Zinc is present at high concentrations in the photoreceptor cells of the retina where it has been proposed to play a role in the visual phototransduction process. In order to obtain more information about this role, the study of the effect of zinc on several properties of the visual photoreceptor rhodopsin has been investigated. A specific effect of Zn$^{2+}$ on the thermal stability of rhodopsin, obtained from bovine retinas and solubilized in docetyl maltoside detergent, in the dark is reported. The thermal stability of rhodopsin in its ground state (dark state) is clearly reduced with increasing Zn$^{2+}$ concentrations (0–50 μM Zn$^{2+}$). The thermal bleaching process is accelerated in the presence of Zn$^{2+}$ with k rate constants, at 55 °C, of 0.028 ± 0.002 min$^{-1}$ (0 μM Zn$^{2+}$) and 0.058 ± 0.003 min$^{-1}$ (50 μM Zn$^{2+}$), corresponding to $t_{1/2}$ values of 24.4 ± 1.6 min and 11.8 ± 0.1 min, respectively. Thermodynamic parameters derived from Arrhenius plots show a significant $E_a$ increase at 50 μM Zn$^{2+}$ for the process, with $\Delta G^\circ$ decrease and increase in $\Delta H^\circ$ and $\Delta S^\circ$ possibly reflecting conformational rearrangements and reordering of water molecules. The stability of the metarhodopsin II intermediate is also decreased and changes in the metarhodopsin II decay pathway are also detected. The extent of rhodopsin regeneration in vitro is also reduced by zinc. These effects, specific for zinc, are also seen for rhodopsin in native disc membranes, and may be relevant to the suggested role of Zn$^{2+}$ in normal and pathological retinal function.

Rhodopsin is the photoreceptor protein of the vertebrate retina (1–3) belonging to the G-protein-coupled receptor (GPCR)$^1$ superfamily (4–6). It is the main protein component of the rod outer segments (ROS) of the retinal photoreceptor cells, and its easy isolation from bovine retinas has made of this receptor a widely used model for the GPCR superfamily. Rhodopsin is a key molecule in the biochemistry of vision and alterations in its sequence have been associated with retinal disease. In particular, a high number of mutations in the opsin gene have been associated with the autosomal form of the retinal degenerative disease retinitis pigmentosa (7, 8). A number of factors have been proposed to be related to retinal function, and among them a possible role for Zn$^{2+}$ in the retina and its metabolism has been proposed (9). Zn$^{2+}$ is present at particularly high concentrations in the retina (10) being a component of the disc membranes in the rod outer segments of the photoreceptor cells (11). Zn$^{2+}$ has been histochemically localized to ROS (12) and it has been shown to copurify with ROS proteins as well (13). Despite this presence, the role of Zn$^{2+}$ in the visual cycle and specifically in its interaction with rhodopsin remains unclear.

Zn$^{2+}$ is required for the function of numerous proteins, serving both as a part of the active site in, for example, metalloenzymes, and acting to stabilize protein domains, such as the Zn$^{2+}$ finger-binding motif in transcription factors (14, 15). The structure of many Zn$^{2+}$-binding sites is known from x-ray crystallography of Zn$^{2+}$-binding proteins and, thus, the geometry of the interaction between Zn$^{2+}$ and different coordinating residues is well characterized (14, 15). This, together with the small size of the zinc (II) ion, makes artificially generated Zn$^{2+}$-binding sites a highly useful approach for probing structure-function relationships in proteins. In GPCRs, for example, construction of bis-His Zn$^{2+}$-binding sites has led to important information about both the organization of the transmembrane helices and their movements during receptor activation to be obtained (16–22). Therefore, engineered Zn$^{2+}$ binding sites created by site-directed mutagenesis is an interesting tool for probing intramolecular interactions in these types of receptors (23). Particularly in the case of rhodopsin, important structural information about helical orientation, connectivities, and the conformational change upon light activation, has been obtained from engineered Zn$^{2+}$-binding sites in this visual photoreceptor (19).

There is experimental evidence indicating the presence of naturally occurring Zn$^{2+}$-binding sites in several GPCR proteins. These include the dopamine transporter (24), D$_1$ dopamine receptor (25) and all D$_2$-like dopamine receptors: D$_2$A, D$_2$B, D$_3$, and D$_4$ (26). Molecules participating in the visual phototransduction process are already known to have Zn$^{2+}$-binding sites, including rhodopsin (27) and cGMP phosphodiesterase (PDE6αβ) (28).

Previous reports showed direct Zn$^{2+}$ binding to rhodopsin, both in ROS membranes and in detergent-solubilized-purified protein systems, by means of $^{65}$Zn competition binding experiments, indicating that there may be coordination sites for Zn$^{2+}$ in native rhodopsin (27). This binding occurs in the dark and it increases upon illumination of the protein (27). Also, previous reports indicated that Zn$^{2+}$ enhanced azido-[α-32P]ATP binding to rat ROS, suggesting a structural effect of Zn$^{2+}$ on rhodopsin (29). Four Zn$^{2+}$ were included in the crystal structure of rhodopsin at 2.8-Å resolution (30, 31). Recently this crystal structure was refined, and seven Zn$^{2+}$ were iden-
Zinc Effect on Rhodopsin

UV/Vis Spectra of Rhodopsin—All measurements were made on a Cary 1E spectrophotometer (Varian, Australia), equipped with water-jacketed cuvette holders connected to a circulating water bath. Temperature was controlled by a Peltier accessory connected to the spectrophotometer. All spectra were recorded, in the 250–650-nm range, with a bandwidth of 2 nm, a response time of 0.1 s, and scan speed of 250 nm/min. The protein sample binding reaction was carried out in the spectrophotometric cuvette at 1 h at 30 °C and was monitored by UV/Vis spectroscopy as described above. These reaction samples were used for photobleaching, thermal stability, and regeneration experiments. The experiments were performed in triplicate. Fourth derivative spectra were obtained, from five absorption spectra coadded to improve the signal-to-noise ratio, by using the Savitzky-Golay algorithm and 200 points with the Grams/32 software (Galactic Industries).

Photobleaching and Acidification of Rho—Samples were bleached with a 150 watt power source equipped with an optic fiber guide and using a 495 nm cut-off filter. Dark-adapted Rho samples with 50 and 100 μM zinc or without zinc were illuminated with a saturating light (with a 25 watt light source and a distance between the sample and the light source of 5 cm) at 5 s intervals. Immediately at the end of each bleaching interval, changes in the absorption spectra were recorded until complete photobleaching was achieved (complete disappearance of the 500-nm chromophoric band).

The formation of MetaII was measured, after Rho illumination for 60 s (saturating illumination), in the presence of different zinc concentrations or without zinc (control) at 15 °C. Complete absorption spectra were recorded in the range studied immediately after illumination and at 2.5-min intervals thereafter. MetaIII formation was monitored at 380 nm. All absorbance values were normalized to the same initial absorbance of 1000 nm before photoconversion.

Thermal Stability Assay—The thermal stability in the dark was followed by monitoring the rate of A160 loss, and the appearance of A380 as a function of time, at constant temperature. Rho, in the presence of 5, 15, 30, 50, 100, and 200 μM zinc, or in the absence of zinc (control), was thermally bleached in the dark at 55 °C. The process was carried out in a similar way as previously described (34) over 3 h. Complete absorption spectra were recorded at 5 min intervals. Other experiments, using the same conditions but with 50 μM calcium and other metal ions, were performed to rule out a possible unspecific charge effect. Thermodynamic parameters for the dark thermal bleaching process were determined as a function of temperature. This method regards thermal bleaching as an irreversible process (34). The thermodynamic parameters were calculated from the decay rate constants versus 1/T (Arrhenius plot) using the values for the slopes of these plots, which were derived from least-squares analysis. For this purpose, Rho samples in the dark and in the presence of 50 μM zinc (or without zinc in the control sample) were thermally bleached in the dark at different temperatures in the range of 50 to 55 °C.

Regeneration of Rhodopsin—The regeneration of Rho was carried out in 15 °C in the dark. 11-cis-Retalin in an ethanolic stock solution was added to a 2.5-fold molar excess over dark Rho in the presence of 50, 100, and 200 μM zinc or without zinc (control). Then, the sample was illuminated for 60 s (light-saturated), and absorption spectra were immediately recorded at 2.5 min cycles over 4 h. Rho regeneration rate constants were determined at 500 nm. These parameters were eventually used to perform the presence of Schiff base-linked species. Regeneration experiments were also carried out with ROS rhodopsin samples essentially in the same way as for the detergent-solubilized samples.

RESULTS

UV Spectroscopic Characterization of the Zn2+ -Rhodopsin Complex—The presence of Zn2+, at a concentration of 200 μM, did not affect the spectral properties of rhodopsin in the dark or in the light (Fig. 1). The characteristic 500-nm visible chromophoric band remained unchanged in the Zn2+ -containing sample (compare Fig. 1, A and B). The presence of Zn2+ did not interfere with the ability of rhodopsin to form the 380-nm band, corresponding to the MetaII intermediate, upon illumination under light-saturating conditions (Fig. 1B) indicating the same
photobleaching behavior than the non-zinc containing sample (Fig. 1A). In both cases the presence of a protonated Schiff base linkage was detected by acidification of the illuminated samples, with the corresponding shift of the band to 440 nm, confirming that the 380-nm band can be attributed to the MetaII species, and indicating that the protonation status of the Schiff base linkage was not affected by Zn$^{2+}$ (Fig. 1B). These results are in agreement with studies using wild-type recombinant rhodopsin in which even in the presence of 5 mM Zn$^{2+}$ similar results were obtained (19), suggesting that the rhodopsin-Zn$^{2+}$ complex is functional, although some differences in the transducin activation ability were detected in the presence of zinc (19). The photobleaching efficiency is not altered by zinc when rhodopsin is illuminated with subsaturating light intensities (Fig. 2A). However, MetaIII formation is decreased in the presence of zinc when compared with the non-zinc-containing sample (Fig. 2B). Furthermore, when Rho samples are acidified at different times after photobleaching a 2-fold decrease under the studied conditions in the MetaII stability can be detected (the determined $t_{1/2}$ values are 30 and 15 min in the presence and in the absence of Zn$^{2+}$, respectively).

Fourth Derivative of the UV Absorption Spectra—Fourth derivative spectroscopy allows separation of the different electronic components corresponding to Phe, Tyr, and Trp residues in proteins. This allows a detailed analysis of the structural environment of these aromatic amino acids to be performed (35). The fourth derivative represents the best compromise between the best resolution and the best signal-to-noise ratio (35). This technique has been successfully applied to the study of conformational changes in native and recombining proteins (36). No significant differences were detected in the fourth derivative UV spectra of rhodopsin in the dark upon Zn$^{2+}$ addition to the sample (Fig. 3, A and B and Table I). The wavelengths of the fourth derivative peaks at 285.6 and 292.2 nm and troughs at 289.0 and 295.5 nm remained unchanged upon zinc addition to the sample (compare $a$ traces in Fig. 3, A and B; see Table I). However, some differences were detected after illumination of the control rhodopsin sample in the absence of zinc (compare $a$ and $c$ traces in Fig. 3A; see Table I). These differences were increased when Zn$^{2+}$ was present in the sample (compare $a$, $b$, and $c$; see Table I) with larger blue-shifts in the 275–300-nm region and increased intensity changes in some of the bands. In contrast, peaks in the 240–275 nm underwent only minor changes when compared with the spectra of the non-zinc-containing samples.

Zn$^{2+}$-induced Decrease in the Thermal Stability of Rhodopsin—The thermal stability of Rho in the presence of different concentrations of Zn$^{2+}$ was determined at 55°C in the dark. The kinetics of the thermal bleaching process of rhodopsin in the dark was spectroscopically monitored by measuring the decay of the 500-nm chromophoric band at constant temperature (55°C) as a function of time (Fig. 4A). At this temperature, a progressive faster decay of this band was observed as a function of time after illumination. The first spectrum corresponds to dark rhodopsin (dark, $a$). Spectra recorded at different times after illumination are shown (traces b and c). Spectrum labeled as light (trace c) was obtained 9 h after illumination. Fourth derivative spectra were obtained from five coadded absorption spectra by using the Savitzky-Golay algorithm as described under “Experimental Procedures.”
The wavelength for the maximum and minimum peak values ($\lambda_{\text{max}}$ and $\lambda_{\text{min}}$) were obtained from UV fourth derivative spectra (see Fig. 3). Tyr and Trp residues of rhodopsin (control, in the absence of Zn$^{2+}$) were derived for the process is $121.6$ min in the dark. These parameters were significantly different for the Zn$^{2+}$ and in the presence of $50 \text{ M}$. Choline was assayed at a concentration of $100 \text{ M}$. The...
is observed for maximal rhodopsin regeneration with increasing zinc concentration. This decrease is about 30% at 200 μM added zinc. Higher zinc concentrations tested indicated further reduction of the regeneration percentage (to very low regeneration level), but the corresponding values could not be reliably obtained due to a high aggregation propensity of the samples with time. Regeneration experiments carried out with rhodopsin in ROS membranes (non-solubilized samples, Fig. 7B) indicated a similar behavior to that found for Rho samples (compare Fig. 7, A and B). In the case of ROS membranes the maximal regeneration observed was slightly lower than that observed for the Rho samples, but the effect of zinc addition was comparable.

DISCUSSION

Previous studies have reported specific Zn$^{2+}$ binding to purified rhodopsin and disc membranes in both dark and light conditions (27, 29). Furthermore, Zn$^{2+}$ was shown to enhance rhodopsin phosphorylation (29). Structurally bound zinc has been also found in the recent crystal structure of rhodopsin (30–32). Despite these reports, a detailed study of the effect of Zn$^{2+}$ on rhodopsin properties has not been previously reported. We find a clear effect of zinc on several rhodopsin properties. Our spectroscopic results indicate that zinc does not alter the retinal binding pocket of rhodopsin and the formation of the MetaII intermediate. Also, the status of the protonated Schiff base is not affected by the presence of zinc ions.

Under physiological conditions, inactive dark state rhodopsin changes almost entirely to the active MetaII conformation which shift the equilibrium to the MetaII side (41). Recently shown that this equilibrium is also influenced by salts, by several experimental conditions (37). Thermodynamic parameters measure macroscopic changes representing the sum of all reaction events of the Zn$^{2+}$-rhodopsin complex formation. The thermal bleaching process is accompanied by a decrease in $\Delta G^\circ$ and an increase in $\Delta H^\circ$ and $\Delta S^\circ$ for the Zn$^{2+}$-containing samples when compared with the values in the absence of Zn$^{2+}$. Changes in enthalpy can be interpreted as reflecting conformational changes, and entropy effects can be interpreted as a reordering of water molecules as proposed previously (23, 24). Similar interpretation has been provided for the binding of zinc to the D2-like dopamine receptor. In this latter case, the fact that the reaction was entropy driven suggested that zinc was essentially chelated by dopamine receptors, possibly resulting in the reordering of hydrogen bonds (23). Thermodynamic changes could reflect a structural rearrangement of native rhodopsin, during the formation of the Zn$^{2+}$-rhodopsin complex, without affecting the retinal binding pocket and the protonated Schiff base linkage in the dark state of rhodopsin. The observed effect, reflecting reordering of water molecules, may be significant in the case of rhodopsin where a functional role for water molecules has been recently proposed (32). In this latter study more than ten water molecules are found structurally associated with each one of the two monomers, and two of these water molecules are in the intradiscal domain of the receptor, where four of the seven zinc atoms are located (32).

The preceding section of the discussion has dealt with results obtained with Rho (rhodopsin solubilized in DM detergent). However, it is known that rhodopsin is more stable in native ROS membranes than in detergent-solubilized form. Thus, it could be possible that the effect observed with Rho would be different when rhodopsin was assayed in disc membranes. Our results show that, although the rhodopsin samples are more stable in membranes, the differential effect found in the case of the zinc-containing sample is maintained, i.e. rhodopsin in membranes with zinc has lower thermal stability than control samples without zinc. Furthermore, the same behavior is detected in the case of the pigment regeneration experiments. In that case the reduction in rhodopsin regeneration induced by zinc is also observed when rhodopsin is found in its native membrane. These findings argue for the physiological relevance of the results obtained.

A structural effect of Zn$^{2+}$ on rhodopsin was also detected in a previous study, using engineered Zn$^{2+}$-binding sites. In this case Zn$^{2+}$ binding to wild-type recombinant rhodopsin resulted in a somewhat reduced transducin activation (19). We find that zinc specifically alters several rhodopsin properties: a definite reduction in thermal stability of ground state dark rhodopsin, reduced stability of the active MetaII conformation, altered pathway of photointermediates after MetaII formation, and reduced pigment regeneration with 11-cis-retinal. These effects are accompanied by conformational changes of the protein that are shown to be specific of zinc and not of other divalent metal ions studied. The effect of zinc on retinal function seems to
strongly depend on the local concentration and the ratio of free to bound cation (10). The proposed effect of Zn\(^{2+}\) on visual function may be also related to effects upon other proteins of the visual phototransduction cascade (28) or upon some component of the retinoid cycle. Furthermore, the proposed increase in rhodopsin phosphorylation caused by Zn\(^{2+}\) (29) could be also related to other processes, like receptor internalization, necessary for its recycling, thus changing the normal turnover of the receptor in the membrane.

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