Calmodulin and Calmodulin Binding Proteins in Amphibian Rod Outer Segments†

Seiji Nagao,‡,§ Akio Yamazaki,*,‡ and Mark W. Bitensky‖

Life Sciences and Physics Divisions, Los Alamos National Laboratory, University of California, Los Alamos, New Mexico 87545

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ABSTRACT: The calmodulin (CaM) content of fully intact frog rod outer segments (ROS) has been measured. The molar ratio between rhodopsin and total CaM in ROS is 800:1. This is in good agreement with the data reported for bovine ROS CaM [Kohnken, R. E., Chafouleas, J. G., Eadie, D. M., Means, A. R., & McConnell, D. G. (1981) *J. Biol. Chem. 256*, 12517–12522]. In the absence of Ca^{2+}, the ROS membrane fraction contains only 4% of total ROS CaM. In contrast, in the presence of Ca^{2+}, 15% of total ROS CaM is found in the membrane fraction. For half-maximal binding of CaM to CaM-depleted ROS membranes, 3×10^{-7} M Ca^{2+} is required. This CaM binding is inhibited by trifluoperazine. CaM binding proteins in the ROS membrane fraction are identified by using two different methods: the overlay method and the use of 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP), a bifunctional cross-linking reagent. Ca^{2+}-dependent CaM binding proteins with apparent molecular weights of 240 000, 140 000, 53 000, and 47 000 are detected in the ROS membrane fraction by the overlay method. Anomalous, Ca^{2+}-independent CaM binding to rhodopsin is also detected with this method, and this CaM binding is inhibited by the presence of Ca^{2+}. With the bifunctional cross-linking reagent, DTSSP, three discrete proteins with molecular weights of 240 000, 53 000, and 47 000 are detected in the native ROS membrane fraction. CaM binding to rhodopsin is not detected with this method. Moreover, while the M_r 140 000 band is not detected with DTSSP, a smeared band with a molecular weight between 78 000 and 93 000 is identified (with DTSSP) in the ROS membrane fraction. These data suggest that both the Ca^{2+}-independent binding of CaM to rhodopsin and the Ca^{2+}-dependent binding of CaM to the M_r 140 000 protein (detected by the overlay method) represent binding of CaM to a site(s) which is (are) exposed only after denaturation. However, Ca^{2+}-dependent CaM binding to the smeared band is detected only in the native conformation. Ca^{2+}-dependent CaM binding in the cytoplasmic fraction is also evaluated with the overlay method. The amount of soluble CaM binding proteins detected in this manner is less than 5% of the total membrane-bound CaM binding proteins. These data suggest that CaM and its binding proteins participate in the regulation of Ca^{2+}-sensitive processes primarily on the ROS disk membranes.

A variety of studies have indicated that several discrete biochemical steps occur between photon capture and the subsequent change in rod outer segment (ROS)[1] membrane current (Matthews & Baylor, 1981). Biochemical studies clearly show the mechanisms of light- and GTP-dependent activation of cyclic GMP phosphodiesterase (Yamazaki et al., 1984). Characterization of cyclic GMP phosphodiesterase indicates a large turnover number coupled with a very rapid rate of activation following rhodopsin bleaching (Yee & Liebman, 1978; Woodruff & Bownds, 1979). Moreover, a series of recent electrophysiological studies indicate that cyclic GMP can directly modulate the Na^+ channels in rod plasma membranes (Miller & Nicol, 1979; Fesenko et al., 1985).

There remains, however, considerable interest in the possibility that light absorption by rhodopsin is associated with changes in cytoplasmic and extracellular [Ca^{2+}] (Yau et al., 1981; Gold & Korenbrot, 1980). Following the initial hypothesis (Yoshikami & Hagins, 1971), a role for calcium in the visual process has been supported by a variety of electrophysiological experiments (Brown et al., 1977; Hodgkin et al., 1984). However, evidence for direct involvement of Ca^{2+} in Na^+ channel regulation is still absent. Most studies of photoreceptor Ca^{2+} dynamics utilize electrophysiological measurements. There are not many biochemical studies of Ca^{2+} in ROS. Ca^{2+} can influence the activity of cyclic GMP phosphodiesterase (Kawamura & Bownds, 1981). In the regulation of rod guanylate cyclase, Ca^{2+} acts as an inhibitory effector, and light-induced changes in intracellular [Ca^{2+}] may regulate the synthesis of rod cyclic GMP (Fleischman & Denisevich, 1979; Lolley & Racz, 1982).

In many cellular systems, CaM is a primary regulator of Ca^{2+}-dependent enzyme activity (Klee et al., 1980; Cheung, 1980; Kakiuchi & Sobue, 1982). A series of recent studies show that the regulation of biological processes by Ca^{2+} and CaM often depends upon the CaM binding proteins (Kakiuchi & Sobue, 1982; Manalen & Klee, 1984). In bovine ROS, the presence of CaM has also been described (Kohnken et al., 1981). The importance of CaM and its binding proteins for Ca^{2+}-dependent cellular, regulation, and the accumulated evidence suggesting that photoreceptor Ca^{2+} levels are dynamically regulated (Korenbrot, 1985; Fain & Schroder, 1985), prompted our interest in CaM and its binding proteins in frog ROS. In this study, we demonstrate the presence and

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* Correspondence should be addressed to this author.

‡ Life Sciences Division.

§ Present address: Department of Biochemistry, Gifu University, School of Medicine, Gifu, Japan.

‖ Physics Division.

[1] Abbreviations: ROS, rod outer segment(s); CaM, calmodulin; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); RIA, radioimmunoassay; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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subcellular distribution of CaM and CaM binding proteins in amphibian ROS and discuss the possible role of CaM binding proteins in photoreceptor physiology.

Experimental Procedures

Materials. Bovine brain acetone powder was purchased from Sigma. Lactoperoxidase was obtained from Calbiochem. The bifunctional cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) was obtained from Pierce Chemical Co. Percoll was obtained from Pharmacia. $Na^{125}I$ (carrier-free) was obtained from ICN. CaM radioimmunoassay (RIA) reagents were purchased from New England Nuclear. All other reagents were of analytical grade.

Preparation of Intact ROS. Intact ROS were isolated from *Rana catesbiana* as described (Adams et al., 1982) with the following modifications. ROS were separated from retinas by shaking in buffer A [10 mM Tris·HCl (pH 7.5), 115 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$] containing 10 mM glucose and 45% Percoll (w/w). Crude ROS (3 mL) were layered between 30% and 50% Percoll in a 17-mL centrifuge tube containing 3 mL of 30%, 5.5 mL of 50%, and 5.5 mL of 65% Percoll (w/w) prepared in buffer A and then centrifuged for 25 min with a Beckman SW 27.1 (3000 rpm, 4 °C). Purified ROS were found at two interfaces (45%/50% and 50%/65%) of the Percoll gradients. ROS found at the lower interface (50%/65%) were intact as described below. All preparations were carried out under infrared light. The absorbance ratio of the intact ROS (A_{280}:A_{500}) was 2.8 in 2% Ammonyx-LO and 0.1 M phosphate buffer, pH 7.0 (De Grip et al., 1980). The intactness of isolated ROS was determined by staining with didansylcysteine and observing cytoplasmic fluorescence under the light microscope (Yoshikami et al., 1974).

ROS Washing. Intact ROS obtained from Percoll gradients were suspended in 50–100 volumes of buffer A or buffer B [10 mM Tris·HCl (pH 7.5), 115 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA]. The suspensions were centrifuged for 30 min (105000*g*, 4 °C), and the pellets were washed with the same buffers. The resulting supernatants were collected, dialyzed (in M_r 2000 cutoff dialysis tubing) against water, and concentrated by lyophilization. These samples were used to examine CaM content and CaM binding proteins. For experiments using DTSSP, the pellets were further washed (3 times) with 50–100 volumes of buffer C [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 115 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA].

Radioiodination of CaM. CaM was purified from bovine brain acetone powder (Kakiuchi et al., 1981b) with slight modifications. Column chromatography was performed with octyl-Sepharose (Gopalakrishna & Anderson, 1982) instead of fluphenazine-Sepharose. Purified CaM was radioiodinated with $Na^{125}I$ and lactoperoxidase (Carlin et al., 1981), and the iodinated CaM was isolated (Richman & Klee, 1978). The specific activity of the iodinated CaM was $\sim 10^6$ cpm/μg.

Measurement of CaM Content. CaM content was determined by RIA as described (Kohnken et al., 1981). Membranous fractions were suspended in 0.1% Triton X-100, 6 mM borate (pH 8.4), and 1 mM EGTA and were sonicated at 5 kilocycle for 30 s with a Branson sonifier. Both soluble and membranous samples were then heated at 90 °C for 5 min, rapidly cooled, and centrifuged for 30 min (105000*g*, 4 °C). Supernatant solutions were subjected to RIA.

Assay of CaM Binding to ROS Membranes. Unless otherwise indicated, washed ROS membranes (0.1–0.2 mg) were incubated for 30 min at 30 °C in 0.5 mL of 10 mM Tris·HCl (pH 7.5) containing 4 mg of bovine serum albumin, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, 115 mM NaCl, 2.5 mM KCl, 0.5 mM EGTA, and an appropriate amount of ^{125}I-CaM. Following incubation, binding was stopped by the addition of 1.5 mL of cold buffer followed by centrifugation for 30 min (105000*g*, 4 °C). The pellet was washed with 2 mL of the same buffer (at 4 °C), and the radioactivity of the membranes was measured. In order to check the Ca^{2+} dependency of CaM binding to disk membranes, Ca^{2+}–EGTA buffer was prepared (Ogawa, 1968). For the purpose of calculating the concentration of free Ca^{2+}, 4.4×10^7 M^{-1} was taken as the apparent binding constant of EGTA for Ca^{2+} (Burtnik & Kay, 1977).

Binding of ^{125}I-CaM to Proteins in the Gel Overlay Procedure. The gel overlay procedure was carried out as described (Carlin et al., 1981) by using a 3–15% gradient acrylamide gel. The fixed gels were equilibrated in buffer D [50 mM Tris·HCl (pH 7.6), 250 mM NaCl, and 1 mM $CaCl_2$] and then incubated with ^{125}I-CaM (2 μg/mL) for 12 h. The gels were then washed by incubation in the same buffer for 2–3 h (8 times) and autoradiographed. To evaluate whether the binding of CaM to its putative binding proteins was dependent on Ca^{2+}, 5 mM EGTA and 1 mM $CaCl_2$ were added in buffer D in place of 1 mM $CaCl_2$. Moreover, in control experiments, 250 μg of unlabeled CaM was added as a "cold chase" in order to test the specificity of CaM binding. In some experiments, the half-gels were dried for autoradiography, and the radioactive bands in the corresponding half-gels were excised and incubated with 300 μL of 30% hydrogen peroxide at 70 °C overnight in sealed containers. Aliquots of the dissolved gels were then quantitated by scintillation spectroscopy.

Cross-Linking of ^{125}I-CaM to ROS Membranes and Extraction of Cross-Linked Proteins. Washed ROS membranes (1.5–2 mg of protein) were incubated with ^{125}I-CaM (6.7 μg) in 240 μL of buffer C in the presence or absence of 2 mM $CaCl_2$ for 30 min at room temperature under various conditions. DTSSP (10 μL) was added (12.5–25 μM final concentrations) and incubated for an additional hour at room temperature. The reaction was stopped by the addition of 100 volumes of buffer C. Aliquots were analyzed by SDS–polyacrylamide gel electrophoresis (3–15% gradient acrylamide gel) and autoradiography. In order to avoid dissociating potential cross-linked CaM binding proteins from ^{125}I-CaM, thiol reducing agents were omitted from electrode and sample buffers.

In some cases, proteins cross-linked to ^{125}I-CaM were extracted electrophoretically (Stephens, 1975). Following electrophoresis of cross-linked complexes on SDS gels, protein bands were visualized with 4 M sodium acetate (Higgins & Dahmus, 1979). Portions of the protein bands were excised, rinsed with water, minced, and placed over SDS–polyacrylamide tube gels (4% acrylamide). Knotted dialysis tube (M_r 3500 cutoff) was attached to the bottom (anodic end). Protein was then eluted electrophoretically into the dialysis tube with a 50-V field for 24 h. The eluted protein was subsequently incubated with 10% 2-mercaptoethanol for 1 h and dialyzed against sample buffer containing 0.1% SDS for the overlay procedure.

Miscellaneous Procedures. Electrophoresis of ROS proteins was carried out on SDS–polyacrylamide gradient gels (Laemmli, 1970). Protein concentrations were measured (Lowry et al., 1951) with bovine serum albumin as the standard.

Results

CaM Content of ROS Preparations. CaM content in ROS was measured by using a preparation of fully intact ROS. Figure 1 shows a phase-contrast micrograph (A) and a

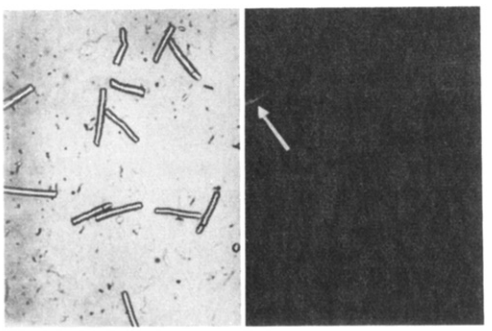

FIGURE 1: Light micrographs of ROS (90×). ROS were isolated and stained for 1 min with 100 μM didansylcysteine, prior to microscopical examination as described. (A) Phase-contrast micrograph. (B) Fluorescence micrograph of the same field. Excitation, 365 nm; fluorescence photographed through a filter which transmits above 450 nm. The arrow shows a single leaky rod which did not exclude the fluorescent probe.

Table I: CaM Content of Total ROS and Suborganelle Fractions[a]

fraction	total ROS protein (mg)	CaM (ng)	ng of CaM/mg of ROS protein
total ROS	2.50 ± 0.31	1052 ± 145	421
EGTA supernatant	0.54 ± 0.05	840 ± 73	1556
EGTA pellet	2.20 ± 0.31	32 ± 4	15
$CaCl_2$ supernatant	0.45 ± 0.06	667 ● 54	1482
$CaCl_2$ pellet	2.30 ● 0.23	97 ± 11	42

[a] ROS were prepared from 40 retinas and washed as described. The resulting supernatant and pellet fractions were assayed for CaM by RIA as described.

fluorescence micrograph (B) of the intact ROS preparations which were found at the interface between 50% and 65% of the Percoll gradient. With these procedures, more than 90% of the ROS had intact plasma membranes (i.e., excluded the fluorescent dye), and the yield of intact ROS was about 30% of the total ROS preparations. It is noteworthy that the preparation of intact frog ROS depended upon the use of Percoll gradients. Efforts to purify intact frog ROS with sucrose or Ficoll gradients were not successful, in contrast to the preparation of intact bovine ROS (Adams et al., 1982; Zimmerman & Godchaux, 1982; Smith & Litman, 1982).

Table I presents the CaM content found in the soluble and particulate fractions of intact frog ROS. Intact ROS contained 421 ng of CaM/mg of total protein. When the intact ROS were disrupted by syringing (3 times) through a 21-gauge needle and then washed with isotonic buffer containing 1 mM EGTA, 96% of the CaM appeared in the supernatant with only 4% sedimenting with ROS membranes. In contrast, however, when the disrupted ROS membranes were washed with isotonic buffer containing 1 mM $CaCl_2$, about 15% of the CaM sedimented with the ROS membranes. These data demonstrate the presence of Ca^{2+}-dependent CaM binding proteins in association with ROS membrane suspensions. It is noteworthy that the extent of Ca^{2+}-dependent CaM binding to the ROS membrane fraction was not affected either by the presence of illuminated rhodopsin or by the ionic strength of the washing buffer. Using a widely accepted estimate (Heitzman, 1972; Robinson et al., 1972; Daemen et al., 1972) that 85% of the protein which remains in the ROS membrane fraction after washing is rhodopsin and based on our total CaM recovery of 318 ng/mg of ROS protein, we calculate the molar ratio of CaM to rhodopsin is 1:800.

CaM Binding to EGTA-Washed ROS Membranes. Table I shows the presence of Ca^{2+}-dependent CaM binding proteins

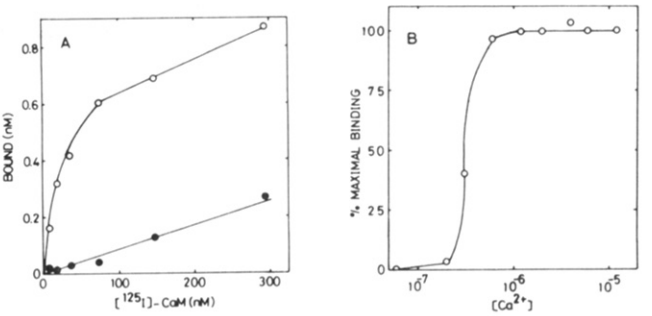

FIGURE 2: Demonstration of CaM binding to ROS membranes. (A) CaM binding to ROS membranes as a function of CaM amount. The binding of ^{125}I-CaM to 100 μg of ROS membrane proteins was determined in an assay mixture of 500 μL. (○) CaM binding in the presence of 0.5 mM EGTA and 0.8 mM $CaCl_2$; (●) CaM binding in the presence of 0.5 mM EGTA. (B) CaM binding to ROS membranes as a function of Ca^{2+} concentration. The Ca^{2+} concentrations shown were achieved by using a Ca^{2+}–EGTA system as described.

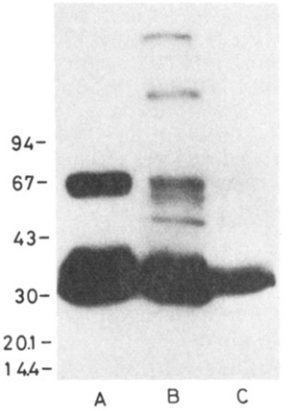

FIGURE 3: Identification of CaM binding proteins in ROS membranes. Following electrophoresis of membrane proteins (200 μg in each lane), the CaM binding proteins were identified by using the gel overlay method. The binding of ^{125}I-CaM was evaluated in the presence of (lane A) 5 mM EGTA, (lane B) 1 mM $CaCl_2$, and (lane C) 1 mM $CaCl_2$ and 250 μg of unlabeled CaM. The binding of ^{125}I-CaM was detected by autoradiography. Molecular weight standards on the left are indicated as $M_r \times 10^{-3}$.

in ROS membranes. To quantitate these proteins, the binding of ^{125}I-CaM to ROS membranes was measured by simple centrifugation. Figure 2A shows a saturation curve of the Ca^{2+}-dependent ^{125}I-CaM binding to 100 μg of EGTA-washed (CaM-depleted) ROS membranes. Approximately 75 nM CaM was required for maximal binding. The nonspecific binding measured in the presence of EGTA was not saturable. Figure 2B shows that ^{125}I-CaM binding to ROS membranes depends upon the free [Ca^{2+}]. Negligible ^{125}I-CaM bound to ROS membranes at less than 0.1 μM [Ca^{2+}]. However, 1 μM [Ca^{2+}] supported maximal binding of ^{125}I-CaM to ROS membranes.

Ca^{2+}-dependent ^{125}I-CaM binding to ROS membranes was inhibited by both unlabeled CaM and trifluoperazine. Binding observed with 75 nM ^{125}I-CaM was 50% displaced by the addition of 125 nM unlabeled CaM and was also 50% inhibited by the presence of 150 μM trifluoperazine. These results further support the observation that ^{125}I-CaM can bind specifically to CaM binding proteins which are present in ROS membranes.

CaM Binding Proteins in ROS Membrane Fractions. A number of CaM binding proteins can be detected in particulate ROS fractions by the gel overlay procedure (Figure 3). This method is based on the observation that CaM binding proteins

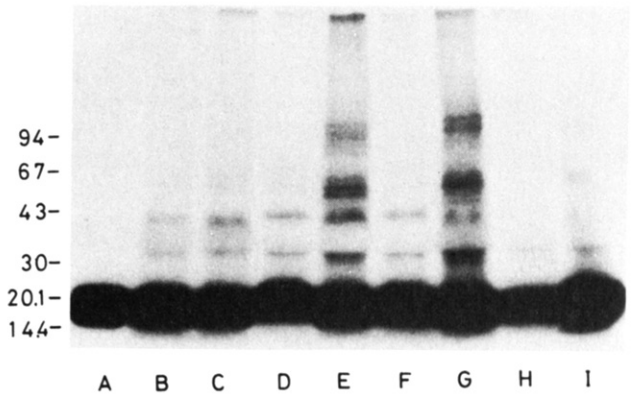

FIGURE 4: Use of a cross-linking method to identify the ROS membrane CaM binding protein. A sulfhydryl reagent dissociable cross-linker (DTSSP) was used to identify ROS membrane CaM binding proteins as described. The cross-linking step was carried out under the following experimental conditions: Lane A contained 50 ng of ^{125}I-CaM alone, and lanes B–I contained ^{125}I-CaM (6.7 μg/250 μL), 1 mM EGTA, and ROS membranes (2 mg of protein/250 μL). In addition: lane B, no DTSSP and no $CaCl_2$; lane C, no DTSSP with 2 mM $CaCl_2$; lane D, 12.5 μM DTSSP without $CaCl_2$; lane E, 12.5 μM DTSSP with 2 mM $CaCl_2$; lane F, 25 μM DTSSP without $CaCl_2$; lane G, 25 μM DTSSP with 2 mM $CaCl_2$; lane H, 335 μg of unlabeled CaM, 25 μM DTSSP, and 2 mM $CaCl_2$; lane I, 500 μM trifluoperazine, 25 μM DTSSP, and 2 mM $CaCl_2$. The binding of ^{125}I-CaM was detected by autoradiography. Molecular weight standards on the left are given as $M_r \times 10^{-3}$.

in the acrylamide gel matrix regain Ca^{2+}-dependent CaM binding when most of the SDS is removed by washing the gel (Lacks & Springhorn, 1980). The method allows rapid and sensitive detection of CaM binding proteins in crude extracts (Watterson et al., 1984). In the presence of 1 mM $CaCl_2$, ^{125}I-CaM bound to six proteins with molecular weights of 240 000, 140 000, 67 000, 53 000, 47 000, and 35 000, respectively (lane B). In the presence of 5 mM EGTA, ^{125}I-CaM was bound to proteins with molecular weights of 67 000 and 35 000. Moreover, binding of ^{125}I-CaM to the M_r 35 000 protein was not completely displaced by 12 μM unlabeled CaM, suggesting the presence of extremely large amounts of the M_r 35 000 protein in ROS. The abundance of the M_r 35 000 protein was clearly apparent in Coomassie blue stained gels. When the sample was heated at 95 °C in electrophoresis buffer for longer periods of time (>5 min), the amount of M_r 67 000 protein was substantially increased, and the amount of M_r 35 000 protein was correspondingly decreased. These data indicate that the M_r 35 000 protein is rhodopsin and the M_r 67 000 protein is the dimer of rhodopsin (Bridges & Fong, 1982). An unexpected result was the finding that ^{125}I-CaM binding to the M_r 35 000 protein (rhodopsin) was clearly inhibited by the presence of Ca^{2+}.

In Table I and Figure 2, the presence of Ca^{2+}-dependent CaM binding proteins is demonstrated in ROS membranes. However, in Figure 3, denatured rhodopsin (a major ROS integral protein) was found to be an abundant CaM binding protein by the overlay procedure. Furthermore, the binding of CaM to rhodopsin (in SDS) was diminished by the addition of $CaCl_2$. To further study ROS CaM binding proteins in their native conformation, a bifunctional cross-linker, DTSSP, was also used to detect putative CaM binding proteins in ROS membranes in the absence of SDS. Figure 4 shows that the cross-linked complexes formed with ^{125}I-CaM were detected at three different regions of the acrylamide gel: at the top of the gel, M_r 95 000–110 000, and M_r 57 000–67 000. Non-Ca^{2+}- and non-cross-linker-dependent complexes with ^{125}I-CaM were also detected in the gel at M_r 40 000 and 33 000. It is obvious from this figure and Coomassie blue staining of the gel (not

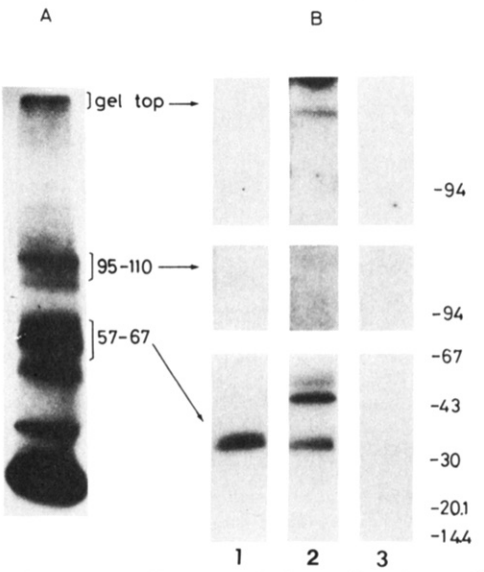

FIGURE 5: Comparison of the cross-linking and overlay methods for identification of CaM binding proteins in ROS membranes. (A) ROS membranes (2 mg of protein/250 μL) were cross-linked with ^{125}I-CaM (6.7 μg/250 μL) in the presence of 25 μM DTSSP and 2 mM $CaCl_2$ and then solubilized and electrophoresed as described. (B) Individual complexes cross-linked with ^{125}I-CaM were isolated electrophoretically from selected gel protein bands. This included the following gel regions: the gel top, 95 000-110 000, and 57 000–67 000 molecular weight regions. The eluted protein complexes were then cleaved with 2-mercaptoethanol and reelectrophoresed. Subsequently, the gel overlay procedure was used for detection of these CaM binding proteins. The binding of ^{125}I-CaM was performed in the presence of (lane 1) 5 mM EGTA, (lane 2) 1 mM $CaCl_2$, and (lane 3) 1 mM $CaCl_2$ and 250 μg of unlabeled CaM. The binding of ^{125}I-CaM was detected by autoradiography. Molecular weight standards are indicated as $M_r \times 10^{-3}$.

shown) that native rhodopsin is not a Ca^{2+}-dependent CaM binding protein. These data indicate that rhodopsin can bind CaM only after treatment with SDS and that this anomalous binding is inhibited by Ca^{2+}.

The CaM binding proteins which were identified by cross-linking to ^{125}I-CaM were also identified by gel overlay after cleavage of the complex with a reducing agent. As illustrated in Figure 5, lane A shows an autoradiogram of the cross-linked complexes between native membrane proteins and ^{125}I-CaM. Lane B illustrates ^{125}I-CaM binding (detected by the overlay procedure) to those proteins which were eluted from the cross-linked complexes with a reducing agent. From the cross-linked complex which remained at the top of the gel, an M_r 240 000 protein was eluted which exhibited Ca^{2+}-dependent binding of ^{125}I-CaM by the overlay procedure. This CaM binding was also displaced by unlabeled CaM. However, from the M_r 95 000–110 000 region, no ^{125}I-CaM binding activity was detected by the overlay procedure. From the M_r 57 000–67 000 region, M_r 47 000 and 53 000 proteins were eluted, and these proteins clearly showed Ca^{2+}-dependent ^{125}I-CaM binding activity. Binding of ^{125}I-CaM to an M_r 35 000 protein (present only in small amounts) was also detected in the gels. We suggest that this protein represents rhodopsin which coelutes with the above proteins, both because of its size and because CaM binding to this protein was inhibited by Ca^{2+}. Taken together, these data suggest that proteins with molecular weights of 240 000, 53 000, and 47 000 show CaM binding activity by two different procedures and that rhodopsin in its native conformation is not a CaM binding protein. Furthermore, a protein in the M_r 95 000–110 000 region appears to bind CaM only in its native configuration and is detected with DTSSP but not with the overlay method.

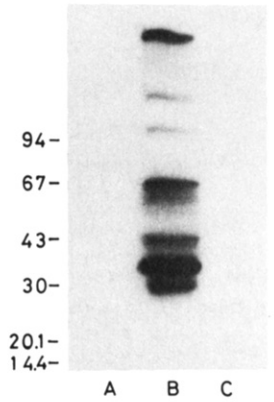

FIGURE 6: Identification of soluble ROS CaM binding proteins. Following electrophoresis of the soluble protein (220 μg in each lane), the CaM binding proteins were identified with the gel overlay method. ^{125}I-CaM binding was evaluated in the presence of (lane A) 5 mM EGTA, (lane B) 1 mM $CaCl_2$, and (lane C) 1 mM $CaCl_2$ and 250 μg of unlabeled CaM. The binding of ^{125}I-CaM was detected by autoradiography. Molecular weight standards on the left are indicated as $M_r \times 10^{-3}$.

CaM Binding Proteins in Soluble ROS Fractions. Ca^{2+}-dependent CaM binding proteins in the soluble fraction were also checked by the overlay procedure. As illustrated in Figure 6 (lane B), more pronounced binding of CaM was found in the molecular weight regions of 240 000, 67 000, 44 000, 35 000, and 29 500. Less intense CaM binding was detected at the molecular weight regions of 140 000, 105 000, and 53 000. All binding activities were removed by the addition of either 5 mM EGTA (lane A) or unlabeled CaM (lane C), indicating that ^{125}I-CaM binds specifically to those proteins in a Ca^{2+}-dependent manner. However, the amount of CaM binding proteins in the soluble fraction appeared to be less than 5% of the CaM binding proteins in the membrane fraction, when radioactive bands were excised from gels and the total radioactivities of CaM binding proteins were compared with those found in the membrane fractions. Moreover, the ratio of these proteins in the different samples was not constant. Finally, we found that neither light nor Ca^{2+} changed the distribution of CaM binding proteins between the soluble and membrane fractions. Our data show that only very small (and variable) amounts of CaM binding proteins are detected in the soluble fraction of ROS.

Discussion

The data presented here demonstrate the presence of CaM- and Ca^{2+}-dependent CaM binding proteins in highly purified, intact amphibian ROS. In bovine ROS, CaM is present at the level of about 450 ng of CaM/mg of protein, and the molar ratio of rhodopsin to CaM is 700:1 (Kohnken et al., 1981). In our study which utilized intact frog ROS, CaM was present at a level of about 420 ng/mg of ROS protein, and the molar ratio of rhodopsin to CaM was about 800:1. The agreement of the data on CaM abundance from two different species suggests that CaM might play a common role in the regulation of Ca^{2+}-sensitive processes in ROS. The stoichiometric ratio of rhodopsin to CaM suggests that Ca^{2+}/CaM-dependent regulatory processes involve protein species of intermediate abundance.

Table II lists the Ca^{2+}-dependent CaM binding proteins in amphibian ROS which are detected by gel overlay and by the formation of cross-linked complexes with ^{125}I-CaM. In the soluble fraction, the amount of CaM binding proteins appears to be very small (less than 5% of the membrane-bound CaM binding proteins). In contrast, CaM binding proteins in the membrane fraction are readily detected. Moreover, the soluble fraction appears to contain the same CaM binding proteins as found in the membrane fraction. These CaM binding proteins may represent contamination from the membrane-bound CaM binding proteins. This hypothesis is compatible with our observation that the ratio of these CaM binding proteins in the different soluble fractions was not constant.

Table II: Summary of Ca^{2+}-Dependent CaM Binding Proteins in ROS

fraction	mol wt ($\times 10^{-3}$)	method of detection: overlay	method of detection: cross-linking
soluble	105	+	ND[c]
	67	+	ND
	44	+	ND
	35	+	ND
	29.5	+	ND
membrane	240[a]	+	+
	140[a]	+	–
	95–110[b]	–	+
	53[a]	+	+
	47	+	+

[a] These proteins are putative minor contaminants of the soluble fraction. [b] This molecular weight includes CaM and its binding protein. [c] ND, not done.

The identification of Ca^{2+}-dependent CaM binding proteins in ROS is a first step in understanding the role of CaM in the regulation of Ca^{2+}-dependent ROS processes. In this study, three independent approaches to the quantitation of CaM binding proteins were employed. These included (1) the gel overlay procedure which depends upon recovery of native conformation after removal of SDS, (2) centrifugation of native ROS membranes to detect bound ^{125}I-CaM in the presence or absence of Ca^{2+}, and (3) the use of a reducible cross-linking agent designed to detect Ca^{2+}-dependent binding of CaM to native ROS membrane proteins. When only one of these methods is employed, there exists the very real possibility of being misled concerning the identity and characteristics of the CaM binding proteins. The gel overlay procedure has the potential of creating artifacts, since some authentic CaM binding proteins may be irreversibly denatured by SDS and/or some proteins may be found to bind CaM only after denaturation (Glenney & Weber, 1983). In this study, rhodopsin was shown to behave like a CaM binding protein by the gel overlay procedure. This binding was suspect not only because it was dependent upon denaturation of rhodopsin by SDS but also because it was stimulated by EGTA. It is noteworthy that with the centrifugation approach, no EGTA-stimulated CaM binding was observed. It should also be noted that the cross-linking approach is not entirely free of artifacts inasmuch as the cross-linking agent also covalently stabilizes some nonspecific and non-Ca^{2+}-dependent binding activity of CaM (Figure 4). The centrifugation approach could also detect nonspecific binding and is incapable of identifying individual species of CaM binding proteins. In this study, we tried to clarify this complicated situation by using three independent ways to evaluate CaM binding proteins. By comparing the data from all three approaches, it becomes possible to identify the authentic CaM binding proteins. For example, rhodopsin does not appear to be an authentic CaM binding protein. A CaM binding protein associated with the membranes in the M_r 95 000–110 000 region was detected by the cross-linking approach but failed to bind CaM with the overlay procedure. Here we suggest that CaM binding to this protein is lost following exposure to SDS.

It is noteworthy that, using the overlay procedure, both M_r 35 000 and 67 000 CaM binding proteins were detected in the

soluble fraction. We do not believe that these proteins represent contamination by rhodopsin monomers and dimers since the pseudo-CaM binding displayed by rhodopsin was inhibited by Ca^{2+} while the M_r 35 000 and 67 000 proteins in the soluble fraction exhibited CaM binding which was entirely dependent upon the presence of Ca^{2+}.

Recently, new sites for regulation of cytoskeletal proteins by Ca^{2+} and CaM have been reported (Kakiuchi & Sobue, 1983; Kakiuchi, 1983). In these studies, CaM controls cytoskeletal proteins through CaM binding proteins. Calspectin/fodrin (M_r 240 000) is one of these CaM binding proteins which are regulated by Ca^{2+} and CaM in a flip-flop manner (Kakiuchi et al., 1981a; Glenney et al., 1982). Moreover, τ factor (M_r 55 000) is also a CaM binding protein (Timasheff & Grisham, 1980), and Ca^{2+}-sensitive microtubule assembly is dependent upon the presence of τ factor (Kakiuchi & Sobue, 1981). Furthermore, an M_r 135 000 protein purified from chicken gizzard smooth muscle is also reported as a CaM binding protein (Sobue et al., 1982). In photoreceptors, there is no independent evidence to show the presence of these proteins. However, it is of interest that membrane-bound CaM binding proteins in the photoreceptor exhibited the same molecular weight as those CaM binding proteins found in association with cytoskeletal components. In vertebrate photoreceptors, several kinds of cytoskeletal proteins have been reported including large intrinsic membrane protein (Papermaster et al., 1978) and microtubular subunits (Ali & Klyne, 1984). Calibrations of the higher molecular weight regions of gradient gels suffer from a certain degree of ambiguity made worse by a sancity of acceptable protein standards in this region. Thus, in the absence of more definitive proof (with specific immunoglobulins), it is not yet possible to definitively identify the higher molecular weight membrane CaM binding protein as either intrinsic membrane protein or calspectin/fodrin. Recently, the light-dependent movements of the photoreceptor itself and the pigment granules of the retinal pigment epithelium, called retinomotor movements, were found to be regulated by Ca^{2+} (Dearry & Burnside, 1984). These findings and the data presented here may suggest an additional feature of the complex function of Ca^{2+} in ROS. We have also not excluded the possibility that the M_r 35 000 Ca^{2+}-dependent CaM binding protein is related to the β subunit of the GTP binding protein in ROS. More data are also needed to fully characterize the putative role of Ca^{2+} in the regulation of ROS cyclic GMP phosphodiesterase and to integrate such effects into the overall events of light-regulated closure of cation channels in ROS plasma membranes.

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Characterization of the Unfolding of Ribonuclease A in Aqueous Methanol Solvents†

Anthony L. Fink* and Brooks Painter
Department of Chemistry, University of California, Santa Cruz, California 95064
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ABSTRACT: The effect of methanol on the thermal denaturation of ribonuclease A has been investigated over the −40 to 70 °C range. The transition was fully reversible to at least 60% (v/v) methanol at an apparent pH of cryosolvent (pH*) of 3.0 and was examined at methanol concentrations as high as 80%. The unfolding transition, as monitored by absorbance change at 286 nm, became progressively broader and occurred at increasingly lower temperatures as the alcohol concentration increased. In 50% methanol, increasing the pH* from 2 to 6 shifted the transition to higher temperature. A substantial decrease in cooperativity was noted at the more acidic conditions. On the other hand, increasing concentrations of guanidine hydrochloride in 50% methanol caused the transition to shift to lower temperatures with little effect on the cooperativity. The observed effects on the cooperativity of the unfolding transition suggest that methanol and lower temperatures may increase the concentration of partially folded intermediate states in the unfolding of ribonuclease. Comparison of the transition in 50% methanol as determined by absorbance or fluorescence, which monitor the degree of exposure of buried tyrosines and hence the tertiary structure, to that determined by far-UV circular dichroism, which monitors secondary structure, indicated that the major unfolding transition occurred at a higher temperature in the latter case. Thus, the tertiary structure is lost at a lower temperature than the secondary structure. This observation is consistent with a model of protein folding in which initially formed regions of secondary structure pack together, predominantly by hydrophobic interactions, to give the tertiary structure. Carboxymethylated, disulfide-reduced ribonuclease was used as a model to determine the effects of methanol and temperature on unfolded ribonuclease.

Many important questions regarding the mechanism of protein folding, and the stability of the native state, remain unanswered. Although it is clear that the amino acid sequence and interactions of the polypeptide with its solvent environment determine the three-dimensional conformation of the protein, the particular factors that determine the folding pathway (in other words the "code" or "rules" for folding) are as yet unknown. Recent results are consistent with the folding pathway involving intermediate, partially folded states (Kim & Baldwin, 1982). In order to shed light on the mechanisms of folding, it will be necessary to obtain information about intermediate partially folded states, especially structural characterization, kinetics of intermediate transformation, and effects of environmental factors, including solvent, pH, and temperature, on the thermodynamic stability of the intermediates.

The native states of proteins are only marginally more stable than the unfolded state; typically the native state is 5–15 kcal mol^{-1} more stable. Under physiological conditions, the lifetimes of intermediates in folding are very short and their concentrations very low. As a consequence, most single domain proteins exhibit adherence to a two-state system, N ↔ U, under equilibrium conditions, due to the low population of the intermediate states. Consequently, it is necessary to use experimental conditions far from the physiological to stabilize and populate partially folded intermediate states for the time periods required to obtain high-resolution structural information. One such approach is to use subzero temperatures, which should permit dramatic decreases in the rates of folding.

Several recent investigations suggest that secondary structure may form early in folding (Kim & Baldwin, 1982). If this is the case, then partially folded intermediates would be expected in which the nonpolar side chains would be exposed

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