Cross-linking of bovine rhodopsin with sulfosuccinimidyl 4-(N1-maleimidomethyl)cyclohexane-1-carboxylate affects its functionality

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Abstract

Rhodopsin is the photoreceptor protein involved in visual excitation in retinal rods. The functionality of bovine rhodopsin was determined following treatment with sulfosuccinimidyl 4-[(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), a bifunctional reagent capable of forming covalent cross-links between suitable placed lysines and cysteines. Denaturing polyacrylamide gel electrophoresis showed that rhodopsin incubated with sulfo-SMCC generated intermolecular dimers, trimers, and higher oligomers, although most of the sulfo-SMCC-treated protein remained as a monomer. Minor alterations on the absorption spectrum of light-activated sulfo-SMCC-treated rhodopsin were observed. However, only about 2% stimulation of the guanine nucleotide binding activity of transducin was measured in the presence of sulfo-SMCC-cross-linked photolyzed rhodopsin. Moreover, rhodopsin kinase was not able of phosphorylating sulfo-SMCC-cross-linked rhodopsin after illumination. Rhodopsin was purified in the presence of either 0.1% or 1% n-dodecyl β-D-maltoside, to obtain dimeric and monomeric forms of the protein, respectively. Interestingly, no generation of the regular F1 and F2 thermolytic fragments was perceived with sulfo-SMCC-cross-linked rhodopsin either in the dimeric or monomeric state, implying the formation of intramolecular connections in the protein that might thwart the light-induced conformational changes required for interaction with transducin and rhodopsin kinase. Structural analysis of the rhodopsin three-dimensional structure suggested that the following lysine and cysteine pairs: Lys<sup>66</sup>/Lys<sup>67</sup> and Cys<sup>316</sup>, Cys<sup>140</sup> and Lys<sup>141</sup>, Cys<sup>140</sup> and Lys<sup>248</sup>, Lys<sup>311</sup> and Cys<sup>316</sup>, and/or Cys<sup>316</sup> and Lys<sup>325</sup> are potential candidates to generate intramolecular cross-links in the protein. Yet, the lack of fragmentation of
sulfo-SMCC-treated Rho with thermolysin is consistent with the formation of cross-linking bridges between Lys$^{66}$/Lys$^{67}$ and Cys$^{316}$, and/or Cys$^{140}$ and Lys$^{248}$. 
Introduction

Rhodopsin (Rho) is a prototypical G protein-coupled receptor (GPCR) that is responsible for dim light vision in retinal rod cells. As other GPCRs, Rho is an integral membrane protein composed of 7 transmembrane-spanning α-helices. Beside the mass that is embedded within the disc membrane, Rho also contains a roughly evenly distributed mass between the intradiscal side and the cytoplasmic or interdiscal side of the membrane. Its N-terminus tail is located on the intradiscal side, and its C-terminus region, which is on the cytoplasmic side of the membrane, features a short amphipathic helix that is parallel to the disc membrane. An 11-cis-retinal chromophore is embedded in the protein hydrophobic region and acts as the light-sensing ligand of the photoreceptor protein. Rho is inactive in the dark, but upon photon absorption, the C₁₁–C₁₂ cis double bond of the chromophore isomerizes to the trans geometry [1], which results in a change of conformation that leads to the formation of the activated receptor state (Rho* or metarhodopsin II). Rho* is now capable of interacting with its associated G protein, transducin (T), and with other downstream proteins of the visual cascade, such as rhodopsin kinase and arrestin-1.

A significant debate prevails about which is the molecular species that is essential for Rho function. On the one hand, many studies have demonstrated that Rho signals light as a monomer [2-5], and monomeric units of Rho have been historically portrayed within the rod outer segment (ROS) disks. Contrarily, a considerable number of reports have revealed that Rho exists as homodimers and higher order oligomers. For instance, Medina et al. [6] found a dimeric quaternary structure for both Rho and Rho*, by using size-exclusion chromatography and
sucrose gradient sedimentation experiments. Imaging of native or illuminated ROS membranes by atomic force microscopy also showed that both Rho and opsin are organized in rows of parallel dimers [7-9]. In addition, Mansoor et al. [10] reported that Rho self-associates in asolectin liposomes, and formed dimers and multimers, by using luminescence resonance energy transfer and fluorescence resonance energy transfer techniques. Moreover, Kota et al. [11] have identified some amino acid residues that appeared to be positioned at the dimeric interface of Rho. Dimeric Rho arrays forming nanodomains have also been reported by cryo-electron tomography of ROS membranes [12], and Zhang et al. [13] have provided in vivo evidence for Rho existing as a dimer. Regardless, the actual organization of Rho within native ROS disks still remains controversial.

Chemical cross-linking procedures has been extensively used for the characterization of inter and intraprotein interactions in protein complexes. In general, a cross-linking reaction involves the formation of covalent bonds between suitable placed amino acid residues. Medina et al. [6] have shown the generation of dimers, trimers and oligomeric forms, when Rho and Rho* were incubated with several bifunctional reagents; however, the formation of these covalently bound polymeric assemblies of Rho was not stoichiometric [6]. Since light activation of Rho causes a change in conformation that allows subsequent protein-protein interactions, we investigated here the effect of the bifunctional agent sulfosuccinimidyl 4-[(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) on the functionality of Rho. More specifically, the aim of this work is to probe three-dimensional proximity relationships between amino acid residues located in the cytoplasmic domain of Rho, and to map intraprotein regions in Rho that participate...
in the light-induced conformational changes required for interaction with other proteins in the signaling cascade. We found that T was not stimulated by sulfo-SMCC-cross-linked Rho under illumination, and rhodopsin kinase did not phosphorylate sulfo-SMCC-treated Rho in the presence of light. In addition to the formation of Rho dimers, trimers and high order multimers, our results also indicated the generation of intramolecular cross-linked connections between properly placed Cys and Lys residues in Rho monomers following treatment with sulfo-SMCC. These findings revealed that the formation of these intraprotein bridges in Rho clearly restrained the light-induced conformational changes required for its interactions with T and rhodopsin kinase, and prevented the transference of signaling information from Rho to downstream proteins in the pathway.

Materials and methods

Materials

Reagents were purchased from the following sources: [8-³H] β,γ-imido-guanosine 5'-triphosphate (GMPpNp, 17.9 Ci/mmol), Amersham Biosciences (GE Healthcare); Opti-Phase HiSafe II (liquid scintillation counting solution), LKB (GE Healthcare); Sephacryl S-300, and concanavalin A-Sepharose 4B, Pharmacia (GE Healthcare); sulfo-SMCC, and alkaline phosphatase (AP)-conjugated secondary antibodies against mouse IgG, Pierce (Thermo Fisher Scientific); Kinase-Glo® luminescent kinase assay reagent, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitro blue tetrazolium (NBT), Promega; n-dodecyl β-D-maltoside (DM), n-octyl β-D-thioglucomoside (OTG), phenylmethylsulfonyl fluoride (PMSF), thermolysin, phenyl isothiocyanate (PITC), N-ethylmaleimide (NEM), and gel filtration molecular
weight protein marker kit, Sigma; diethylaminoethyl cellulose DE52, Whatman; nitrocellulose membranes (0.45 μm), Advantec. The monoclonal antibody 1D4, which is directed against the last COOH-terminal 18 residues of Rho, was kindly provided by Dr. Barry Knox (State University of New York, Syracuse, USA). All other chemicals were of the highest quality grade available.

**Extraction of retinas from bovine eyes and preparation of ROS and dark-depleted ROS membranes**

Bovine eyes were acquired from the nearest slaughterhouse (Beneficiadora Diagon, C.A., Matadero Caracas, Venezuela). Eyes were dissected in the dark to remove the retinas, which were maintained frozen at -70 °C. ROS membranes were isolated from frozen retinas as described by Bubis [14]. Dark-depleted ROS membranes were prepared according to Medina et al. [6]. Both biological samples were stored in the dark at –70 °C.

**Purification of Rho**

Rho was purified according to the extraction procedure reported by Okada et al. [15]. Briefly, dark-depleted ROS membranes were solubilized under dim red light with OTG using a detergent to Rho molar ratio of 350, in the presence of 50 mM Mes (pH 5.0) and 80 mM zinc chloride. Following vigorous agitation for ~ 2 min and incubation for 4 hours at room temperature, samples were centrifuged at 100,000 x g, for 30 min, at 4 °C, and Rho was released in the corresponding supernatants in a highly selective manner.
In some experiments, Rho was extracted from the ROS membranes in the dark with 1% DM in Rho buffer [50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes, pH 6.6), 140 mM NaCl, 20% glycerol, 3 mM MgCl₂, and 2 mM CaCl₂]. The sample was then diluted 10 times with Rho buffer to reduce the concentration of DM to 0.1% and centrifuged at 100,000 x g, for 30 min, at 4 °C. Rho was purified from the resulting supernatant by batchwise affinity chromatography on concanavalin A-Sepharose [16], using DM instead of n-octyl-β-D-glucopyranoside as the detergent. Affinity-purified Rho was employed to raise polyclonal anti-Rho antibodies in ascitic fluid from Balb/C mice.

**Purification of T**

T was isolated from ROS membranes prepared under room light, at 4 °C, following the affinity procedure reported by Kühn [17]. GTP (~ 100 µM) was used to elute T from the washed illuminated ROS membranes, and T was further purified to homogeneity by anion exchange chromatography on a diethylaminoethyl cellulose DE52 column [18,19].

**Preparation of an enriched-fraction of rhodopsin kinase**

A partially purified fraction of rhodopsin kinase was acquired according to Medina et al. [6]. Briefly, freshly isolated ROS were extensively washed in the dark with 100 mM potassium phosphate (pH 6.8), 5 mM magnesium acetate, 5 mM β-mercaptoethanol, and 0.1 mM PMSF. Then, the isotonically washed ROS were hypotonically extracted in the dark with 5 mM Tris-HCl (pH 7.4), 5 mM magnesium...
acetate, 5 mM β-mercaptoethanol, and 0.1 mM PMSF. An enriched fraction of rhodopsin kinase was obtained in the supernatant following centrifugation at 100,000 × g, for 30 min, at 4 °C.

**Cross-linking of Rho with sulfo-SMCC**

Samples of dark-depleted ROS membranes or purified Rho were incubated in the dark with 5 mM sulfo-SMCC for 1 or 2 h, at room temperature or 37 °C, in 10 mM sodium phosphate (pH 7.2), and 5 mM magnesium acetate. As a control, parallel experiments were performed using just the vehicle [10 mM sodium phosphate (pH 7.2), and 5 mM magnesium acetate].

**Blockage of cross-linking with sulfo-SMCC by specific modification of cysteyl and lysyl residues in Rho**

Purified Rho (3 µg) was pre-incubated with 5 mM of either NEM, or PITC, or a combination of both, in 10 mM sodium phosphate (pH 7.2) and 5 mM magnesium acetate for 1 h, at 37 °C, in the dark, and under constant agitation. Then, sulfo-SMCC (5 mM) was added and the reactions were further incubated under identical conditions.

**T activation**

The light-dependent guanine nucleotide binding activity of T was measured by Millipore filtration using [³H] GMPpNp [20,21], in the presence of either Rho or sulfo-SMCC-cross-linked Rho.
Phosphorylation was evaluated using the Kinase-Glo® luminescent kinase kit from Promega, which screens ATP:phosphotransferase activity by quantitating the amount of ATP remaining in solution following a kinase reaction. In a multiwell plate, Rho or sulfo-SMCC-cross-linked Rho samples (~5 µg) were incubated with 50 mM Tris-HCl (pH 7.4), 15 mM MgCl₂, 20 mM KF, and 1 µM ATP, in the presence of a 50-µl aliquot of an enriched fraction of rhodopsin kinase. Following incubation for 30 min at room temperature, under illumination, a volume of Kinase-Glo® reagent equal to the volume of the completed kinase reaction was added, and the luminescence was measured using a Tecan microplate reader.

Preparation of Rho dimers and Rho monomers

Dimeric and monomeric forms of Rho were prepared in the presence of low (0.1% or 1.96 mM) and high (1% or 19.6 mM) concentrations of DM, respectively. Briefly, dark-depleted ROS membrane samples containing ~0.8 mg of Rho were solubilized with 1% DM in Rho buffer. Rho is known to be monomeric at a concentration of 1% DM, while the protein remained in its dimeric state after reducing the concentration of DM to 0.1% [22]. Therefore, the monomeric Rho was acquired by leaving the ROS membranes at 1% DM, whereas the dimeric Rho was attained after diluting the sample 10 times with Rho buffer. Both detergent-treated ROS membrane samples were centrifuged at 100,000 x g, for 30 min, at 4 °C, to obtain the solubilized Rho in the supernatant fraction.

In order to demonstrate the dimeric or monomeric states of Rho, the samples were individually applied to a Sephacryl S-300 size exclusion column.
previously equilibrated with Rho buffer in the presence of either 1% DM (for the monomeric form) or 0.1% DM (for the dimeric form). The separation was performed under dim red light, at 4 °C, and the eluting fractions were simultaneously monitored at 280 nm and 500 nm, and subsequently separated by SDS-PAGE. The elution of Rho was also immunologically monitored by immunoblotting using the anti-Rho monoclonal antibody 1D4. A mixture of protein markers of different molecular weights were employed to calibrate the gel filtration column.

**Digestion of Rho with thermolysin**

Thermolysin cleaves Rho at the C-terminal side of Ser\(^{240}\) and generates two fragments, F1 (~ 25 kDa), from the NH\(_2\)-terminal residue (Asn\(^{2}\)) to Ser\(^{240}\), and F2 (~ 12 kDa), from Ala\(^{241}\) to Pro\(^{327}\) [23]. In addition, two small fragments from the protein carboxyl-terminal remaining region are originated (Leu\(^{328}\) to Lys\(^{339}\) and Thr\(^{340}\) to Ala\(^{348}\)). Rho or sulfo-SMCC-labeled Rho samples, either in its dimeric or monomeric forms, were digested with thermolysin as described [24,25], using a Rho:thermolysin ratio of 5:1 or 20:1 (w/w). Reaction mixtures were analyzed by SDS-PAGE after an overnight incubation (16-20 h), at room temperature, under constant agitation. In some experiments, the proteolytic digestion of Rho and sulfo-SMCC-modified Rho was also followed by western blot using mouse polyclonal anti-Rho antibodies.

**Additional Procedures**

Protein concentration was assayed according to Bradford [26], using bovine serum albumin as protein standard. SDS-PAGE was carried out on 1.5-mm thick slab gels.
containing 10 or 12% polyacrylamide [27]. For Western blot analyses, the proteins were electrotransferred from the gels to nitrocellulose filters [28]. For immunodetection, the filters were incubated either with the monoclonal antibody 1D4 (dilution 1:60,000) or with polyclonal anti-bovine Rho antibodies produced in mice ascitic fluid (dilution 1:5000). The membranes were then treated with AP-conjugated secondary antibodies against mouse IgG, at a dilution of 1:2000, and the immunoreactive bands were visualized with BCIP and NBT. Ultraviolet/visible absorption spectra of Rho and Rho* (or the corresponding sulfo-SMCC-treated samples) were recorded in the dark and after illumination with a 150-watt light source, respectively. The molar extinction coefficient of Rho (40,700 M\(^{-1}\)cm\(^{-1}\) at 500 nm) was employed to determine the concentration of the photoreceptor protein [29]. Molecular distances between amino acid residues were calculated using the UCSF Chimera program, version 1.11.2 (https://www.cgl.ucsf.edu/chimera/) on the three-dimensional crystal structure of inactive bovine Rho (PDB ID = 1U19, https://www.ncbi.nlm.nih.gov/protein/), which has been solved with a resolution of 2.2 Å [30], and has been refined (https://gpcrdb.org/structure/homology_models/1U19_refined).

**Results**

Fig. 1 analyzes untreated Rho and sulfo-SMCC-treated Rho by SDS-PAGE. As previously reported [6], dimers, trimers and oligomeric forms of Rho were generated when the purified photoreceptor protein was incubated with sulfo-SMCC, showing some predominance for the formation of cross-linked Rho dimers (Fig. 1). However, an incomplete formation of cross-linked products was generally
observed, and plenty of the monomeric form of Rho still remained in the sample. Immunostaining using 1D4 monoclonal antibodies confirmed the generation of dimers, trimers and oligomeric forms of Rho, in addition to the Rho monomeric species (data not shown).

Both Rho and sulfo-SMCC-cross-linked Rho showed the typical absorption spectrum in the dark with a maximum peak at 500 nm (Fig. 2). Following illumination for 7 min, at room temperature, this band was shifted to 380 nm that corresponded to the metarhodopsin II activated state (Fig. 2). Therefore, the ultraviolet/visible absorption spectra of sulfo-SMCC-modified Rho (dark state) and sulfo-SMCC-modified Rho* (light-activated state) were essentially identical to those of the control untreated sample. However, in contrast to untreated Rho that revealed an isosbestic point at ~ 425 nm between the dark and light-activated species, an altered isosbestic point was observed for the sulfo-SMCC-cross-linked Rho sample (Fig. 2), which appears to indicate that more than two species were in equilibrium after photolysis in the sample modified with the bifunctional reagent.

With the intention of tracking spectral modifications in the cross-linked sample, the absorption spectrum of sulfo-SMCC-treated Rho was measured at 4 °C using a spectrophotometer that was refrigerated with an in-line cooling circulating water bath. As shown in Fig. 3, an altered spectrum was obtained for sulfo-SMCC-cross-linked Rho*, at 4 °C, and after 1 min of illumination. Instead of a peak absorbing at 380 nm, a photointermediate species absorbing at about 470 nm was detected (Fig. 3), with a concomitant reduction of the dark species that absorbs at 500 nm. Apparently, the incorporation of the covalently bound cross-linking reagent seems
to hinder the light-induced conformational change in the photoreceptor protein, probably stabilizing an inactive photointermediate.

Given that sulfo-SMCC is capable of forming bridges between suitable located Cys and Lys residues, purified Rho samples were pre-incubated either with NEM to modify cysteines, or with PITC to modify lysines, or with both compounds, in order to determine whether these site-specific compounds hindered the formation of cross-linking bridges in the photoreceptor protein. No formation of oligomeric species was observed when these site-specific labeling reagents were employed (Fig. 1), demonstrating that both NEM and PITC, either individually or in combination, protected against the formation of sulfo-SMCC-dependent cross-linked products in Rho. As described above, the formation of cross-linked oligomeric species of Rho was not stoichiometric, and most of the sulfo-SMCC-treated protein remained as a monomer. This result suggested that the bifunctional agent might also be affecting the monomeric state of the protein by either modifying individual cysteyl or lysyl residues, without the formation of linking bridges, or by generating intramolecular cross-links between suitable located Cys and Lys in each Rho monomer. Interestingly, the sulfo-SMCC-cross-linked Rho monomeric band slightly ran at a lower migration distance than the untreated Rho band, which implied that the higher size was probably due to the modification of individual cysteyl or lysyl residues in the protein (Fig. 1).

The ability of sulfo-SMCC-cross-linked Rho* to stimulate the guanine nucleotide binding activity of T was assayed as shown in Fig. 4A. $[^3H] \text{GMPpNp}$ binding by bovine T was measured after light-dependent activation of either untreated or sulfo-SMCC-treated Rho. As anticipated, Rho* was capable of
activating T (Fig. 4A). However, only about 2% stimulation of the guanine nucleotide binding activity of T was detected in the presence of sulfo-SMCC-cross-linked Rho* (Fig. 4A). These results clearly showed that treatment with sulfo-SMCC affected the light-dependent capability of the photoreceptor protein to activate T.

The C-terminal tail of Rho possesses a cluster of Ser and Thr residues that are exposed following illumination, and can be rapidly phosphorylated by rhodopsin kinase [31,32]. The ability of sulfo-SMCC-cross-linked Rho* to become phosphorylated by rhodopsin kinase was measured in Fig. 4B. As expected, this specific protein kinase was able of phosphorylating untreated Rho*. However, rhodopsin kinase was not capable of phosphorylating sulfo-SMCC-cross-linked Rho* (Fig. 4B), indicating that the modified photoreceptor protein did not serve as a substrate for this kinase.

Since Rho can be internally cleaved by thermolysin into two fragments known as F1 and F2, sulfo-SMCC-treated Rho was incubated with this protease in order to evaluate whether the Rho digestion pattern was maintained or altered in the cross-linked sample. SDS-PAGE analyses revealed the formation of the F1 and F2 fragments in the untreated Rho sample (Fig. 5A). However, no fragmentation of the cross-linked sample was acquired after incubation with thermolysin since a polypeptide band that migrated with an apparent molecular mass of about 35 kDa was obtained (Fig. 5A). An additional experiment was carried out with the purpose of determining whether sulfo-SMCC was capable of covalently linked the F1 and F2 digestion fragments, and form an artificially reconstituted product holding a similar apparent size than Rho. As seen in Fig. 5A,
thermolysin-digested Rho was not capable of joining back the F1 and F2 pieces following sulfo-SMCC treatment. Immunoblotting using polyclonal anti-Rho antibodies also showed that the control sample of untreated Rho was digested, as a F1 fragment of ~25 kDa was generated (Fig. 5B). In contrast, almost no limited proteolysis with thermolysin was perceived in the sulfo-SMCC-cross-linked Rho sample (Fig. 5B). No F2 fragment was observed because it accidentally ran out of the gel as it co-migrates with the dye front. The monomeric and dimeric Rho bands (R1 and R2) migrated faster in the thermolysin-treated samples than in the untreated control samples, given that small fragments from the protein carboxyl-terminal region can be generated following incubation with the proteolytic enzyme. It is known that up to 21 amino acids from the Rho C-terminal tail can be cleaved following thermolysin treatment [23].

Rho was prepared from dark-depleted ROS membranes in the presence of either 0.1% DM or 1% DM to obtain dimeric and monomeric states of the photoreceptor protein, respectively [22]. Size-exclusion chromatography of both samples of DM-solubilized Rho on a Sephacryl S-300 column resulted in the elution of sharp peaks absorbing at 500 nm that overlapped with single peaks absorbing at 280 nm (data not shown). The Rho-containing fractions from both separations were verified by SDS-PAGE and immunoblotting employing the 1D4 anti-Rho monoclonal antibody (data not shown). Since the Sephacryl S-300 column was calibrated with a mixture of protein standards of known molecular weights, the corresponding sizes of the samples of Rho solubilized with either 0.1% or 1% DM were estimated (Fig. 6). The sizes of the Rho-DM complexes were empirically determined to be 126,254 Da and 87,815 Da, when the
chromatography was carried out in the presence of 0.1% DM or 1% DM, respectively. Since the molecular weight of DM micelles has been calculated to be ~ 50,000 [33], the molecular masses of Rho prepared in 0.1% DM and Rho prepared in 1% DM were assessed by subtracting 50,000 from the total size of the protein-detergent complexes, yielding 76,254 Da and 37,815 Da, respectively (about 76 kDa and 38 kDa). Therefore, the presence of 1% DM clearly dissociated the dimers of Rho into monomers, and the dimeric and monomeric states of both DM-solubilized Rho samples were verified by gel filtration chromatography.

In an attempt to assess whether intramolecular cross-links can be generated following incubation of Rho with sulfo-SMCC, samples of unmodified Rho dimers and monomers were first incubated with sulfo-SMCC followed by digestion with thermolysin. Unlike untreated monomeric or dimeric Rho, which was clearly digested after incubation with thermolysin (Fig. 7), no limited proteolysis with thermolysin was detected in the sulfo-SMCC-cross-linked monomeric or dimeric Rho samples (Fig. 7). These results demonstrated the presence of intramolecular cross-linking bridges in the sulfo-SMCC-treated Rho samples, which may either hinder the availability of the C-terminal side of Ser^{240} that is the target site for the proteolytic enzyme, or may covalently link the digested fragments even if the protein was cleaved after reaction with thermolysin.

Discussion

Although the generation of cross-linked oligomeric species of Rho was not stoichiometric, the incubation of Rho with sulfo-SMCC affected its functionality and hindered its interactions with T and rhodopsin kinase. Obviously, the formation of
Rho dimers, trimers and higher oligomers following sulfo-SMCC incubation do not account for the functional inactivation of the protein. Then, it is plausible that the bifunctional agent was also capable of affecting the monomeric state of the protein by either modifying individual cysteiny or lysyl residues, or by forming intramolecular bridges between suitable placed Cys and Lys in the Rho monomers. Our results actually revealed that the band corresponding to the monomeric sulfo-SMCC-cross-linked Rho ran at a lower migration distance than the untreated Rho band following separation by SDS-PAGE, implying that some modification of individual cysteiny or lysyl residues in the protein was occurring. However, we have previously shown that chemical labeling of Cys [19,29] or Lys residues [34] of Rho with site-specific reagents did not affect the functionality of the photoreceptor protein, given that the modified protein was capable of interacting with T and induce its light-dependent exchange of guanine nucleotides. Therefore, the effects of sulfo-SMCC on the functionality of Rho cannot be rationalized just by the individual labeling of cysteiny and lysyl residues, but by the formation of intramolecular bridges between appropriate Cys and Lys in the Rho monomers.

We demonstrated that sulfo-SMCC-dependent intramolecular cross-linking of Rho thwarted its light-induced interactions with downstream proteins in the phototransduction cascade. Albeit no clear effect on the absorption spectra of Rho and sulfo-SMCC-modified Rho was obtained either in the dark or upon illumination, some alterations were evident when the time course of formation of the light-activated state of the protein was measured. Focusing on the isosbestic point, it was apparent that more than two species were in equilibrium in the photolyzed sulfo-SMCC-cross-linked Rho sample. Moreover, when the absorption spectrum of
sulfo-SMCC-treated Rho was measured at 4 °C, a peak absorbing at ~ 470 nm was revealed, suggesting that a Rho photointermediate different than metarhodopsin II was stabilized following cross-linking. It is well-known that after illumination, the 11-cis-retinal chromophore of Rho is isomerized to its all-trans configuration [1]. This process leads to the generation of high-energy Rho photointermediates that are distinguished by their maximum wavelength of absorption [photorhodopsin (570 nm), bathorhodopsin (543 nm), blue shifted intermediate or BSI (477 nm), lumirhodopsin (497 nm), metarhodopsin I (478 nm), and metarhodopsin II (380 nm)] [35-38]. The metarhodopsin II species, which corresponds to the activated form of the receptor, decays into opsin and free all-trans-retinal on a timescale of minutes, and the release of retinal from its binding site, inactivates the receptor. An alternative pathway of receptor deactivation is provided by the formation of metarhodopsin III, another inactive photoproduct with intact retinal protonated Schiff base that absorbs at 465-470 nm [39,40]. Metarhodopsin III eventually decays to opsin and all-trans-retinal but on a timescale of hours. It is conceivable that the ~ 470 nm-intermediate obtained for the sulfo-SMCC-treated Rho* sample might correspond to one of the regular inactive Rho photointermediate precursors, which could be chemically stabilized following cross-linking with the bifunctional reagent, perhaps BSI (477 nm) or metarhodopsin I (480 nm). Alternatively, cross-linking of Rho with sulfo-SMCC might facilitate the formation of the inactive metarhodopsin III (465-470 nm), which regularly is generated after the decay of the activated metarhodopsin II state. Stabilization by cross-linking of any of these three photoproducts could easily explain the effect of the modification by sulfo-SMCC on the functionality of Rho. In
spite of this, we also showed that the ~ 470 nm-intermediate did not remain stationary, but progressed toward the formation of the metarhodopsin II activated photointermediate. Therefore, the generation of intramolecular bridges in Rho does not seem to prevent its light-induced conformational change required for activation. Instead, it appears that by stopping the helix movements responsible of causing the conformational changes in the cytoplasmic face of the activated protein, light-activated sulfo-SMCC treated Rho is not capable of interacting with downstream proteins of the visual cascade, such as T and rhodopsin kinase. Although in vivo experiments were not performed, our in vitro findings can be extended or extrapolated to what occurs in the native cellular environment given that the signaling steps in phototransduction have been clearly established.

Choe et al. [41] have reported the crystal structure of metarhodopsin II (PDB ID: 3PXO, https://www.ncbi.nlm.nih.gov/protein/), and showed that the elongation of retinal after its isomerization to the all-trans configuration, and the interaction of its β-ionone ring moiety with transmembrane helices TM5 and TM6 induced a rigid body-type rotational tilt of TM6 and motion of TM5. Plausibly, sulfo-SMCC is connecting amino acid residues that are located in or in the vicinity of these helices in the inactive state of Rho, hindering in turn the light-dependent conformational transitions of the photoreceptor protein. Interestingly, limited digestion with thermolysin of sulfo-SMCC-cross-linked Rho did not generate the regular fragmentation of the protein into its F1 and F2 pieces, because a polypeptide with an apparent molecular weight of about 35,000 was obtained by SDS-PAGE, which is very similar to the size of the monomeric form of Rho. No generation of thermolytic F1 and F2 Rho fragments was attained under conditions that
maintained the dimeric and monomeric forms of sulfo-SMCC-treated Rho by changing the concentrations of DM [22]. These findings clearly demonstrated that the incubation of Rho with sulfo-SMCC produced not only intermolecular but also intramolecular cross-linked bridges between Cys and Lys residues located at the right distances in the protein monomer. Perhaps, the sulfo-SMCC-dependent formation of intramolecular cross-linking bridges in Rho may protect against thermolysin digestion just by steric hindrance or structural obstruction. On the other hand, even if the fragments were generated after treatment with the proteolytic enzyme, the thermolytic F1 and F2 polypeptide pieces may remain covalently fused via intramolecular cross-linking bridges created by the bifunctional agent. If that is the case, the intramolecular links require to be formed between cysteyl and lysyl residues located at each site of the thermolysin cutting point in Rho, which is the C-terminal side of Ser^{240}.

Sulfo-SMCC is a bifunctional compound with a length of 11.6 Å, and a spacer arm of 8.3 Å. In an attempt to identify candidates for cysteyl and lysyl residues’ pairing preferences, bioinformatics screening on the crystal structure of bovine Rho was employed to examine the molecular distances between all 10 Cys and 11 Lys contained in the protein. We specifically used a crystal structure of Rho that was solved with a resolution of 2.2 Å [30]. This structure was identified with the PDB ID: 1U19 (https://www.ncbi.nlm.nih.gov/protein/), and has been refined (https://gpcrdb.org/structure/homology_models/1U19_refined). It is known that Cys^{140} and Cys^{316} are located at the cytoplasmic face of Rho and contain the most chemically reactive sulphhydryl groups to site-specific modification reagents [42,43]. Moreover, Cys^{316} appears to be more responsive than Cys^{140} [44-46]. Cys^{140} is
located at the end of helix TM3 and/or beginning of the C2 intracellular loop, and

Cys$^{316}$ is at the middle of the amphipathic helix VIII that follows helix TM7. The rest

of the protein thiol groups are either involved in disulfide bonding (Cys$^{110}$ and

Cys$^{187}$), or modified by palmitoylation (Cys$^{322}$ and Cys$^{323}$), or structurally hindered

(Cys$^{167}$, Cys$^{185}$, Cys$^{222}$, and Cys$^{264}$), and as such they are not accessible to sulfo-

SMCC modification or cross-linking. It is also known that the 11-cis-retinal

cromophore is covalently attached to the protein by means of a protonated Schiff

base to the ε-NH$_2$ group of Lys$^{296}$ in helix TM7. For that reason Lys$^{296}$ is not

available either for modification or cross-linking with the bifunctional agent. On the

basis of the three-dimensional structure of Rho, several intraprotein cross-linking

connections can then be envisioned using these two reactive cysteines and various

lysine residues that are positioned at distances approximately comparable to the

size of the sulfo-SMCC compound in order to allow its insertion. The distances

were measured from the sulfur of the thiol group of the cysteines’ side chain to the

nitrogen of the ε-NH$_2$ group of the lysines’ side chain. For example, pairing of

Cys$^{140}$ and Lys$^{141}$, Lys$^{311}$ and Cys$^{316}$ and/or Cys$^{316}$ and Lys$^{245}$ are all possible given

that these residues are placed at distances of 11.9 Å, 12.1 Å and 10.1 Å apart,

respectively, which are suitable for the formation of Rho intraprotein cross-linking

bridges following treatment with sulfo-SMCC (Fig. 8). However, neither of these

cross-links will explain the apparent absence of formation of the usual thermolytic

F1 and F2 fragments in the sulfo-SMCC-treated protein. On the other hand, Cys$^{316}$

and two lysines of the C1 cytoplasmic loop, Lys$^{66}$ and Lys$^{67}$, are placed at

distances of 11.1 Å and 9.8 Å apart, respectively; and Cys$^{140}$ and Lys$^{248}$, a lysyl

residue that is positioned at the beginning of helix TM6, are separated by a
distance of 12 Å (Fig. 9). In all these cases, the separating distances between the
candidate amino acid residues are very close to the cross-linker’s length of 11.6 Å,
which suggests that sulfo-SMCC can fit and be easily inserted. Interestingly, the
thermolysin cleaving site (carboxylic side of Ser\textsuperscript{240}) is also contained within the
region that ligates these last pairs of potential targeted residues. Hence, if these
residues are covalently connected intramolecularly by sulfo-SMCC-mediated
cross-linking bonds, the F1 and F2 fragments will not be separated even if they are
generated after hydrolysis with the thermolysin protease. As said before, another
possible option is that the intramolecular cross-linking bridge formed in Rho after
sulfo-SMCC treatment may protect the protein against digestion by obstructing the
accessibility of the protease to the C-terminal side of the Ser\textsuperscript{240} residue that is its
target site. Therefore, the pairing of these Lys and Cys residues, i.e. Lys\textsuperscript{66}/Lys\textsuperscript{67}
and Cys\textsuperscript{316}, and Cys\textsuperscript{140} and Lys\textsuperscript{248}, are the best candidates that might participate in
the formation of sulfo-SMCC intraprotein cross-links in Rho. A model summarizing
all these findings is shown in Fig. 10. Several of the postulated cross-linking
connections can be formed simultaneously.

Consistently, amino acid residue 65, which is adjacent and vicinal to Lys\textsuperscript{66}
and Lys\textsuperscript{67} and is located near the cytoplasmic end of helix TM1, has been shown to
be in close proximity to Cys\textsuperscript{316} in the dark inactive state of Rho [47]. Specifically,
Yang et al. [47] generated a mutant protein that contained the naturally occurring
Cys\textsuperscript{316}, a replacement of the second reactive cysteine at position 140 for serine
(Cys\textsuperscript{140}/Ser), and a substitution that incorporated a new cysteine at position 65
(His\textsuperscript{65}/Cys). Under appropriate conditions, they found that the mutated Cys\textsuperscript{65} and
the native Cys\textsuperscript{316} were disulfide bonded [47]. Klein-Seetharaman et al. [48] also
focused on determining the proximity between Cys$^{316}$ and engineered cysteines at amino acid positions 55-75 in the C1 cytoplasmic loop connecting helices TM1 and TM2. A rapid spontaneous formation of disulfide bonds was observed between Cys$^{316}$ and engineered cysteines at positions 65 and 68, which are very close to Lys$^{66}$ and Lys$^{67}$, the two lysyl residues that have been suggested here as potential targets for cross-linking with Cys$^{316}$ following incubation with sulfo-SMCC.

However, Cai et al. [49] reported that the formation of disulfide bonds between Cys$^{65}$ and Cys$^{316}$ did not affect neither T activation nor the phosphorylation of Rho by rhodopsin kinase, which implied that the relative motion between the regions containing both of these residues appeared to be not essential for recognition of T and rhodopsin kinase.

In order to probe the proximity of the residues that are located at the cytoplasmic ends of Rho helices TM3 and TM6, Farrens et al. [50] used a rhodopsin mutant in which the native cysteines at position 140, 316, 322 and 323 were replaced by serine, and generated double substitution mutants that contained one cysteine at position 139 (Val$^{139}$Cys) that is located adjacent to Cys$^{140}$ at the end of helix TM3, and engineered cysteines at amino acid positions 247-252 in the cytoplasmic end of helix TM6. This region includes Lys$^{248}$, the lysyl residue that has been suggested here as a potential target to form a cross-link with Cys$^{140}$ following incubation with sulfo-SMCC. These Rho double mutants were then incubated with copper phenanthroline as an oxidizing reagent to induce the formation of disulfide cross-links. Disulfide bridges were formed by all double mutants except for the Cys$^{139}$/Cys$^{252}$ mutant protein [50], demonstrating the proximity between both of these regions. Moreover, all double mutants that formed...
disulfide cross-links did not activate T, suggesting a functional importance for the
movement of helix TM6 [50]. Interestingly, Cai et al. [49] also showed that disulfide
bonds between Cys\textsuperscript{139} and Cys\textsuperscript{248} and between Cys\textsuperscript{139} and Cys\textsuperscript{250} abolished both T
activation and phosphorylation by rhodopsin kinase, which is comparable to what
we found here following sulfo-SMCC treatment of Rho. On the basis of the findings
of Choe et al. [41] that revealed conformational changes in metarhodopsin II that
involved the protein helix TM6 where Lys\textsuperscript{248} is located, and since i) residue 139 is
contiguous to Cys\textsuperscript{140}, ii) the mutated Cys\textsuperscript{248} was replacing Lys\textsuperscript{248} in the double
mutant, and iii) residue 250 is vicinal to Lys\textsuperscript{248}, one may speculate that an
intramolecular cross-linking bridge between Cys\textsuperscript{140} and Lys\textsuperscript{248} after sulfo-SMCC
treatment is probably the best Lys/Cys pair capable of causing all the effects seen
here on the functionality of the photoreceptor protein.

Recently, a cryogenic electron microscopy structure of a fully functional
light-activated Rho-T complex was solved in the absence or presence of a G
protein-stabilizing nanobody [51] (PDB IDs: 6OY9 and 6OYA, respectively,
https://www.ncbi.nlm.nih.gov/protein/). These structures revealed that light-
activated Rho forms extensive contacts with T through its helix TM3, C2
cytoplasmic loop, helix TM5, helix TM6, and amphipathic helix VIII. As mentioned
before, Cys\textsuperscript{140} is located at the end of helix TM3 and/or beginning of the C2
intracellular or cytoplasmic loop, and Lys\textsuperscript{248} is located at the beginning of helix TM6
(near the cytoplasmic end of helix TM6). Therefore, it is easy to imagine that
bridging these two residues by forming covalent bonds with sulfo-SMCC would
hamper the light-dependent conformational changes in the cytoplasmic face of Rho
that are required to have a productive interaction with T. No structural interpretation
of our data regarding the lack of interaction between sulfo-SMCC-cross-linked Rho
and rhodopsin kinase can be made yet, given that no structure of a complex
between light-activated Rho and rhodopsin kinase has been solved to date.

Besides mapping intramolecular interfaces in proteins, cross-linking studies
have other applications in the field of protein research. Basically, bifunctional
reagents are useful tools to investigate protein-protein interactions, i.e. specific
physical contacts established between two or more protein components. Protein-
protein interactions are essential to the generation of protein networks and
multimolecular complexes, which are responsible of most physiological processes
within a cell. These interactions can be either strong (high affinity) or transient (low
affinity), but both kinds can be trapped by using chemical cross-linkers. Studying
multimeric protein assemblies by means of traditional structural methods, such as
X-ray crystallography, nuclear magnetic resonance, or electron microscopy
techniques can be challenging, due to their size, heterogeneity, or resistance to
crystallization. Since structural constrains that depend on the length of the cross-
linker are incorporated into the interaction partners, a measure of the proximity of
the participating amino acid residues can be inferred, offering in turn information on
the folds of proteins and the topology of their complexes. Therefore, cross-linking
experiments can provide structural insights and useful low resolution information
on proteins and protein complexes. Moreover, cross-linking experiments can
actually be carried out without disrupting the regular subcellular environment where
the interactions occur. In addition, chemical cross-linking coupled with mass
spectrometry has emerged as a valuable strategy to improve the study of protein-
protein interactome networks in biological organisms and identify proximal amino
acid residues within proteins and protein complexes [52-56]. When using this combined methodology, proteins are first reacted with the bifunctional cross-linking agent, for example sulfo-SMCC, which physically tether spatially proximal amino acid residues through the formation of covalent bonds. The cross-linked proteins are then enzymatically digested with specific proteases, and the resulting peptide mixtures are separated and analyzed via liquid chromatography with tandem mass spectrometry. Subsequent database searching identifies cross-linked peptides and their linkage sites. In future investigations, it would be worthwhile to use chemical cross-linking combined with mass spectrometry to precisely identify the cross-linking sites in sulfo-SMCC-treated Rho.

In conclusion, the work presented here provides direct evidence that the cross-linking of Rho with sulfo-SMCC affects its functionality. On the one hand, the interaction between Rho and T was hindered when the cross-linked sample was employed. Likewise, rhodopsin kinase was not able of phosphorylating sulfo-SMCC-treated Rho after illumination, indicating that the modified protein was not an appropriate substrate for this kinase. Limited digestion with thermolysin of sulfo-SMCC-cross-linked Rho revealed the formation of intramolecular bridges in the photoreceptor protein that appeared to prevent the light-induced conformational changes at its cytosolic side that are required for interaction with both T and rhodopsin kinase. Finally, bioinformatics analysis of the Rho three-dimensional structure mapped potential lysines and cysteines that are properly located to form intramolecular covalent cross-links in the protein.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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**Author contributions**

R.M., D.P., C.M., and J.B. performed research and analyzed data. J.B. designed research and wrote the paper. All authors read and approved the final manuscript.

**Abbreviations:** Rhodopsin (Rho), activated Rho (Rho*), G protein-coupled receptor (GPCR), rod outer segment (ROS), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), transducin (T), alkaline phosphatase (AP), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), n-dodecyl β-D-maltoside (DM), n-octyl β-D-thioglucoside (OTG), phenylmethylsulfonyl fluoride (PMSF), β,γ-imido-guanosine 5′-triphosphate (GMPpNp), N-ethylmaleimide (NEM), phenyl isothiocyanate (PITC), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), 2-(N-morpholino)ethanesulfonic acid (Mes), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), nonylphenoxy polyethoxylethanol (NP-40), blue shifted intermediate (BSI).
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Figure captions

Figure 1. Protection against the sulfo-SMCC-dependent cross-linking of Rho by pre-incubation with specific cysteine and lysine modification reagents.

Rho was purified from bovine ROS membranes using a combination of OTG (molar ratio of OTG/Rho = 350) and 80 mM zinc chloride [15]. Following pre-incubation of Rho (3 µg) with 5 mM of either NEM (Pre-NEM), or PITC (Pre-PITC), or a combination of both (Pre-NEM & PITC), samples were treated with 5 mM sulfo-SMCC. Formation of Rho cross-links was determined by SDS-PAGE, and the gel was silver stained. A sample of Rho treated only with 5 mM sulfo-SMCC is shown in the first lane. Samples of purified Rho (Control) and Rho incubated with the vehicle [Control (vehicle)] were also included as controls. Incubation in the absence or presence of sulfo-SMCC is illustrated by the – and + signs, respectively. All experiments were performed in the dark. R1, R2, R3, and Rn = monomeric, dimeric, trimeric, and higher oligomeric species of Rho.

Figure 2. Ultraviolet/visible absorption spectra of Rho and sulfo-SMCC-cross-linked Rho at various illumination times.

Aliquots (50 µl) of dark-depleted ROS membranes containing 7.4 mg/ml of Rho were incubated in the absence (A) or presence (B) of 5 mM sulfo-SMCC in 10 mM sodium phosphate (pH 7.2) and 5 mM magnesium acetate for 1 h, at room temperature, in the dark. Reactions were terminated by using 50 mM dithiothreitol, and the modified dark-depleted ROS membrane were washed with the same buffer to eliminate the excess of free reagents. Following solubilization by vigorous agitation with 12.8 mM DM, 23.2 NP-40 and 1.5 M Genapol® X-100, the final
Volumes of the reaction mixtures were diluted 10 times with a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM magnesium acetate and 5 mM β-mercaptoethanol. Absorption spectra (250-650 nm) were determined in the dark (0) and after different illumination times (10'', 1', 2', 3', 5' and 7').

**Figure 3. Ultraviolet/visible absorption spectra of sulfo-SMCC-treated Rho at low temperature.**

A sample of dark-depleted ROS membranes was incubated in the dark in the absence (A) or presence (B) of with 5 mM sulfo-SMCC in a buffer containing 10 mM sodium phosphate (pH 7.2) and 5 mM magnesium acetate for 2 h, at 37 ºC, under constant agitation. The reaction was terminated by using 10 mM dithiothreitol. After eliminating excess of free reagents by washing three times the labeled dark-depleted ROS membranes with the same buffer, Rho was selectively solubilized using a combination of OTG (molar ratio of OTG/Rho = 350) and 80 mM zinc chloride [15]. Absorption spectra were measured at 4 ºC using a spectrophotometer that was refrigerated with an in-line cooling circulating water bath. Shown are spectra for sulfo-SMCC-cross-linked Rho in the dark (Dark) and after 1 min of illumination (Light).

**Figure 4. Cross-linking of Rho with sulfo-SMCC affects its functionality.**

A, Determination of the [³H] GMPpNp binding activity of purified bovine T after light-dependent activation of either untreated (Rho) or sulfo-SMCC-cross-linked Rho (Rho + sulfo-SMCC). Sulfo-SMCC-cross-linked Rho was prepared as described in Fig. 4, and Rho was prepared in a similar way but in the absence of...
the bifunctional reagent. B, Determination of the phosphorylation by rhodopsin kinase of Rho (Rho + RhoK) or sulfo-SMCC-cross-linked Rho (Rho + sulfo-SMCC + RhoK) by using the Kinase-Glo® kit from Promega. Dark-depleted ROS membranes were incubated in the absence or presence of 5 mM sulfo-SMCC, and the reactions were terminated by adding 10 mM dithiothreitol. Rho and sulfo-SMCC-cross-linked Rho were affinity-purified using chromatography on concanavalin A-Sepharose [16] in the presence of 0.1% DM. Assays using only rhodopsin kinase (RhoK) or just buffer (Control) were included as controls. An enriched fraction of bovine rhodopsin kinase was employed as the source of the kinase enzyme. RLU = relative light or luminescence units.

Figure 5. Treatment of Rho and sulfo-SMCC-cross-linked Rho with thermolysin.

Evaluation by SDS-PAGE (A) and western blot (B) of the proteolytic products generated after incubating Rho (Rho) or sulfo-SMCC-cross-linked Rho (Rho + sulfo-SMCC) overnight with thermolysin (+), using a Rho:thermolysin ratio of 20:1 (w/w). An additional experiment was performed in which Rho was digested first with thermolysin and then incubated with sulfo-SMCC (Digested Rho + sulfo-SMCC). Control experiments of Rho and sulfo-SMCC-treated Rho in the absence of thermolysin (-) were also included. The gel in A was stained with Coomassie blue R-250, and the blot in B was revealed using mouse polyclonal anti-Rho antibodies. R1, R2, and Rn = monomeric, dimeric, and higher multimeric species of Rho. F1 and F2 = fragments generated in Rho by limited digestion with thermolysin. M = molecular weight markers.
Figure 6. Gel filtration chromatography of Rho prepared in the presence of 0.1% DM and 1% DM.

Rho was solubilized from dark-depleted ROS membranes in the presence of either 0.1% DM or 1% DM. Each sample was individually applied to a Sephacryl S-300 size exclusion column previously equilibrated in the presence of either 0.1% DM or 1% DM. Separations were performed under dim red light, at 4 °C, and the eluting fractions were simultaneously monitored at 280 and 500 nm, and subsequently separated by SDS-PAGE. The elution of Rho was also monitored by western blot using the anti-Rho monoclonal antibody 1D4. The calibration curve for the Sephacryl S-300 column was established with β-amylase (β-Am, 200 kDa), alcohol dehydrogenase (ADH, 150 kDa), dimeric bovine serum albumin (Dim BSA, 134 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (Ov, 43kDa), chymotrypsinogen A (Chym, 25 kDa), myoglobin (Myo, 17 kDa), ribonuclease A (RNase, 13.7 kDa), and cytochrome C (Cyt C, 12.3 kDa). Shown is the plot of the partition coefficient Kav value of each standard versus the logarithm of its molecular weight (Log M). Represented are the averages of the Kav values ± the standard deviation obtained for each protein marker under both conditions. Dim Rho = dimeric Rho; Mon Rho = monomeric Rho.

Figure 7. Treatment of dimeric and monomeric Rho and sulfo-SMCC-cross-linked Rho with thermolysin.

SDS-PAGE analyses of dimeric Rho (Dim Rho, A) and monomeric Rho (Mon Rho, B), either untreated (Rho) or cross-linked with sulfo-SMCC (Rho + sulfo-SMCC),
that have been incubated overnight with thermolysin (+) using a Rho:thermolysin ratio of 5:1 (w/w). Control experiments of Rho and sulfo-SMCC-treated Rho in the absence of thermolysin (-) were also included. R1, R2, and Rn = monomeric, dimeric, and higher oligomeric species of Rho. F1 and F2 = fragments generated in Rho by limited digestion with thermolysin. M = molecular weight markers.

**Figure 8. Potential intraprotein cross-linking connections between Cys\textsuperscript{140} and Lys\textsuperscript{141}, Lys\textsuperscript{311} and Cys\textsuperscript{316}, and Cys\textsuperscript{316} and Lys\textsuperscript{325} in Rho.**

Based on the three-dimensional structure of inactive Rho, cross-linking bridges between Cys\textsuperscript{140} and Lys\textsuperscript{141} (A), Lys\textsuperscript{311} and Cys\textsuperscript{316} (B), and Cys\textsuperscript{316} and Lys\textsuperscript{325} (C) can be hypothesized after sulfo-SMCC treatment, given that these residues are placed at distances of 11.9 Å, 12.1 Å and 10.1 Å apart, respectively. Top, ribbon diagrams showing the location of the crucial residues. Bottom, zoom of the demarcated area containing the various Lys/Cys pairs. Highlighted are the amino acids that are prospectively involved in sulfo-SMCC-mediated cross-linking.

**Figure 9. Potential intramolecular cross-linking bridges between Lys\textsuperscript{66}/Lys\textsuperscript{67} and Cys\textsuperscript{316}, and Cys\textsuperscript{140} and Lys\textsuperscript{248} in Rho.**

On the basis of the three-dimensional structure of inactive Rho, cross-linking bridges between Lys\textsuperscript{66} and Cys\textsuperscript{316} (A), Lys\textsuperscript{67} and Cys\textsuperscript{316} (B), and Cys\textsuperscript{140} and Lys\textsuperscript{248} (C) can be predicted following incubation with sulfo-SMCC since these residues are placed at distances of 11.1 Å, 9.8 Å, and 12 Å apart, respectively. Top, ribbon diagrams showing the location of the crucial residues. Bottom, zoom of the
demarcated area containing the various Lys/Cys pairs. Highlighted are the residues that potentially participate in the formation of cross-links.

Figure 10. A, Two-dimensional model of Rho illustrating amino acids that can be intramolecularly cross-linked by sulfo-SMCC, and are flanking the internal thermolysin cleavage site (Ser^{240}).

The diagram is a representation of the secondary structure of Rho. The amino terminal tail and the intradiscal side are toward the top, and the carboxyl-terminal tail and cytoplasmic (or interdiscal) side are toward the bottom. The transmembrane α-helices TM1 to TM7 (1-7), the cytosolic connecting loops, the intradiscal connecting loops, and amphipathic helix HVIII (VIII) are also shown. Lys^{66}, Lys^{67}, Cys^{140}, Lys^{248}, and Cys^{316} are mapped onto the model using the single letter amino acid code, and the structure of the chemical group that can be covalently inserted following sulfo-SMCC treatment is also indicated. Scissors (✂) symbolize the sites where the protein can be digested by thermolysin.

B, Graphic illustration of the F1 and F2 fragments of Rho that can be generated following thermolysin digestion.

No formation of the F1 and F2 pieces can be seen in sulfo-SMCC-cross-linked Rho since the internal thermolysin cleaving site (S^{240}) is contained within the region that is intramolecularly ligated by the bifunctional reagent. The small thermolytic fragments generated from the Rho carboxyl-terminal tail are also shown. \( \text{X—X} = \) cross-linking bridge inserted by sulfo-SMCC; ▼ = sites that are susceptible to cleavage by thermolysin. ✂ = thermolysin proteolysis.
A

$[^3]H$ GMPpNp binding activity (cpm)

Rho  Rho + sulfo-SMCC

B

RLU

Rho + RhoK  Rho + sulfo-SMCC  RhoK  Control
