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TARGET SIZE ANALYSIS OF RHODOPSIN IN RETINAL ROD DISK MEMBRANES

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SUMMARY: Radiation inactivation of rhodopsin *in situ* using high-energy electrons gave a value for M_r of 20,200 by spectral assay, but 47,100 by assay of rhodopsin regeneration from opsin and 11-*cis*-retinal (sequence M_r=38,840). No light/dark differences were seen. We conclude: (a) radiation inactivation measures the size of the functional unit, and the single hit hypothesis does not hold in our experiments; (b) 500 nm absorbance requires only about half the rhodopsin molecule to be intact, but reconstitution of rhodopsin from opsin requires the whole molecule; (c) we find no evidence for functional interactions between rhodopsin monomers in darkness or light.

Target size analysis is one of the very few techniques that can be used to estimate the size of protein complexes *in situ* in membranes (1). The underlying assumption is the single hit hypothesis which states that a single hit by a high energy electron (from a linear accelerator) will completely destroy a polypeptide, and often a multisubunit protein complex, by internal energy transfer regardless of where on the protein the electron hits. This hypothesis has recently been challenged (2-5).

We wished to use target size analysis to investigate the size of rhodopsin in intact rod outer segment disk membranes. Rhodopsin has been suggested to be monomeric (6-9) or polymeric (10-16), and it has been proposed that it may form multimeric complexes which catalyse ion transport across disk membranes in response to light (17-19). The issue of the aggregation state of rhodopsin *in situ* is therefore not resolved, and is potentially of importance in understanding the mechanism of vision.

In this paper we report that the target size of rhodopsin *in situ* depends on the method of assay. This shows that the single hit hypothesis does not hold in this system. We find no evidence for functionally interdependent rhodopsin polymers in either darkness or light.

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EXPERIMENTAL

Rod outer segments were prepared from fresh bovine eyes by a sucrose/Ficoll 400 flotation procedure as described previously (20,21). They were stored in darkness on ice. $A_{280/500}$ was routinely 2.7-3.5 and yield 2-5 nmol rhodopsin/retina.

Target size analysis. Irradiations were carried out as described by Hughes & Brand (21). Samples were pre-incubated in 1.5 ml Sarstedt tubes for 5 min either in darkness or in daylight at 21°C. They were then rapidly frozen in liquid nitrogen and freeze-dried in darkness in medium supplemented with 10 mM KH_2PO_4 and 5 mM dithiothreitol, then placed in light- and air-tight containers and evacuated to under 0.1 mm Hg. Irradiation with 16 MeV electrons from the Phillips MEL SL75-20 linear accelerator at New Addenbrooke's Hospital, Cambridge was in an air-cooled apparatus in vacuo in darkness. The dose administered to the sample was accurately calibrated (21). After irradiation samples were resuspended in H_2O to the volume present before freeze-drying and assayed.

Rhodopsin assay. $A_{500}-A_{650}$ was measured in a Perkin-Elmer 557 spectrophotometer. The sample was then bleached by exposure to room light and $A_{500}-A_{650}$ was remeasured. The absorbance at 500 nm due to rhodopsin was calculated according to: $(A_{500}-A_{650})_d-(A_{500}-A_{650})_l$, where d is before and l is after full bleaching.

Regeneration of Rhodopsin. After rhodopsin bleaching the all-trans-retinal formed detaches from opsin leaving the chromophore site empty. If 11-cis-retinal is added to the system it recombines with opsin reforming the rhodopsin peak at 498 nm (22). Subsequent addition of 50 mM hydroxylamine converts free excess 11-cis-retinal to retinaloxime which has no significant absorbance at 498 nm. Hence addition of 11-cis-retinal to a fully bleached sample will allow the assay of all the remaining functional opsin molecules. Freeze-dried bleached rod outer segments were resuspended in 2% digitonin (w/v), 67 mM KH_2PO_4, pH 6.4 to a rhodopsin concentration of 0.05-0.15 mM. An aliquot containing 1-3 nmol rhodopsin was then added to 1 ml of 10 mM Hepes(K), pH 7.0. Excess 11-cis-retinal prepared as in (23) was added and samples incubated at 21°C for 3 hours. 50 mM hydroxylamine was then added and after 30 min samples were assayed for rhodopsin. Fig. 1 shows quantitative regeneration of rhodopsin after a

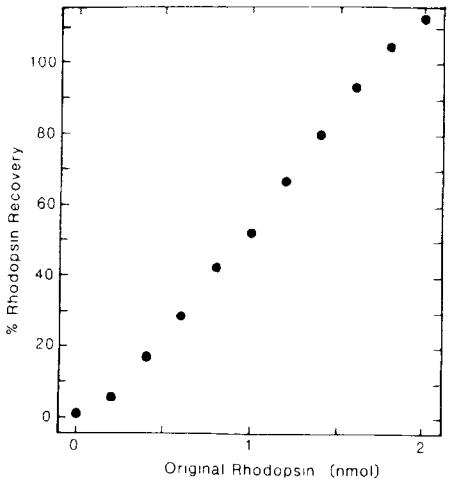

Fig. 1. Regeneration of Rhodopsin. Freeze-dried, bleached rod outer segments were resuspended as described in the text. Aliquots containing various amounts of bleached rhodopsin (calculated from A_{500} of the original preparation) were added to 1 ml of 10 mM Hepes(K), pH 7.4. They were then regenerated to rhodopsin by addition of 11-cis-retinal followed by hydroxylamine and assayed for rhodopsin as described in the text. $A_{500}-A_{650}$ for 2 nmol original rhodopsin was 0.081.

full bleach. In fact recovery was 112%, probably because of the pool of opsin present even in dark-adapted rod outer segments.

Treatment of data. Individual points are averages of all estimates at that dose. Lines are least squares fits to the individual estimates. The D_{37} values were calculated by interpolation. 95% confidence limits of D_{37} were calculated taking into account errors in both slope and intercept of the interpolated line.

RESULTS

Assay by A_{500}. Irradiation of rod outer segments gave rise to an unexpected absorbance at short wavelengths which obscured the 380 nm signal, so direct spectral assay was restricted to A_{500}, i.e. unbleached samples. Fig. 2a shows that unbleached rhodopsin had a target size of 20,200 ± 4,600 M_r (mean ± 95% confidence limit) when assayed by A_{500}

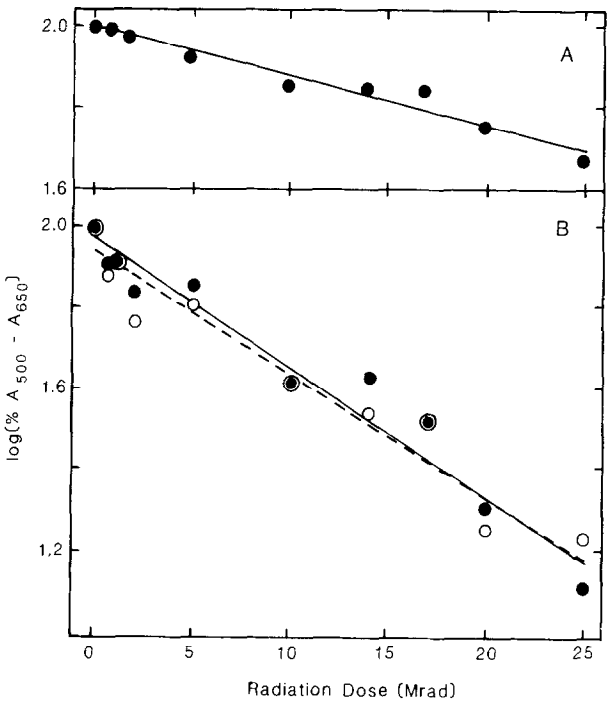

Fig. 2. Target size analysis of rhodopsin. A: Rod outer segments (1-3 nmol rhodopsin) were pre-incubated in darkness, frozen, freeze-dried and irradiated with various doses as described under 'Experimental', then resuspended in 0.1 ml of 67 mM KH_2PO_4, pH 6.4, 2% (w/v) digitonin. Rhodopsin remaining after irradiation was assayed spectrally as described under 'Experimental' by addition of 0.025 ml to 1 ml of 10 mM Hepes(K), pH 7.0. In both A and B points are averages of triplicates from 4 separate experiments. The line represents M_r=20,200. B: Rod outer segment suspension from A after rhodopsin assay was regenerated as described under 'Experimental' and regenerated rhodopsin was assayed (closed circles, solid line, M_r=51,800). Rod outer segments (1-3 nmol rhodopsin) were pre-incubated in daylight for 5 minutes to bleach all rhodopsin present then frozen, freeze-dried, irradiated and resuspended as above. Opsin remaining after irradiation was measured after regeneration as described under 'Experimental' (open circles, dashed line, M_r=53,700).

remaining after irradiation. However from the primary sequence it is known that rhodopsin has an M_r of 38,840 (24), a value which must be increased by 5% owing to glycosylation in vivo. Clearly, assay by remaining A_{500} does not depend on the intactness of the whole rhodopsin molecule, suggesting that only when a critical domain is destroyed is there loss of this signal.

Assay by reconstitution of rhodopsin from opsin and 11-cis-retinal. The regeneration of rhodopsin from 11-cis-retinal and opsin is known to be a better indicator of opsin integrity than the existence of the A_{500} spectral peak (25,26), although careful proteolysis can fragment rhodopsin without preventing regeneration (27). We therefore used the regeneration method to assess the opsin remaining after irradiation. The rod outer segment samples used in Fig. 2a were regenerated by the addition of 11-cis-retinal to reform the 500 nm peak. This was then measured exactly as for the simple assay. (Fig. 2b, closed circles). The target size was 51,800 ± 16,400, substantially higher than was found by the simple spectral assay.

The target size of rhodopsin after light pre-incubation, assayed after regeneration was 53,700 ± 19,700 (Fig. 2b, open circles). Both target sizes were slightly higher than the expected value of about 41,000. Closer examination of the data in Fig. 2b shows that the lines intercept the vertical axis below 100%. This may indicate either a small amount of a higher M_r rhodopsin aggregate or a small loss of rhodopsin due to a free radical effect as seen by Jarvis et al (28). When the target sizes were recalculated assuming that the true 100% value was the intercept point (i.e. that the lines seen represented a single species) the dark M_r was 47,100 ± 14,000 and the light M_r was 43,800 ± 12,400. We have also assessed the target size of bleached rhodopsin in the presence and absence of 5'-guanylylimido-diphosphate (Gpp(NH)p) which removes guanine nucleotide binding protein from rhodopsin (29). We saw no difference in target size in four such experiments (results not shown). Thus there is no significant size change induced by light as detected by this technique.

DISCUSSION

The value of 20,200 for the M_r of unbleached rhodopsin before regeneration appears anomalous, and was not obtained in our preliminary studies (32). It suggests that the single-hit hypothesis is not holding in this experiment. Variation of freeze-drying period or pre-incubation media or the use of various assay regimes did not alter the low target size observed. Perhaps only a small region of the rhodopsin molecule is involved with chromophore binding and generation of the spectrum. Indeed, it is known (27,31,32) that cleavage of the opsin chain and even removal of small portions of the protein with proteases (33) do not alter its spectrum greatly. Hence electrons incident on the large cytoplasmic domain of the protein or some of the alpha-helices not involved in chromophore binding may not destroy the 500 nm absorbance.

The M_r values from the reconstitution assay are close to the M_r expected from sequence data (24), showing that for successful regeneration the whole rhodopsin molecule must be intact. We conclude that the single hit hypothesis is inadequate to account for our results, and that radiation inactivation, at least under some conditions, measures the size of the functional entity needed for the subsequent assay of the remaining activity of the protein. This agrees with similar conclusions reached for invertase (2), glutamate dehydrogenase (3) and (Na,K)-ATPase (4,5). The maximum size of rhodopsin in our studies was that of the monomer. This shows that there is no functional interaction between rhodopsin monomers which affects A_{500} or reconstitution from opsin. Neither is there great energy transfer between monomers of any putative polymer. However, we cannot exclude the possibility that rhodopsin polymers exist *in situ* but remained undetected in our experiments.

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