 5% (w/v) sucrose (1.2 ml retina). The suspension was centrifuged for 10 min at 4,000 \(\times\) g and the supernatant was filtered through a nylon net (pore size, 100 \(\mu\)m). The filtered supernatant, containing mainly ROS, was diluted 1:2 with buffer A and centrifuged again for 10 min at 4,000 \(\times\) g. Each pellet was resuspended in 1 ml buffer A containing 18.5% (w/v) sucrose. Aliquots containing 3–6 mg of rhodopsin were pipetted onto Eppendorf tubes and centrifuged for 30 min at 16,000 \(\times\) g. The supernatants were discarded and the ROS pellets were stored frozen under argon at -80 °C.

Preparation of Soluble ROS Proteins—Hypotonic dark extracts from ROS of dark-kept eyes were prepared under dim red light by homogenizing ROS pellets with 5 mM Hepes/KOH, pH 7.2, 1 mM dithiothreitol (5 mg of rhodopsin/ml), followed by centrifugation at 50,000 \(\times\) g for 30 min. The supernatants were centrifuged again at 50,000 \(\times\) g for 45 min to remove any residual membranous material.

Isotonic dark-extracts from ROS of dark-kept eyes were prepared in the same way as hypotonic dark-extracts, except using 10 mM Hepes/KOH, pH 7.4, 100 mM KCl as suspending buffer. Arrestin-depleted extracts from ROS of dark-kept eyes were obtained from ROS which were illuminated for 30 min at 30 °C with white light (Osram lamp, 150 W) in the presence of 5 mM ATP and 1 mM MgCl\(_2\), leading to the binding of arrestin to photoexcited and phosphorylated rhodopsin. Isotonic extracts from these bleached ROS and extracts from control ROS, which were illuminated in the absence of ATP, were prepared like the isotonic dark-extracts.

Isotonic protein extracts from illuminated eyes were prepared by suspending the ROS pellets in 10 mM Hepes/KOH, pH 7.4, 100 mM KCl (0.6 mg of rhodopsin/ml) using gentle homogenization. The suspension was left in darkness for 30 min at 28 °C and 150 min at 4 °C to allow arrestin to dissociate from rhodopsin. Under dim red light this suspension was centrifuged twice like the other extracts. Arrestin-depleted extracts from illuminated eyes were prepared by illumination of isotonic extracts for 15 min at 30 °C with white light (Osram lamp, 150 W) in the presence of a 3-fold excess of phosphorylated and regenerated isorhodopsin (see below). After illumination the extracts were centrifuged twice at 50,000 \(\times\) g for 45 min.

Re-extracts from membranous pellets of isotonic dark extracts were prepared under dim red light. The pellets obtained after the first centrifugation of isotonic dark-extracts were resuspended and gently homogenized in 5 mM Hepes/KOH, pH 7.2 (2 ml/pellet), and left in
the dark for 30 min on ice. The re-extracts were centrifuged two times at 50,000 x g for 45 min. All extracts containing trace amounts of EDTA were washed by ultrafiltration with Centricon 30 microconcentrators (Amicon) at 5,000 x g. During filtration the sample volume is reduced thereby in a sonication bath (Branson Sonifier B-12, output 60 W).

**Purification of Arrestin**—Arrestin from bovine retina was purified either by column chromatography (14) or via its light-dependent binding to photoexcited and phosphorylated rhodopsin (15), resulting in crude preparations of ~80-95% purity. Crude arrestin was further purified to homogeneity by FPLC-anion exchange chromatography or FPLC-chromatofocusing (16). Purity of arrestin preparations was checked by densitometry of Coomassie Blue-stained SDS gels and by silver staining as reported previously (15). The purified arrestin was washed and centrifuged with H2O by ultrafiltration (Centricon 30 microconcentrators, Amicon), frozen in liquid nitrogen, and stored at -80°C. Before use arrestin was mildly sonicated three times for 5 s in a sonication bath (Branson Sonifier B-12, output 60 W).

**Radiative Labeling of Proteins with "Ca"**—CaCl2 in H2O (0.4–1.3 Ci/mmol Ca2+) was purchased from Amersham Corp. and stored at -20°C. The protein samples were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (16) and then electrophorezed on a nitrocellulose membrane (pore size, 0.2 µm; NitroScreen West, Du Pont-New England Nuclear) (17). The transfer was performed at a constant current of 70 mA at 4°C for 90 min with 15 mM Tris, 120 mM glycine as the transfer buffer. After transfer, the membrane was equilibrated for 30–60 min in 60 mM KCl, 0.1 mM MgCl2, 10 mM imidazole/HCl, pH 6.8, with three times repeated exchange of the buffer. Then the membrane was incubated for 30 min at 37°C and agitated for another 60 min at room temperature in the same buffer containing "CaCl2 (10–15 µM Ca2+)", specific activity 900–1200 cpm/pmol). The membrane was washed three times with H2O for 3 min each, dried at room temperature, and exposed to Hyperfilm-MP X-ray film (Amersham Corp.) at -80°C using Kodak X-Omatic intensifying screens.

**Phosphorylation and Regeneration of Rhodopsin**—Membranes with phosphorylated and regenerated rhodopsin were prepared by incubating ROS from dark-kept eyes in 100 mM sodium phosphate, pH 7.0, 3 mM ATP, 1 mM MgCl2, and 0.1 mM EDTA (0.5 mg of rhodopsin/ml) for 3 h at 30°C under continuous illumination through an orange Plexiglas filter (λ > 540 nm) (18). The resulting phosphorylated opsins was regenerated to phosphorylated isorhodopsin (90–105% regeneration) by incubating with a 3-fold molar excess of 9-cis-retinal in the dark for 15 h. The membranes were washed three times with water and then once with 10 mM Hepes/KOH, pH 7.4, 100 mM KCl by centrifugation (45 min at 50,000 x g).

**Titration Measurements with Arsenazo III**—The Ca2+ concentration of different protein solutions was monitored at 652 nm with a Cary 17 spectrophotometer (Varian) using the Ca2+-sensitive dye arsenazo III (60 µM). Arsenazo III was chromatographically purified using DEAE-cellulose as described (19). The samples in the cuvette (1 ml) always contained 10 mM Hepes/KOH, pH 7.4, 100 mM KCl, and were stirred and thermostated at 20°C. Sample and reference were initially identical except for the presence of 2 mM EDTA in the reference cuvette; the cuvettes were carried out in the absence and presence of Ca2+-binding proteins.

The titration procedure was carried out as follows: First, the absorption of arsenazo III was measured without added Ca2+. Then, absorption changes of arsenazo III were measured successively (six times) in response to 10-µl additions of 1 mM CaCl2 containing 0.01 µCi of Ca2+ in the sample buffer (arsenazo III was present to avoid dilution effects of the indicator). Finally, arsenazo III was saturated with Ca2+ by adding 1 mM CaCl2, followed by addition of 2 mM EDTA (Ca2+ desaturation of arsenazo III).

The Ca2+ concentration was determined spectrophotometrically from the maximal absorption change to be measured at 652 nm between Ca2+-saturated and Ca2+-free arsenazo III using the differential extinction coefficient of 28,500 M-1 cm-1 at pH 7.4 (19).

To evaluate these titrations, we use the relation

\[
[Ca^{2+}]_{tot} - ([AI][Ca^{2+}] - [P-Ca^{2+}]) = \frac{K}{([AI][Ca^{2+}] + [AI][Ca^{2+}]_{tot})}
\]

where [P-Ca2+] denotes the concentration of protein-bound Ca2+. In order to obtain a given free Ca2+ concentration, which is determined by Equation 1, one has to add more Ca2+ in the presence than in the absence of Ca2+-binding proteins, viz. according to Equations 1 and 2:

\[
[Ca^{2+}]_{tot} - ([AI][Ca^{2+}] - [P-Ca^{2+}]) = \frac{K}{([AI][Ca^{2+}] + [AI][Ca^{2+}]_{tot})}
\]

Hence, when plotting [Ca2+]tot vs. [AI][Ca2+] vs. [AI][Ca2+]/[AI][Ca2+]tot the ordinate values between protein-containing and protein-free solutions yields for a fixed absorbance value the concentration of protein-bound Ca2+. This evaluation does not require the knowledge of the dissociation constant, K, of arsenazo III, but it is only feasible as long as the ratio of Ca2+ bound to Ca2+ free arsenazo III can be reliably determined, i.e. in the pH range of the indicator arsenazo III, 0.5–5.0 µM.

It is obvious from Equations 1 and 2 that the titration curves [Ca2+]tot vs. ([AI][Ca2+] - [P-Ca2+]) versus ([AI][Ca2+]/[AI][Ca2+]tot must go through the origin. Practically, there is unavoidably a small Ca2+ concentration of a few micromolar in each solution which contributes to the total Ca2+ concentration:

\[
[Ca^{2+}]_{tot} = ([AI][Ca^{2+}] + [AI][Ca^{2+}]_{tot})
\]

We use the titrated Ca2+ concentration, [Ca2+]tot, in the plot above instead of the total Ca2+ concentration (which is a priori unknown). The curve titration intercepts the ordinate at some negative value between -1 and -3 µM, which corresponds to the concentration of "contaminating" Ca2+. Since the ordinate is linear in the Ca2+ concentration the correct titration curve is obtained by adding the concentration of contaminating Ca2+ to all ordinate values, i.e. by shifting the titration curve in ordinate direction. After this correction, the titration curves originate at zero and the difference between protein-containing and protein-free solutions measures the protein-bound Ca2+ (see Equation 3). It should be stressed that this correction procedure allows to carry out Ca2+ titrations in the absence of a Ca2+ buffer.

**Miscellaneous**—The content of individual proteins in extracts was determined by densitometry of Coomassie Blue-stained SDS gels, the content of protein extracts and of purified proteins (calmodulin, arrestin) by Bradford's assay (20) using in each case bovine serum albumin (Bio-Rad) as a standard. Rhodopsin concentration was determined from its light-sensitive absorbance at 500 nm using an extinction coefficient of 41,000 M-1 cm-1. Calmodulin was purchased from Calbiochem and 9-cis-retinal from Sigma.

**RESULTS**

**Ca2+ Titration Experiments**—We measured Ca2+ binding of soluble proteins spectroscopically by Ca2+ titration using the indicator arsenazo III. The concentration difference of the total Ca2+ (which is the added plus the contaminating Ca2+; see "Experimental Procedures") minus the Ca2+-bound arsenazo III is plotted versus the ratio of Ca2+ bound to Ca2+-free arsenazo III (Fig. 1). Since arsenazo III binds Ca2+ at micromolar Ca2+ concentrations with a stoichiometry of 1:1 (19), the ordinate is simply equal to the free Ca2+ concentration if there are no other Ca2+-binding compounds present than arsenazo III (control, open circles). In the presence of additional Ca2+-binding compounds, the ordinate values are higher due to Ca2+ binding of these compounds, and the deviation of the ordinate values from the control values measures directly the concentration of Ca2+-bound compounds other than arsenazo III (see "Experimental Procedures").
Ca⁺ Binding of Cytoplasmic Proteins from Rod Photoreceptors

The reliability of this titration method was verified using the Ca⁺-binding protein calmodulin which binds Ca⁺ in the micromolar range (Fig. 1, inset). The values for the stoichiometry, 1.407 ± 0.27 (S.D., n = 4), and for the dissociation constant, $K = 2.42 ± 0.19$ µM (S.D., n = 4), obtained with calmodulin concentrations between 1 and 2.5 µM, are in good agreement with previously reported values obtained by other methods (stoichiometry, 1.4 and $K = 2.4$ µM) (21).

**Hypotonic and Isotonic Dark-extracts—**All extracts obtained from ROS, which were purified in the presence of 1 mM EDTA, were washed extensively by ultrafiltration which reduces their initial concentration of EDTA (about 10 µM) by a factor of $>10^3$. Extracts obtained from ROS prepared in the absence of calmodulin; the curve in the presence of 1.5 µM calmodulin (solid circles) is shifted along the ordinate to greater values. This shift is due to the decrease of Ca⁺-bound calmodulin when the sample contains other Ca⁺-binding compounds. The samples contained 10 mM Hepes/KOH, pH 7.4, 100 mM KCl, 60 µM arszenazo III ± 1.5 µM calmodulin. The titration curve in the absence of proteins (control) deviates slightly from linearity; this curvature is presumably due to the presence of differently protonated forms of arszenazo III with different Ca⁺ affinities at pH 7.4 ($pK$ for protonation is 7.0) (10). This observation does not complicate the evaluation of the experiments since the $pK'$ of arszenazo III is not used (see “Experimental Procedures”). Inset, the difference between these two traces (along the ordinate) which is due to Ca⁺ binding of 1.5 µM calmodulin is plotted versus the free Ca⁺ concentration, $[\text{Ca}^+]_{\text{free}}$. The fitted curve corresponds to a mass-action-law process with a dissociation constant of 2.4 µM and an asymptotic value of 6 µM, implying a stoichiometry of the Ca⁺ binding of calmodulin of 1:4 ([CaM-Ca⁺]/CaM).

**Arrestin-containing and Arrestin-depleted Extracts—**In contrast to transducin and PDE we observed that the absence of arrestin reduces markedly the concentration of Ca⁺ binding sites. Illumination of ROS in the presence of ATP and Mg²⁺ leads to the binding of arrestin to photoexcited phosphorylated rhodopsin (11); therefore, isotonic protein extracts from ROS illuminated in the presence of ATP contain only trace amounts of arrestin (arrestin-depleted light-extracts; Fig. 3, inset B). Illumination of ROS in the absence of ATP does not result in phosphorylation of rhodopsin and

![Fig. 2. Hypotonic re-extracts from isotonic membraneous pellets](http://www.jpcl.org/)
Fig. 3. Ca\(^{2+}\) binding curves of isotonic extracts from ROS of illuminated eyes. The arrestin-containing extract (A, open circles) has a greater Ca\(^{2+}\) binding capacity than the arrestin-depleted extract (B, solid circles). The difference in the concentration of arrestin between these two extracts is 1.5 \(\mu\)M; the difference in the Ca\(^{2+}\) binding of these extracts is 1.6 \(\mu\)M. The binding curves are plotted like those in Fig. 2. Inset, densitograms of the isotonic extracts, whose Ca\(^{2+}\) binding curves are shown. The ordinate of these densitograms (not shown) represents the relative absorbance at 580 nm of Coomassie Blue-stained SDS gels. The arrestin-depleted extract (A, 0.79 mg of protein) shows a markedly lower amount of arrestin (arrows) than the arrestin-containing extract (A, 0.87 mg of protein). The lower Ca\(^{2+}\) binding capacity of arrestin-depleted extracts is explainable with the lower amount of arrestin in these extracts. MW, molecular weight.

thus arrestin does not bind (arrestin-containing light-extracts; Fig. 3, inset A).

The arrestin contents of arrestin-containing light-extracts and isotonic dark-extracts are similar. The same Ca\(^{2+}\) binding capacity was observed for arrestin-containing light-extracts and isotonic dark-extracts. However, the Ca\(^{2+}\) binding capacity of arrestin-depleted light-extracts is definitively lower, namely 2.81 ± 0.57 nmol of Ca\(^{2+}\)/mg protein (S.D., n = 3), although the amount of proteins in arrestin-depleted light-extracts is slightly reduced due to the lack of arrestin in these extracts (arrestin makes up about 10% of the protein mass in arrestin-containing light-extracts). Since the Ca\(^{2+}\) binding capacity of arrestin-depleted light-extracts is only one-half as high as that of arrestin-containing light-extracts, this finding suggests that about one-half of the Ca\(^{2+}\) binding capacity of arrestin-containing light-extracts is due to arrestin.

To enlarge the concentration of arrestin in ROS, we prepared ROS from illuminated eyes since arrestin is shifted in a light-dependent manner from the inner to the outer segment of rods (22). The isotonic extracts from these ROS contained about three times as much arrestin as isotonic extracts from ROS of dark-kept eyes. Arrestin-depleted extracts were prepared by removal of arrestin from arrestin-containing extracts via its light-dependent binding to photoexcited and phosphorylated isorhodopsin (see "Experimental Procedures"). The difference in the Ca\(^{2+}\) binding between these arrestin-containing and arrestin-depleted extracts (Fig. 3) was about three times higher than that of extracts from dark-kept eyes, i.e. similar to the increase in arrestin content in these preparations. Fig. 3 shows Ca\(^{2+}\) binding curves of arrestin-containing and arrestin-depleted extracts. The difference curve which is due to the Ca\(^{2+}\) binding of arrestin (Fig. 4, open circles) yields a dissociation constant of 3.45 ± 0.82 \(\mu\)M (S.D., n = 6) and a stoichiometry for Ca\(^{2+}\) binding to arrestin of 1:1.07 ± 0.11 (S.D., n = 6). These results strongly suggest that arrestin contains one Ca\(^{2+}\) binding site with micromolar affinity.

The Ca\(^{2+}\) binding to arrestin was further detected by autoradiography (Fig. 5) of ROS proteins revealing binding of \(4^{4}\)Ca\(^{2+}\) to arrestin and presumably to the separated \(\alpha\)-subunit of transducin and to rhodopsin. This presumption was supported by autoradiography of protein extracts containing primarily transducin and PDE and of hypotonically washed ROS membranes (data not shown). The more intensive labeling of arrestin in ROS obtained from illuminated eyes (Fig. 5A, lane 5) as compared to ROS from dark-kept eyes (lane 4) corresponds to the higher amount of arrestin in these preparations, suggesting that the Ca\(^{2+}\) binding is not due to a comigrating protein. This inference is corroborated by the Ca\(^{2+}\) binding of crude (lane 2) and FPLC-purified (lane 3) arrestin.

Purified Arrestin—Ca\(^{2+}\) binding of arrestin was established for both crude (purity 80–95%) and FPLC-purified (purity >98%) arrestin. This finding was obtained for arrestin isolated from many retinae and from single retinae of individual animals. Fig. 4 (solid circles) shows that one Ca\(^{2+}\) is bound per arrestin, independent of whether crude (1:1.03 ± 0.16, S.D., n = 10) or FPLC-purified arrestin was measured (1:1.03 resp. 1:1.01, n = 2). The dissociation constant calculated from the Ca\(^{2+}\) binding curves is 3.21 ± 0.81 \(\mu\)M (S.D., n = 10; crude arrestin) and 1.78 resp. 2.23 \(\mu\)M (n = 2; FPLC-purified arrestin).

Since arrestin can be fractionated in different isoelectric subtypes, we investigated the Ca\(^{2+}\) binding capacity of the main subtypes of arrestin (pI ~ 5.8) from pooled retinae. We obtained one Ca\(^{2+}\) binding site per arrestin, implying that the various arrestin subtypes probably do not differ in their molar ratio of Ca\(^{2+}\) binding site per molecule.

DISCUSSION

The Ca\(^{2+}\) titration technique used throughout this study can be performed in about 30 min, detects micromolar concentrations of Ca\(^{2+}\) binding sites, and yields quantitative values for the Ca\(^{2+}\) binding capacity in the micromolar Ca\(^{2+}\).
concentration range. Since arszenazo III binds Ca\(^{2+}\) in the micromolar range, it is more suited to measure Ca\(^{2+}\) affinities in this range than other indicators like Quin 2 (24) or tetramethylmurexide (25) which do not have micromolar dissociation constants. Beeler et al. (26) reported that binding of arszenazo III to muscle proteins may occur which affects the Ca\(^{2+}\) affinity of the indicator. In our experiments, binding of arszenazo III to one or more protein components is unlikely for the following reasons: (a) we found the correct Ca\(^{2+}\) affinity and binding stoichiometry for calmodulin; (b) no systematic deviations were observed between the titration curves of protein-free (control) and protein-containing solutions of PDE (up to 1.9 \(\mu\)M) and transducin (up to 13.4 \(\mu\)M); (c) binding of arszenazo III to proteins could not be detected with equilibrium dialysis of arszenazo III in the presence of the investigated proteins (ROS extracts, calmodulin). Therefore, this titration technique measures correctly the Ca\(^{2+}\) binding capacity of ROS extracts and purified arszenazo.

Although Ca\(^{2+}\) plays an important role in photoreceptors (6-8), presumably through sensitivity control, only little is known about Ca\(^{2+}\)-binding proteins in ROS. Calmodulin has known about Ca\(^{2+}\)-binding proteins in ROS. Calmodulin has been identified in very low amounts in bovine and frog ROS but its role in visual excitation processes is questionable (27, 28). Recently, the guanylate cyclase activity of bovine ROS has been reported to be controlled by nanomolar Ca\(^{2+}\) concentrations presumably by a Ca\(^{2+}\)-dependent regulatory protein (10). We report here that the concentration of Ca\(^{2+}\) binding sites with micromolar affinity is in the range from 2 to 6 nmol of Ca\(^{2+}\)/mg protein in protein extracts from ROS of dark-kept eyes and that at least half of these Ca\(^{2+}\) binding sites can be attributed to arszenazo. Transducin and PDE, the major proteins in hypotonic extracts from ROS displayed no micromolar Ca\(^{2+}\) affinity. This does not exclude the possibility, however, that these proteins may adopt conformational states which allow Ca\(^{2+}\) binding as it is indicated for the separated \(\alpha\)-subunit of transducin by autoradiography.

Remarkably, analysis of the amino acid sequence of arszenazo (29) displays no typical Ca\(^{2+}\) binding domain of the EF-hand type which comprises normally 12 amino acids (30). This does not exclude Ca\(^{2+}\) binding sites of other types, though. In fact, comparison of the amino acid sequences of Ca\(^{2+}\) binding sites of calmodulin (Asp\(^{120}\)-Glu\(^{126}\)), parvalbumin (Asp\(^{120}\)-Glu\(^{126}\)), and troponin C (Asp\(^{127}\)-Glu\(^{126}\), Asp\(^{33}\)-Glu\(^{33}\)) with the arszenazo amino acid sequence yields a conspicuous conservation or conserved substitution of four from five negatively charged amino acids in the amino acid stretch 362-373 of arszenazo (Asp-X-Asp-X-X-Glu-X-X-X-Asp-Glu). Although the glycine residue which is conserved in many Ca\(^{2+}\) binding sites does not occur in this sequence we suggest tentatively that this amino acid stretch is involved in Ca\(^{2+}\) binding since negatively charged amino acids are essential to form Ca\(^{2+}\) binding sites (30).

One may suspect that Ca\(^{2+}\) binding to arszenazo influences its binding to photoexcited and phosphorylated rhodopsin; however, we did not observe an influence of micromolar Ca\(^{2+}\) concentrations on the binding of arszenazo to photoexcited and highly phosphorylated rhodopsin.\(^3\)

Several lines of evidence indicate that Ca\(^{2+}\) buffering of arszenazo might be important in photoreceptors: (a) arszenazo is one of the major proteins in ROS, and its molar ratio to rhodopsin in whole photoreceptor cells was reported to be close to 1:1 (22); (b) the molar ratio of arszenazo to rhodopsin in ROS is about 1:10 (81); (c) arszenazo binds one Ca\(^{2+}\) with an affinity of about 3 \(\mu\)M, and at least one-half of the Ca\(^{2+}\) binding capacity with micromolar affinity of isotonic dark-extracts is due to arszenazo (this work). Given the above molar ratio of arszenazo to rhodopsin in ROS one estimates that the arszenazo concentration may well be in the millimolar range, in particular since the cytoplasmic volume of ROS is largely restricted by the disk stack. This means that Ca\(^{2+}\) buffering of arszenazo may become substantial. High local concentrations of arszenazo may thus readily explain the electrophysiological observation (5) that about 95% of the Ca\(^{2+}\) entering a rod cell is bound and is not accessible to injected aequorin. Of course, other Ca\(^{2+}\) binding sites, as those reported here, may add to the Ca\(^{2+}\) buffering capacity as well. Intracellular Mg\(^{2+}\) may also influence the effective Ca\(^{2+}\) affinity of arszenazo; however, autoradiography indicates that arszenazo binds Ca\(^{2+}\) even in the presence of a 7-10-fold excess of Mg\(^{2+}\) over Ca\(^{2+}\). The Ca\(^{2+}\) buffering capacity of ROS is further increased upon illumination since arszenazo is shifted from the inner to the outer segment of rods after light-activation of the photoreceptor (e.g. Ref. 22). Aside from its function to prevent transducin activation by competitive binding to light-activated rhodopsin we suggest that a second physiological function of arszenazo is probably to act as an internal Ca\(^{2+}\) buffer in photoreceptors.

Acknowledgment—We thank Dr. K.-W. Koch for critical reading of the manuscript.

\(^3\) B. Hupperts, unpublished experiments.


REFERENCES

1. Kühn, H. (1984) Prog. Retinal Res. 3, 123-156
2. Stryer, L. (1986) Annu. Rev. Neurosci. 9, 87-119
3. Kaupp, U. B., Hanke, W., Simmoteit, R., and Lühring, H. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 407-415
4. Yau, K.-W., and Baylor, D. A. (1989) Annu. Rev. Neurosci. 12, 289-327
5. McNaughton, P. A., Cervetto, L., and Nunn, B. J. (1986) Nature 322, 961-963
6. Torre, V., Matthews, H. R., and Lamb, T. D. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7109-7113
7. Matthews, H. R., Murphy, R. L. W., Fain, G. L., and Lamb, T. D. (1988) Nature 334, 67-69
8. Nakatani, K., and Yau, K.-W. (1988) Nature 334, 69-71
9. Fleischman, D., and Denisevich, M. (1979) Biochemistry 18, 5060-5066
10. Koch, K.-W., and Stryer, L. (1988) Nature 334, 64-66
11. Kühn, H., Hall, S. W., and Wilden, U. (1984) FEBS Lett. 176, 473-478
12. Wilden, U., Hall, S. W., and Kühn, H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1174-1178
13. Bauer, P. J. (1988) J. Physiol. (Lond.) 401, 309-327
14. Torre, V., Cervetto, L., and Faure, J. P. (1982) Ophthalmic Res. 14, 249-265
15. Wilden, U., Wüst, E., Weyand, I., and Kühn, H. (1986) FEBS Lett. 207, 292-296
16. Laemmli, U. K. (1970) Nature 227, 680-685
17. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) J. Biochem. (Tokyo) 95, 511-519
18. Wilden, U., and Kühn, H. (1985) Biochemistry 24, 3014-3022
19. Bauer, P. J. (1988) Anal. Biochem. 170, 67-72
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
21. Means, A. R., Tash, J. S., and Chafouleas, J. G. (1982) Physiol. Rev. 62, 1-39
22. Broekhuyse, R. M., Tolhuizen, B. F. J., Jannsen, A. P. M., and Winkens, H. J. (1988) Curr. Eye Res. 4, 613-618
23. Rochette-Egly, C., and Daviaud, D. (1985) Electrophoresis 6, 235-238
24. Ojewo, B. B., and Storm, D. R. (1989) Biochemistry 24, 8081-8086
25. Ogawa, Y., and Taneokura, M. (1984) J. Biochem. (Tokyo) 95, 12-28
26. Beeler, T. J., Schibeci, A., and Martonosi, A. (1980) Biochim. Biophys. Acta 629, 317-327
27. Kohnken, R. E., Chafouleas, J. G., Eadie, D. M., Means, A. R., and McConnell, D. O. (1981) J. Biol. Chem. 256, 12517-12522
28. Nagno, S., Vamaraki, A., and Riftenski, M. W. (1987) Biochemistry 26, 1659-1665
29. Shinohara, T., Dietzschold, B., Craft, C. M., Wistow, G., Early, J. J., Donovan, L. A., Horsitz, J., and Tao, R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6975-6979
30. Kreisiker, R. H. (1976) Annu. Rev. Biochem. 45, 239-266
31. Kühn, H., and Wilden, U. (1987) Discussions Neurosci. 4, 75-79
Ca\textsuperscript{2+} binding capacity of cytoplasmic proteins from rod photoreceptors is mainly due to arrestin.

B Huppertz, I Weyand and P J Bauer

*J. Biol. Chem.* 1990, 265:9470-9475.

Access the most updated version of this article at [http://www.jbc.org/content/265/16/9470](http://www.jbc.org/content/265/16/9470)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/16/9470.full.html#ref-list-1](http://www.jbc.org/content/265/16/9470.full.html#ref-list-1)