Direct zinc binding to purified rhodopsin and disc membranes

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Using the radionuclide ^{65}Zn , we have demonstrated the direct binding of zinc to purified rhodopsin. ^{65}Zn is eluted with detergent-solubilized rhodopsin from concanavalin A columns and remains bound to the visual pigment through a subsequent gel-filtration step. Zinc binding to purified disc membranes is highly specific and, of the ions tested, copper is the best competitor. Equilibrium-dialysis experiments indicate that zinc binding to detergent-solubilized forms of rhodopsin may increase on bleaching the photopigment. These results may have important implications for studies that indicate that zinc plays a role in retinal degeneration and normal photoreceptor physiology.

INTRODUCTION

For decades, investigators have been interested in potential roles for zinc in the retina since it has been shown that ocular tissues, and in particular the retina and choroid, contain high concentrations of the bivalent cation (Galín *et al.*, 1962; Eckhert, 1979). Reports have indicated that zinc copurifies with rod outer segment proteins (Tam *et al.*, 1976) and is a normal component of the disc membrane (McCormick, 1985), that zinc deficiencies may be involved in photoreceptor degeneration in the rat (LeureduPree, 1981; LeureduPree & Bridges, 1982; LeureduPree & McClain, 1982) and cat (Tucker *et al.*, 1984), and that zinc in the presence of taurine may have a protective effect on photoreceptor outer segments (Pasantes-Morales & Cruz, 1984). Newsome & Rathman (1987) investigated the uptake of zinc in human retinal epithelium cultures and discussed the potential importance of this ion in the visual system.

We have found previously that azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ can bind to rhodopsin in rat rod outer segment preparations, particularly in the presence of zinc (Shuster *et al.*, 1987, 1988a). This zinc-enhanced azido-ATP binding is specific, as other bivalent cations do not promote binding to the extent that zinc does. These results imply that zinc either alters the conformation of rhodopsin by binding directly to the visual pigment or that it forms a complex with azido-ATP which is then able to bind to the protein. An earlier publication (Tam *et al.*, 1976) suggested that zinc is normally associated with rod outer segment proteins.

With these results in mind we decided to investigate whether ^{65}Zn could bind to the visual pigment, rhodopsin. The results presented here indicate that ^{65}Zn binds directly and specifically to both disc membranes and purified rhodopsin in both light and dark conditions. Equilibrium-dialysis experiments show that zinc binding to detergent-solubilized rhodopsin may be enhanced by light. Our experiments indicate that binding of zinc most likely occurs on the intradiscal portion of the protein.

EXPERIMENTAL

Preparation of bovine rod outer segment discs

Intact bovine discs were prepared from frozen dark-adapted retinas using the technique of Smith & Litman (1982). All steps were done on ice under dim red light. Occasionally the discs were

washed in high-ionic-strength buffer and then water to decrease further any contaminating proteins.

Solubilization of disc proteins

Typically, 6–8 mg of disc protein was solubilized in 4–5 ml of TBS (Tris-buffered saline; 20 mM-Tris/HCl/500 mM-NaCl, pH 7.5) containing 2% cholate or Triton X-100 and bivalent cations (most often 2 mM- MgCl_2 , and both CaCl_2 and MnCl_2 at 0.5 mM). Solubilization was done at 0–4 °C in the dark for 2–4 h. The solution was then centrifuged for 45 min in an SS-34 Sorvall rotor at 12000 rev./min (17000 g) and the supernatant was used as solubilized disc proteins. Other buffers at various pHs were also used with similar results. If significant, the buffer used is listed in the Figure legends.

Concanavalin A (Con A) chromatography

Purification of rhodopsin from dark-adapted solubilized disc proteins by Con A chromatography was carried out following a procedure similar to that described by other investigators (e.g. van Breugel *et al.*, 1977). Briefly, the resin (Con A-agarose or Con A-Sepharose 4B; Sigma) was equilibrated in TBS containing 2% of the appropriate detergent and bivalent cations (MnCl_2 and CaCl_2 , both at 0.5 mM, and usually MgCl_2 at 2 mM). Solubilized disc protein was then either batch-adsorbed to the Con A resin and poured into one or more small columns or the resin was placed into a small column and solubilized disc protein was allowed to flow slowly through it so the rhodopsin could bind (all under dim red light with ice-cold buffers). Each column was washed with 20–30 column vol. of wash buffer (TBS with either 0.5% cholate or 1% Triton X-100, and bivalent cations as mentioned above). When appropriate, 20–40 μM - ^{65}Zn in 1 bed vol. of wash buffer was applied to the column at this point and allowed to bind to rhodopsin with the flow stopped for approximately 15 min. The total mol of zinc applied to the rhodopsin-Con A column was always less than the total mol of rhodopsin bound to the column (e.g. zinc/rhodopsin usually 0.2–0.5). The column was again washed with 20–30 vol. of wash buffer to eliminate unbound ^{65}Zn . Bound protein was then eluted in wash buffer containing 0.2–0.3 M-methyl α -mannoside. The methyl α -mannoside was added to the column in 1 bed vol. and allowed to displace protein with the flow stopped for a few minutes. Column flow was then resumed and 0.5–1.0 ml fractions were collected. Columns normally were 1–3 ml total volume. Samples were taken for protein assay, liquid-scintillation

Abbreviations used: TBS, Tris-buffered saline; Con A, concanavalin A; ROS, rod outer segment.

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counting, analysis by SDS/PAGE and further chromatography by gel filtration. When this procedure was done to purify rhodopsin, no ^{65}Zn was applied to the column but the protein was applied to and eluted from the column as discussed above.

Protein assay

The protein concentration of various fractions was determined by the method of Peterson (1977).

Gel electrophoresis SDS/PAGE

Gels were run essentially as previously described (Walter *et al.*, 1986).

Gel-filtration chromatography

When appropriate, fractions were chromatographed on a Superose 6 f.p.l.c. column (Pharmacia, Uppsala, Sweden). This column has an optimal separation range for proteins of M_r 5000–5000000 and was run at 0.5 ml/min which is near the optimal flow rate for separation. Total column volume was 20 ml. Before the experiments, the column was equilibrated in the appropriate buffer with added detergent and cations. Chromatography was performed at room temperature under dim red light and all solutions were degassed and filtered before use. Samples of each fraction were analysed for protein concentration, protein profile and ^{65}Zn content when appropriate. The column was calibrated using the same buffers as those used for rhodopsin solubilization, i.e. detergents etc. were present when the column was calibrated with protein standards, so running conditions were identical for standards and rhodopsin.

Zinc-binding assays

Filtration-based binding assays were used to assay binding to disc membranes and were done on ice. Typically, 1 ml final volume contained TBS with 0.1 or 1 μM - ^{65}Zn and 100 μM -competing ions (when appropriate). The specific radioactivity of ^{65}Zn in these binding assays and during chromatography was 0.5–1.0 mCi/0.8 mg of Zn. Any deviations from these conditions or concentrations are mentioned in the Figure legends. The reaction was started by addition of 15–30 μg of disc membrane protein. After equilibrium had been reached, the reaction was stopped by adding 2.5 ml of TBS (ice-cold) and pouring the solution through either a Millipore HAWP02500 or HVLP02500 filter (the latter was preferred), both with 0.45 μm average pore size. The filter was then washed with 2×2.5 ml of TBS, and ^{65}Zn bound to the filter was quantified by liquid-scintillation counting. All assays were done in duplicate or triplicate and each assay condition had a protein blank (no disc membrane) to determine non-specific binding to the filter. These types of assays were generally done only at low concentrations of zinc (around 1 μM), since zinc binds to the filters thereby increasing the background considerably at higher concentrations.

Equilibrium-dialysis experiments

Equilibrium dialysis was done similarly to established techniques and was used to assay zinc binding to solubilized photopigment. Rhodopsin from dark-adapted disc preparations was either solubilized in cholate or Triton X-100 and used directly or was purified further using Con A chromatography. Experiments were conducted in dim red light unless otherwise noted, and all solutions were cold. Spectra/por 7 membranes with a 10000- M_r cut-off were used during all dialysis experiments. Typically, eight to ten 1 ml bags were dialysed against approximately 1 litre of buffer for at least 12 h. The final concentration of rhodopsin inside the dialysis bag was 2–8 μM , or as mentioned in the Figure legends. Time studies were done to establish that solutions had equilibrated across the membranes

(in about 2 h). Typically, an experiment was as follows: (1) solubilized rhodopsin and dialysis buffer were added to dialysis bags; (2) the experiment was allowed to reach equilibrium overnight (cold, dark with constant stirring); (3) some bags were sampled at approximately 16 and 18 h to determine that equilibrium had been reached, to establish the ^{65}Zn concentrations inside and outside the dialysis bag and to determine the protein concentration within the sample. To determine the effects of bleaching, the dialysis was continued under the same conditions but with lights on for another 6 h or more with sampling every 1–2 h to establish when equilibrium had been reached. A typical buffer used was 20 mM-Bistris propane with 150 mM-NaCl, 0.2 mM- MgCl_2 , 0.2 mM- CaCl_2 and 0.1% Triton X-100, all at pH 6.75 at 4 °C.

Electrophoretic transblotting

Proteins that had been separated by SDS/PAGE were electrophoretically transblotted to nitrocellulose as described (Shuster *et al.*, 1988b).

Proteolysis of disc membrane proteins

Disc membrane proteins were subjected to treatment with various proteinases to investigate how removal of the cytoplasmic portion of rhodopsin would alter the zinc-binding properties. Typically, 1 mg of disc protein and 10 μg of proteinase were mixed together in a final volume of 1 ml (in 20 mM-Hepes/150 mM-NaCl/1 mM- MgCl_2 /1 mM- CaCl_2 at pH 7.0), at room temperature under dim red light. This suspension was incubated for 0.5–1.0 h and the membranes were pelleted (10000 $g \times 45$ min). The membrane pellet was then resuspended in TBS containing 5 M-urea, incubated for 15 min, and centrifuged as above. The pellet was then resuspended in TBS and centrifuged. This proteinase-treated disc membrane pellet was then dissolved in 2% cholate (in 20 mM-Mops/150 mM-NaCl/0.25 mM- MgCl_2 /0.2 mM- CaCl_2 , pH 7.0) for 1–2 h (on ice, in darkness), then centrifuged to clear the solution. The supernatant from this centrifugation was subjected to equilibrium dialysis to determine if the proteolytically altered rhodopsins still bound ^{65}Zn . The procedure was monitored by SDS/PAGE. Samples from (1) the initial incubation, (2) just before cholate solubilization and (3) after equilibrium dialysis were analysed for extent of proteolysis. All proteolysis was stopped by the urea wash. These samples were also analysed for Con A binding after transblotting, to establish which portion of the protein was lost to proteolysis.

Con A binding to proteolysis samples

This was accomplished by transblotting SDS/PAGE-separated proteins and using Con A-peroxidase to probe the proteolytic fragments as described previously (Shuster *et al.*, 1988b). Briefly, nitrocellulose transblots were blocked in BSA, incubated for a few hours in Con A-peroxidase, washed, and the Con A-peroxidase was visualized with hydrogen peroxide and 4-chloro-1-naphthol.

RESULTS

Because azido-ATP labelling experiments (Shuster *et al.*, 1988a) indicated a potential alteration in the conformation of rhodopsin in the presence of zinc, we sought to determine whether zinc would bind directly to the protein. Our approach was to bind ^{65}Zn to rhodopsin already bound to a Con A column. Although Con A binds bivalent cations, requiring calcium and transition elements such as manganese, it is possible to separate the ion-binding properties of Con A from the zinc-binding properties of rhodopsin by studying the elution profiles from Con A-agarose in the absence and presence of rhodopsin.

Fig. 1. Elution of ^{65}Zn from Con A-Sepharose in the absence (□) and presence (◆) of rhodopsin

It is shown that ^{65}Zn flows through the column in the absence of rhodopsin and that no more ^{65}Zn is eluted by the addition of 0.3 M-methyl α -mannoside at the time indicated by the arrow. ◆ shows the elution profile of ^{65}Zn in the presence of rhodopsin (which is co-eluted with the ^{65}Zn). Rhodopsin was solubilized in 2% Triton X-100 from dark-adapted bovine discs as described. ^{65}Zn was applied to the column at fraction 0. Also present were 0.5 mM- CaCl_2 and 0.5 mM- MnCl_2 . For this particular column, a 4-fold molar excess of rhodopsin over ^{65}Zn was applied.

An example of this type of experiment is shown in Fig. 1. Two Con A-Sepharose columns were treated identically except that rhodopsin solubilized in Triton X-100 was applied to one column. Fig. 1 demonstrates that although Con A retards the elution of ^{65}Zn , the radionuclide is washed through the column in 20 column vol. and no more is released when methyl α -mannoside is applied. In contrast, when Triton-solubilized rhodopsin is bound to the column before application of ^{65}Zn , virtually no radionuclide washes through the column and both rhodopsin and ^{65}Zn are eluted in the presence of the competitor. During these experiments, the concentration of rhodopsin applied to the column was always two to five times the concentration of ^{65}Zn applied to the column.

Triton is acceptable for studying the properties of solubilized dark-adapted rhodopsin, and the rhodopsin-dependent ^{65}Zn -binding patterns have been obtained in the presence of millimolar concentrations of magnesium, manganese and calcium, as well as 0.5 M- NaCl . However, it was necessary to determine if our elution profiles depended on detergent type. Thus the same experiment was conducted using rhodopsin solubilized with cholate, an ionic detergent. The ^{65}Zn elution pattern obtained with cholate-solubilized rhodopsin was essentially identical with that obtained with Triton-solubilized rhodopsin, demonstrating that the zinc elution profiles from Con A columns are not a function of detergent type.

Rhodopsin represents 98% of the disc membrane protein (Hargrave, 1986) and is the major Con A-binding protein in disc membranes. In addition, the disc membranes contain a 220000- M_r rim protein (Molday & Molday, 1979; Papermaster *et al.*, 1978) which also binds Con A. To determine whether the 220000- M_r protein might be binding zinc, and to characterize further binding with respect to rhodopsin, the Triton-solubilized protein eluted from the Con A column with ^{65}Zn bound (Fig. 1) was chromatographed on an f.p.l.c. gel-filtration column (Superose 6) capable of separating the two proteins. The column was equilibrated with buffer containing 1% Triton X-100 and

Fig. 2. F.p.l.c. gel filtration (Superose 6) of the ^{65}Zn -rhodopsin fraction eluted from the Con A-Sepharose column run as in Fig. 1

TBS buffer for the gel-filtration column included 1% Triton X-100, 2 mM- MgCl_2 , 0.5 mM- CaCl_2 and 0.5 mM- MnCl_2 . The column was run at 0.5 ml/min and both ^{65}Zn and rhodopsin peaked at fraction 32. The column was calibrated with the same buffer (including detergent) with IgG (I), ovalbumin (O), myoglobin (M) and vitamin B_{12} (B). The 220000- M_r rim protein, if present, would be eluted at fraction numbers lower than IgG. □, Radioactivity; ×, protein.

Fig. 3. Binding of ^{65}Zn to bovine disc membranes in the presence of non-radioactive zinc and calcium

Values are $\pm$ S.D. of a typical experiment. Column A contains no competitor of ^{65}Zn (total zinc present is 1 μM). In addition to ^{65}Zn , column B contains 10 μM -zinc, column C 10 μM -calcium, column D 100 μM -zinc and column E 100 μM -calcium.

calibrated with protein standards in the same buffer. Fig. 2 shows that ^{65}Zn continued to bind to rhodopsin through this chromatographic step. Analysis of the eluted fractions by SDS/PAGE indicated that the protein in the ^{65}Zn -containing fractions was rhodopsin and no 220000- M_r rim protein was apparent in these fractions on heavily loaded gels. Rhodopsin-containing fractions were also identified by their red colour which bleached after exposure to light. The ratio of ^{65}Zn to rhodopsin was 1:5 in the protein peak in Fig. 2. It is unlikely that this quantity of zinc could be binding to a protein that was undetectable on our gels.

Comparison of the protein-elution profile with the zinc profile showed that zinc trailed slightly but was within the rhodopsin peak (i.e. there was not a constant ratio of zinc to rhodopsin across the peak). This is a property of ligands that can associate

Table 1. Ability of other ions to compete for the zinc-binding site on light-adapted rhodopsin in the disc membrane

Values are given as the percentage of ^{65}Zn bound to the membranes in the presence of the competitor ion listed.

Competitor	Zn bound (%)
None	100
Cadmium	62
Calcium	98
Cobalt	64
Copper	6.7
Lead	83
Magnesium	101
Manganese	99
Zinc	5.4
EDTA	5.3

Table 2. Equilibrium dialysis of Triton X-100-solubilized rhodopsin against ^{65}Zn -containing buffer

Buffer used was Pipes at pH 6.75 with univalent and bivalent cations as discussed in the Experimental section. Rhodopsin was added to the bag in the dark (to $5\text{ }\mu\text{M}$), the dialysis was allowed to reach equilibrium overnight and multiple samples (two to four) from several bags were taken from both inside and outside of the tubing for analysis. Dialysis was then continued with the same dialysis samples under identical conditions except room lights were turned on. The bags were samples over time and ^{65}Zn both inside and outside of the bags was quantified by liquid-scintillation counting. Results are given as ^{65}Zn (c.p.m.) in a $200\text{ }\mu\text{l}$ sample taken from inside the bag (with rhodopsin) or outside (no rhodopsin). Additionally, the molar ratio of zinc to rhodopsin is indicated in parentheses. Results are from a typical experiment repeated several times. Means $\pm$ S.D. are given. The free Zn^{2+} concentration was $0.5\text{ }\mu\text{M}$.

	^{65}Zn (c.p.m./ $200\text{ }\mu\text{l}$)	
	Outside bag	Inside bag
Dark-adapted	953 ± 8.8	1746 ± 99 (0.17)
Bleached	942 ± 7.5	2810 ± 192 (0.39)

and dissociate from proteins. Bound zinc released from rhodopsin at the leading edge of the peak is slightly retarded with respect to the protein and rebinds to free rhodopsin in the latter part of the protein peak. An alternative interpretation of these results is that zinc causes a conformational change in rhodopsin which slightly retards the protein migration through the gel matrix. The same elution profile of ^{65}Zn bound to rhodopsin is obtained when the experiment is run with rhodopsin in the presence of both soluble and Triton-soluble total retinal proteins.

^{65}Zn binding to disc membrane proteins was then compared with calcium binding since calcium is a major bivalent cation present within discs. Results presented in Fig. 3 indicate that the zinc binding is not sensitive to the presence of calcium. Experiments using ^{45}Ca demonstrate substantially less calcium binding to disc membranes than that observed with zinc. The ability of other ions to compete for the zinc-binding site is shown in Table 1. Zinc binding is very specific, with copper being the best competitor. Cobalt, cadmium and lead were also able to decrease zinc binding to some extent. The relative abilities of these ions to compete for the site may change under different conditions. Experiments such as this indicate that the K_D for zinc binding to the proteins in disc membranes is in the low (2–10) μM

range. This value compares favourably with results obtained with purified rhodopsin (discussed below).

To quantify zinc binding to purified rhodopsin more accurately, we conducted equilibrium-dialysis experiments using Triton X-100-solubilized rhodopsin in the dark. The pH of the buffer was varied, but results presented in Table 2 were obtained with buffers at or below pH 7 to minimize loss of Zn^{2+} through reaction with hydroxy ions. Total zinc present was $1\text{ }\mu\text{M}$ although the actual Zn^{2+} concentration was decreased. Zirino & Yamamoto (1972) showed that at pH 7, in solutions of high ionic strength, the Zn^{2+} concentration was decreased to half of the total zinc present. In subsequent calculations, we assumed that the same decrease in Zn^{2+} concentration occurred under the conditions of our assays. Results from a typical experiment are shown in Table 2. The total ^{65}Zn inside the dialysis bag (with rhodopsin) was much higher than the ^{65}Zn in the dialysis buffer. Using these data, and assuming that free Zn^{2+} concentration was decreased to $0.5\text{ }\mu\text{M}$, we can calculate (Segel, 1976) a dissociation constant (zinc to rhodopsin) of approximately $2.6\text{ }\mu\text{M}$ in the dark (with the use of Triton X-100 to solubilize rhodopsin).

To determine how bleaching rhodopsin altered zinc binding, we continued our dialysis with the room lights turned on. Samples were taken at various times to establish a lights-on ^{65}Zn concentration inside the dialysis bag. When the lights were turned on, the concentration of zinc associated with rhodopsin (inside the bag) increased substantially (Table 2). The light-induced increase in binding reached equilibrium in less than 2 h. During the time of the light exposure, under the conditions used in our experiments, the solution of rhodopsin solubilized in Triton X-100 remained clear. Centrifugation of the dialysed Triton/rhodopsin solution at $30000g$ did not pellet a protein- ^{65}Zn precipitate, indicating that trapping of zinc in large insoluble aggregates is not the basis for the light-generated increase in zinc binding. Using the data of Table 2 and making the same assumptions regarding the Zn^{2+} concentration as discussed above, we calculate a K_D of approximately $0.7\text{ }\mu\text{M}$ in the light, considerably lower than that obtained in the dark. Triton X-100 is efficient for extracting rhodopsin, and in the dark, the protein is quite stable in the detergent. However, rhodopsin bleached in this detergent does not regenerate readily. Cholate does support opsin regeneration, however, and we have obtained similar light-enhanced binding of zinc using cholate-solubilized rhodopsin. Similar experiments have been done with control proteins. With known non-zinc-binding proteins such as ovalbumin, the concentration of zinc inside the bag (plus protein) was similar to outside and, of course, there was no change with light.

An examination of the pH-dependence of zinc binding to rhodopsin indicates that binding begins to increase at pH 6, reaches 50% at pH 7.2, and is maximal at pH 8.3. These results were obtained in a mixed Mes, Tes and Bistris/propane buffer ranging in pH from 5.5 to 9.1, with similar concentrations of univalent and bivalent cations as discussed above.

We wanted to determine whether zinc was binding to the intradiscal part of rhodopsin, or whether zinc binding was occurring on the C-terminal portion of the protein, where phosphorylation, G-protein activation, etc. occur. To do this, we proteolytically cleaved rhodopsin in the dark, washed away the proteinases, solubilized the protein in cholate and conducted equilibrium dialysis, all at a constant volume with respect to a control. We monitored proteolysis by SDS/PAGE, and tested whether a decrease in M_r was due to loss of the cytoplasmic portion of the protein by testing for an intact Con A-binding region (N-terminal) on rhodopsin as discussed in the Experimental section. Our results, some of which are shown in Fig. 4, indicate that the zinc-binding site is not on the C-terminal tail

Fig. 4. Con A binding to electrophoretically transblotted samples

Proteinase-treated disc membranes were washed and solubilized in cholate as described in the Experimental section. These samples were subjected to equilibrium dialysis and to analysis by SDS/PAGE. After SDS/PAGE, the resolved proteins were transblotted and probed for Con A-peroxidase binding. Lane C contains a rhodopsin control (no proteinase, but same conditions), lane A contains rhodopsin treated with subtilisin and lane B contains rhodopsin treated with Pronase. The bars indicate where prestained M_r markers of 18 500, 27 500, 32 500, 49 500, 80 000 and 106 000 migrated (bottom to top).

and is probably on the intradiscal portion of the protein. The data presented show that rhodopsin can be proteolytically clipped to a 28 000- M_r subunit with no loss of Con A binding in our transblots, and with very little loss of zinc binding. The molar ratios of zinc to total rhodopsin or rhodopsin fragments shown in Fig. 4 during equilibrium dialysis are 0.47 (lane A), 0.53 (lane B) and 0.56 (control, lane C) with Zn^{2+} present at 1.5 μM . These calculations are based on protein assays and Con A binding which indicate that the molarity of rhodopsin and the proteinase-generated rhodopsin fragments is comparable. The proteinases used to generate Fig. 4 (subtilisin, lane A; Pronase, lane B) will totally hydrolyse available peptides so the cytoplasmic tail has most likely been completely removed. The complete loss of the cytoplasmic tail of the protein is further supported by our protein assays which show that even though the proteolysed proteins are similar in zinc and Con A binding (indicating molar equivalency), protein concentration within the dialysis bag has been decreased by 15–20% in samples A and B after washing of the disc membranes, supporting loss of the C-terminal. We do not know that rhodopsin transmembrane loops VI and VII have been dissociated from the remaining 28 000- M_r rhodopsin fragment. These loops could remain associated with the major fragment and might contribute to zinc binding. While we cannot exclude the possibility that the other short cytoplasmic loops participate in zinc binding, it is unlikely, since few cysteine or histidine residues exist in those loops. The intradiscal portion of rhodopsin, on the other hand, does contain several residues which might participate in zinc binding.

Experiments were also conducted to demonstrate saturable binding of zinc to rhodopsin. With increasing concentrations of zinc (concentration range 0.05–50 μM - Zn^{2+}) during equilibrium dialysis utilizing Triton X-100-solubilized rhodopsin in the dark, zinc binding to rhodopsin reached saturation at approximately 20 μM -zinc and, at saturation, the molar ratio of bound zinc to

Fig. 5. Scatchard analysis of ^{65}Zn binding to rhodopsin solubilized in cholate detergent

The dialysis buffer contained 20 mM-Hepes, 150 mM-NaCl, 0.5 mM- $MgCl_2$, 0.25 mM- $CaCl_2$ and 0.5% cholate, as well as various concentrations of ^{65}Zn . Rhodopsin was present at 7.7 μM and the experiment was conducted in the dark or under dim red light. The y -intercept of $n(E_i)/K_D$ yields a K_D of approximately 1.4 μM . The x -intercept of $n(E_i)$ indicates $n = 1$. Results shown are from a typical experiment. Each point is the average of several values which did not vary by more than 5%.

photoreceptor molecule was approximately equal to one. Scatchard analysis utilizing cholate-solubilized rhodopsin also demonstrates that there is one binding site per rhodopsin molecule. The data in Fig. 5 show a typical dark experiment using cholate-solubilized rhodopsin with the intercept on the x -axis indicating one binding site per rhodopsin ($n \times E_t$ is the intercept at 7.7 μM -rhodopsin). The y -intercept of $n(E_i)/K_D$ indicates a dissociation constant of 1.4 μM . The binding constant calculated above for disc membranes compares favourably with the K_D obtained with this technique. Equilibrium dialysis has been used by other investigators to establish similar K_D values for zinc binding to other receptor proteins (Cunningham *et al.*, 1990).

DISCUSSION

The results presented in this paper show that zinc can bind directly to rhodopsin. This binding occurs at low (micromolar) concentrations of zinc in the presence of relatively high (millimolar) concentrations of other bivalent cations. Copper, cobalt, cadmium and, to some extent, lead were able to displace zinc from its binding site under our test conditions. Since small amounts of non-radioactive zinc contaminate most preparations, making detection of endogenous zinc in pure protein preparations an uncertain indicator, ^{65}Zn binding may be the best way to determine if zinc will bind to a protein.

The binding constants we obtained using discs and various solubilized-rhodopsin preparations are in agreement if similar assumptions regarding free Zn^{2+} concentrations are made. Our calculated dissociation constants in dark and light are indicators that zinc binds at micromolar concentrations, and that the binding may increase when the protein is bleached (in detergent solutions). The significance of this observation requires further investigation since the behaviour of rhodopsin in detergent solution is not necessarily the same as occurs in a phospholipid environment. Results from Scatchard analysis and saturation

binding of zinc to rhodopsin solubilized in cholate and Triton X-100 respectively support only one binding site per protein.

The K_D that we estimate for zinc binding to rhodopsin is similar to constants estimated for other proteins known to bind and exchange the cation. Zinc has been shown to mediate the interaction between a hormone (prolactin) and its receptor (Cunningham *et al.*, 1990). The K_D for zinc binding to the prolactin receptor, also established by equilibrium dialysis, is in the same range as the K_D that we find for zinc binding to bleached rhodopsin. Zinc is also known to have a procoagulant effect, and both fibrin and fibrinogen bind the cation with K_D values of 8–18 μM (Marx, 1988). Albumin is probably responsible for transporting most of the zinc in plasma and it has a binding constant of 0.1–0.2 μM for zinc (Marx, 1988).

To help determine where the zinc-binding site exists on rhodopsin, we initially attempted to generate proteolytically fragments of the protein, separate those fragments by SDS/PAGE, electrophoretically transblot the resolved polypeptides to nitrocellulose, block, and expose the transblot to ^{65}Zn . Although this technique has been used successfully for other proteins, we found that the zinc-binding site on rhodopsin did not renature during transblotting. We can readily demonstrate substantial binding of zinc to rhodopsin when compared with ovalbumin during equilibrium dialysis, yet both proteins give similar ^{65}Zn binding after SDS/PAGE (denaturation) and transblotting. Our equilibrium-dialysis experiments with proteinase-treated rhodopsin were successful, however, in that they indicate that a large portion of the cytoplasmic tail can be removed without alteration of zinc binding to the protein. This suggests that zinc binds to the intradiscal portion of the protein, a highly conserved region which does not yet have a well-defined role but has been implicated in degenerative disease states such as retinitis pigmentosa (Dryja *et al.*, 1990).

The normal concentrations of zinc present in rod outer segments is still not firmly established. Tam *et al.* (1976) published data indicating that zinc is a normal component of retina, rod-outer-segment (ROS) discs and emulphogene extracts of ROS discs. Their data suggested that zinc was present in discs at concentrations approaching those of calcium and as high as 2.1 g-atoms of zinc/mol of rhodopsin. The data of McCormick (1985), however, indicate that there is only 1 zinc per 6 or 7 rhodopsins.

Tam *et al.* (1976) were able to show a light-dependent increase in zinc in the void volume of gel-filtration columns. They were, however, running emulphogene-solubilized ROS components through a detergent-equilibrated Sephadex G-25 column. Since bleached forms of detergent-solubilized rhodopsin are unstable and usually denature and aggregate if subjected to chromatography, it is difficult to interpret their data. Our data support both of these previous investigations in that we show, by using ^{65}Zn , that rhodopsin can directly bind the bivalent cation and that the binding may be increased by bleaching the solubilized photopigment.

The physiological significance of the rhodopsin binding site for zinc requires investigation. Zinc has been shown to be an important cofactor for many proteins, some of which play important roles in the retina. There is suggestive evidence from diseases that lower metabolic zinc concentrations alter dark adaptation (Morrison *et al.*, 1978; McClain *et al.*, 1979) and that

zinc is involved in dark/light adaptation. Zinc deficiency has been shown to cause degenerative changes in photoreceptors (Leure-duPree & Bridges, 1982; Leure-duPree & McClain, 1982; Tucker *et al.*, 1984) and to decrease the extent of rhodopsin regeneration (Dorea & Olson, 1986). Potential competitors, such as lead, have also been shown to disrupt rod function (Fox & Farber, 1988; Fox & Chu, 1988).

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