S-modulin controls rhodopsin phosphorylation in a calcium-dependent manner, and it has been suggested that it modulates the light sensitivity of the photoreceptor cell. S-modulin binds to the ROS membrane at high \( \text{Ca}^{2+} \) concentration, and N-terminal myristoylation is necessary for this property (the calcium-myristoyl switch). S-modulin has four EF-hand motifs, of which two (EF-2 and -3) are functional. Here, we report on the roles of EF-2 and -3 in S-modulin function (calcium binding, membrane association, and inhibition of rhodopsin phosphorylation) by site-directed mutants (E85M and E121M). Surprisingly, E121M, which has a mutation in EF-3, neither binds \( \text{Ca}^{2+} \) nor inhibits phosphorylation. In contrast, E85M binds one \( \text{Ca}^{2+} \) and has the same membrane affinity as wild-type S-modulin, but has lost the ability to inhibit rhodopsin phosphorylation. It is suggested that the binding of \( \text{Ca}^{2+} \) to EF-3 is probably required for EF-2 to be a functional Ca\(^{2+}\)-binding site and to induce exposure of the myristoyl group; and that the binding of \( \text{Ca}^{2+} \) to EF-2 is important for the interaction with rhodopsin kinase.

In vertebrate rod photoreceptors, cGMP-gated cation channels are opened in the dark-adapted state (1, 2). Light activates rhodopsin and triggers the phototransduction cascade, which results in the closure of cation channels in the rod outer segment (ROS) and blocks the influx of Ca\(^{2+}\). As intracellular Ca\(^{2+}\) is continuously pumped out by a Na\(^+\)-K\(^+\)/Ca\(^{2+}\) exchanger in the outer segment (3, 4), the cytoplasmic Ca\(^{2+}\) concentration decreases in light-adapted photoreceptors. This decrease of Ca\(^{2+}\) concentration is the underlying mechanism of light adaptation of vertebrate photoreceptors (5, 6).

Phosphorylation of rhodopsin plays a role in shutting off the activation of transducin (7, 8), and the efficiency of phosphorylation is regulated, in a Ca\(^{2+}\)-dependent manner, by a Ca\(^{2+}\)-binding protein, S-modulin, in frogs (9, 10) or recoverin, its bovine homologue (11). At high Ca\(^{2+}\) concentrations (dark-adapted state), S-modulin inhibits phosphorylation of light-activated rhodopsin, but does not interfere at low Ca\(^{2+}\) concentrations (light-adapted state). Therefore, S-modulin and recoverin contributes to increased light sensitivity in the dark-adapted state.

S-modulin and recoverin are Ca\(^{2+}\)-binding proteins which contain covalently attached fatty acyl groups at their N terminus (12). The binding of Ca\(^{2+}\) to these proteins induces exposure of the fatty acyl groups, which enables them to associate with ROS membrane (13, 14). This property is the so-called “Ca\(^{2+}\)-myristoyl switch” (13). There are four EF-hand motifs in S-modulin and recoverin, but only two of them (EF-2 and -3) are thought to be able to bind Ca\(^{2+}\) (15). Therefore, S-modulin (inhibition of frog rhodopsin phosphorylation) is mediated by the binding of Ca\(^{2+}\) ions to EF-2 and -3.

We made site-directed mutants of S-modulin that lack Ca\(^{2+}\) binding ability in their EF-2 or -3. The present study describes the Ca\(^{2+}\) binding properties, membrane-association, and inhibitory effects on rhodopsin phosphorylation of wild-type S-modulin and these mutants. The results suggest that EF-3 first binds Ca\(^{2+}\), which enables S-modulin to associate with ROS membrane and to bind Ca\(^{2+}\) at EF-2. Subsequent EF-2 binding of Ca\(^{2+}\) ions probably causes a conformational change permitting interaction of S-modulin with rhodopsin kinase, which inhibits rhodopsin phosphorylation.

**Experimental Procedures**

*Site-directed Mutagenesis—Oligonucleotides, 5'-GOTCATATGTAGCGTATCATGTACATTTAA-3' and 5'-TTGAAATGCATTGGATATTCAAGCACCATTITTTIT-3' were used as antisense primers to generate site-directed mutants, E85M and E121M, respectively. The S-modulin cDNA fragment (10) and the SMD-NTP primer (16) were used as a template and sense primer, respectively, for the polymerase chain reactions. cDNA fragments encoding E85M and E121M were inserted between the Ncol and XhoI sites of pET-16b (Novagen) plasmid vector, designated pET-E85M and pET-E121M, respectively.*

**Expression and Purification of Recombinant Proteins—** The procedures for expression and purification of recombinants follow Hisatomi *et al.* (16). Briefly, the expression vectors, pET-Smd (16), pET-E85M, and pET-E121M were transfected into *Escherichia coli* BL21DE3 (Novagen) with (+myr) or without (-myr) pBBS13, an expression vector of N-myristoyltransferase. The recombinant proteins were expressed by the addition of 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside. Recombinant proteins were solubilized in 8 M urea buffer, refolded by dialysis, and applied to a DEAE-Sephadex column. The fraction containing recombinant S-modulins was dialyzed against the buffer containing 5 mM Ca\(^{2+}\) and applied to a phenyl-Sepharose column. Recombinant S-modulins were eluted with 5 mM EGTA.

**HPLC—** Purified recombinant proteins were injected onto a reverse-phase C-18 column. Recombinant S-modulin was eluted with a linear gradient of 0–80% acetonitrile (1.5%/min) in 0.1% trifluoroacetic acid at a flow rate of 1.5 ml/min, monitoring absorbance of the eluate at 280 nm.

**Ca\(^{2+}\) Binding Assay—** Binding of Ca\(^{2+}\) ions to S-modulin or mutant proteins was evaluated by ultrafiltration (17). Purified proteins were extensively dialyzed against 25 mM Tris-HCl (pH 8.0) to remove EGTA and calcium, then 20 \(\mu\)M of each calcium-free protein in 1 ml of 25 mM Tris-HCl (pH 8.0) was then added, and the solution was thoroughly mixed. The calcium-protein mixtures were then centrifuged, and the amounts of calcium in the filtrated fractions were measured by atomic absorption (Shimazu AA-660).
Tryptophan Emission Spectrum—Spectroscopic measurement was carried out as described by Hisatomi et al. (16). Briefly, fluorescence emission spectra were recorded from 300 to 400 nm with a fluorescence spectrophotometer (Hitachi, F-4500) at an excitation wavelength of 290 nm, in a mixture containing 2 mM recombinant protein, 100 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EGTA, and 100 mM HEPES (pH 7.0). The free Ca\(^{2+}\) concentration was adjusted by adding 1 M CaCl\(_2\).

Ca\(^{2+}\)-dependent Membrane Association of Recombinant Proteins—Frog ROS were isolated by flotation with 45% sucrose in gluconate buffer (40 mM potassium gluconate, 2.5 mM KCl, 2 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM EGTA, and 10 mM HEPES, pH 7.5), and washed with gluconate buffer containing 4 M urea to eliminate endogenous S-modulin, s26 (cone homologue of S-modulin) and other peripheral proteins. Urea-stripped ROS membranes were mixed with gluconate buffer containing 1% bovine serum albumin to prevent nonspecific binding of the recombinant proteins to the ROS membrane and tube. After washing with gluconate buffer containing various concentrations of Ca\(^{2+}\) (Ca\(^{2+}\) gluconate buffer), the ROS membranes were resuspended in Ca\(^{2+}\) gluconate buffer containing recombinant proteins (120 pmol). The mixtures were incubated at room temperature for 30 min, and the soluble and membrane fractions after centrifugation (37,000 \(\times\) g for 5 min) were analyzed by SDS-polyacrylamide gel electrophoresis. The integrated densities of Coomassie Brilliant Blue-stained bands of the recombinant proteins were quantified by a two-dimensional densitometer (The Discovery Series, pdi Inc.).

**Phosphorylation Assay**—Phosphorylation of rhodopsin was measured by the methods of Kawamura (9) and Sanada et al. (18). For the phosphorylation assay, ROS were isolated in phosphorylation buffer (115 mM potassium gluconate, 2.5 mM KCl, 2 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM EGTA, 10 mM HEPES, pH 7.5) in complete darkness, and washed with the buffer to eliminate endogenous S-modulin, s26, and ATP. The reaction was carried out in 25 \(\mu\)l of the mixture containing 10 \(\mu\)M (final concentration) rhodopsin and various concentrations of S-modulin and/or its mutants in phosphorylation buffer. The free calcium concentration in the mixture was adjusted by adding 1 M CaCl\(_2\) solution. The reaction mixtures were exposed to light for 2 min, and the reaction was initiated by addition of a mixture of ATP (0.1 mM final concentration), [\(\gamma\)-\(\text{P}\)]ATP (168 TBq/\(\mu\)mol, 0.25 \(\mu\)M), and GTP (0.5 mM).

| Assay   | No. of calcium ions bound |
|---------|---------------------------|
| Wild-type| 2.1 ± 0.10                |
| E86M    | 0.97 ± 0.02               |
| E121M   | 0.02 ± 0.04               |

**TABLE I**

The number of Ca\(^{2+}\) ions bound to myristoylated wild-type (+myr), E85M (+myr), and E121M (+myr) S-modulins (per molecule) in the presence of 0.1 mM Ca\(^{2+}\).

**FIG. 1.** HPLC profiles of recombinant S-modulins. The HPLC pattern of wild-type (+myr) (a), wild-type (+myr) (b), E85M (+myr) (c), and E121M (+myr) (d).

**FIG. 2.** Tryptophan fluorescence emission spectra of myristoylated S-modulins. Curve 1, S-modulin (+myr); curve 2, E85M (+myr); curve 3, E121M (+myr) in the presence of 1 nM (upper panel) or 0.1 mM (lower panel) free Ca\(^{2+}\). The dashed line indicates the spectrum of wild-type (+myr).

**FIG. 3.** Membrane binding properties of wild-type and mutant S-modulins. ROS membrane affinity of wild-type (+myr) (open squares), E85M (+myr) (filled squares), and E121M (+myr) (open circles) at various Ca\(^{2+}\) concentrations. Bars represent standard deviations (n = 2).
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Fig. 4. Inhibition of rhodopsin phosphorylation by wild-type and mutant S-modulins. $^{32}$P incorporation of rhodopsin in the presence of $[gamma-^{32}$P]ATP at low (1 nM; filled squares) and high (0.1 mM; open squares) Ca$^{2+}$ concentration. Experiments were carried out in the presence of various concentrations of S-modulins (wild-type (+myr), E85M (+myr), or E121M (+myr)). Bars represent standard deviations ($n = 2$).

Fig. 5. The effect of E85M (+myr) on the inhibition of rhodopsin phosphorylation by wild-type (+myr). The effects on $^{32}$P incorporation by rhodopsin were tested in the presence of $[gamma-^{32}$P]ATP at low (1 nM; filled triangles and circles) and high (0.1 mM; open triangles and circles) Ca$^{2+}$ concentrations. Experiments were carried out in the presence of 0.5 $\mu$M (triangles) and 1 $\mu$M (circles) wild-type. Bars represent standard deviations ($n = 2$).

After 2 min of incubation at room temperature, the reaction was terminated by adding 150 $\mu$l of 10% trichloroacetic acid. After centrifugation (10,000 × g for 5 min) of the reaction mixture, the precipitates were washed with 500 $\mu$l of phosphorylation buffer and subjected to SDS-polyacrylamide gel electrophoresis. The amount of $^{32}$P incorporated into rhodopsin was quantified by using an image analyzer (BAS 2000, Fuji Film).

RESULTS

Myristoylation of Recombinant S-modulin—It has been established that the conserved glutamic acid at position 12 of the Ca$^{2+}$-binding loop is important for coordinating Ca$^{2+}$ (19, 20). To investigate the role of each Ca$^{2+}$-binding site, EF-2 or EF-3 was inactivated by replacing glutamic acid with the hydrophobic amino acid, methionine. Myristoylated wild-type (+myr) and mutant S-modulins, E85M (+myr) and E121M (+myr), and unmyristoylated wild-type (−myr) were expressed and purified as described in the experimental procedures. Myristoylated recombinant proteins eluted from a C-18 column at almost the same retention time (Fig. 1, b, c, and d), which is longer than that of wild-type (−myr) (Fig. 1a) and suggests that these recombinants expressed with N-myristoyltransferase are in fact myristoylated.

Ca$^{2+}$ Binding of S-modulin and Mutants—The number of Ca$^{2+}$ ions bound to each of these proteins was quantified in the presence of 0.1 mM Ca$^{2+}$ (Table 1). As expected, wild-type (+myr) and E85M (+myr) bind two and one Ca$^{2+}$ per molecule, respectively. On the other hand, E121M (+myr) can not bind Ca$^{2+}$. These results indicate that Ca$^{2+}$ binding to EF-3 is necessary for EF-2 to bind Ca$^{2+}$ at physiological Ca$^{2+}$ concentrations. It has been reported that EF-3 has the conformation of a classic EF-hand, but EF-2 is rather different (21). The conformational change induced by Ca$^{2+}$ binding to EF-3 may be required to raise the Ca$^{2+}$ affinity of EF-2, in order for EF-2 to become functional.

Fluorescence Properties of S-modulin and Mutants—E85M (+myr) and E121M (+myr) can be purified in the same way as for wild-type S-modulin, so it seems that the structure of S-modulin is not largely disrupted by the mutagenesis. Fig. 2 shows the tryptophan emission spectra of the wild-type and mutant S-modulins. Wild-type (+myr), E85M (+myr) and E121M (+myr) showed almost the same spectrum in the presence of 1 mM Ca$^{2+}$ (Fig. 2, upper panel), but different from wild-type (−myr). This suggests that E86M (+myr) and E121M (+myr) are in fact myristoylated in a similar way to the wild-type (+myr), and that the mutations of glutamic acid to methionine in EF-2 and EF-3 do not significantly change the environment of the three tryptophan residues in the Ca$^{2+}$-free form of these proteins. However, the emission spectra of mutants were different from that of wild-type at a concentration of 0.1 mM Ca$^{2+}$ (Fig. 2, lower panel). The spectrum of the wild-type is red-shifted by an increasing Ca$^{2+}$ concentration (16); that of E85M (+myr) shows a smaller red shift; and that of E121M (+myr) was not affected by Ca$^{2+}$ concentration. The red shift observed in E85M (+myr), which can bind one Ca$^{2+}$ ion per molecule, is probably caused by the binding of Ca$^{2+}$ to EF-3.

Membrane Association of Wild-type, E85M, and E121M S-modulins—Each myristoylated recombinant, (wild-type (+myr), E85M (+myr), or E121M (+myr)) was mixed with urea-stripped ROS membranes at various Ca$^{2+}$ concentrations and separated by centrifugation into membrane and soluble fractions. The soluble fraction (containing proteins free from ROS membranes) and the membrane fractions (containing proteins bound to ROS membranes) were subjected to SDS-polyacrylamide gel electrophoresis. The densities of Coomassie Brilliant Blue-stained bands of the wild-type and mutant S-modulins were analyzed quantitatively, and the ratio of membrane-bound protein, (membrane fraction)/(membrane + soluble fraction), was plotted against Ca$^{2+}$ concentration (Fig. 3). This shows that E85M (+myr) has almost the same membrane affinity as the wild-type. As exposure of the myristoyl group is essential for membrane binding (13, 14), our results suggest that binding of Ca$^{2+}$ to EF-3 induces exposure of the myristoyl group. It has been reported that ejection of the myristoyl group is required for rotation at Gly-42, unclamping of the myristoyl group, and melting of part of the N-terminal helix (21). Ca$^{2+}$...
binding to EF-3 may induce these changes until the level necessary for membrane association.

As E121M (+myr) can not bind Ca$^{2+}$ at concentrations less than 0.1 mM Ca$^{2+}$, it can hardly bind to the ROS membrane at all, although in the presence of 1 mM Ca$^{2+}$, E121M (+myr) does bind slightly. This may be explained by the binding of Ca$^{2+}$ to EF-2 or inactivated EF-3 at very high (more than 1 mM) Ca$^{2+}$ concentration, which is well above normal physiological Ca$^{2+}$ concentrations.

Inhibition of Rhodopsin Phosphorylation by Wild-type or Mutant S-modulins—Fig. 4 shows the incorporation of $^{32}$P-labeled phosphoric acid into rhodopsin in the presence of various concentrations of wild-type (+myr), E85M (+myr), or E121M (+myr) S-modulins. Wild-type (+myr) inhibits rhodopsin phosphorylation at a high (0.1 mM) Ca$^{2+}$ concentration, but neither E85M (+myr) nor E121M (+myr) can inhibit rhodopsin phosphorylation even at high Ca$^{2+}$ concentrations. This suggests that Ca$^{2+}$ binding to EF-2 is important for the inhibitory activity of rhodopsin phosphorylation.

To investigate whether E85M (+myr) can interact with rhodopsin kinase or not, its effect on rhodopsin phosphorylation by wild-type (+myr) was investigated. Fig. 5 shows the incorporation of $^{32}$P-labeled phosphoric acid into rhodopsin plotted against the concentration of E85M (+myr) in the presence of wild-type (+myr). The inhibitory effect of wild-type (+myr) is not affected by E85M (+myr), indicating that E85M (+myr) does not compete with wild-type (+myr) for binding with rhodopsin kinase. This strongly suggests that the Ca$^{2+}$ binding to EF-2 is important for recognition of rhodopsin kinase.

**DISCUSSION**

Ca$^{2+}$ Binding Cooperativity—Ames et al. (23) reported that unmyristoylated recoverin exhibits heterogeneous and uncooperative binding of two Ca$^{2+}$ ions, but these two Ca$^{2+}$ ions bind cooperatively to myristoylated recoverin with a Hill coefficient of 1.75. One explanation of this difference between myristoylated and unmyristoylated recoverins is that the myristoyl group accommodated within the protein moiety may reduce the Ca$^{2+}$ affinity of EF-3 to a level lower than that of the EF-2 of unmyristoylated S-modulin. When Ca$^{2+}$ concentration is high, EF-3 binds Ca$^{2+}$, which causes the myristoyl group to be exposed and EF-2 to be functional, upon which it binds Ca$^{2+}$.

The Hydrophobic Region Exposed by Ca$^{2+}$ Binding—As with myristoylated wild-type (+myr), unmyristoylated wild-type (−myr) binds to a phenyl-Sepharose column in a Ca$^{2+}$-dependent manner. This suggests that a hydrophobic region of the protein moiety, in addition to the myristoyl group, is exposed by binding of Ca$^{2+}$ (13). In our preliminary experiments, both unmyristoylated E85M (−myr) and E121M (−myr) lose affinity for phenyl-Sepharose. We conclude that exposure of the hydrophobic region is probably caused by Ca$^{2+}$ binding at EF-2.

**Structural Changes of S-modulin Induced by Ca$^{2+}$ Binding**—The upper part of Fig. 6 illustrates the conformational changes of S-modulin deduced from our present analysis. The lower part of Fig. 6 represents the corresponding three-dimensional structure of bovine recoverin during Ca$^{2+}$ binding (15, 21, 22). The structure of N-terminal region in the single Ca$^{2+}$-bound form is different from that in the Ca$^{2+}$-free form but similar to the double Ca$^{2+}$-bound form. It is consistent with our model that the conformational change induced by the Ca$^{2+}$ binding to the EF-3 (shown in blue) may expose the N-terminal myristoyl group. The structure of EF-2 (shown in red) in the single Ca$^{2+}$-bound form is also largely different from that in the Ca$^{2+}$-free form. This difference is probably important for EF-2 to be a functional Ca$^{2+}$-binding site.

One of the largest conformational differences is shown at the region corresponding to amino acids from 180 to 186 (shown in green) between single Ca$^{2+}$-bound and double Ca$^{2+}$-bound forms. In the Ca$^{2+}$-free and single Ca$^{2+}$-bound forms, this region appears to form an α-helix, which undergoes a conformational change to a random coil when Ca$^{2+}$ to EF-2. The conformational change of this region may induce an interaction with rhodopsin kinase.

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