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THE REDUCTION OF RHODOPSIN WITH SODIUM BOROHYDRIDE UNDER NON-BLEACHING CONDITIONS

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SUMMARY

At 35° or in 2.0 M urea at 3°, an aqueous micellar suspension of rhodopsin will not bleach in the absence of light. However, two sulfhydryl groups previously unreactive with *p*-hydroxymercuribenzoate became reactive, and the bond between the chromophore and the backbone of the molecule may be slowly reduced with $NaBH_4$. Under the mildest conditions, 0.10 M potassium phosphate buffer of pH 7.2 at 35°, the reduction product is *N*-retinylphosphatidylethanolamine. With 0.33 M potassium phosphate buffer of pH 7.2 at 35°, or 2.0 M urea in 0.10 M potassium phosphate buffer of pH 7.2 at 3°, the reduction product is unextractable with organic solvents, suggesting that it is bound to the protein portion of the molecule. This suggests that the bond between the chromophore and the backbone of the molecule is very labile, but does not involve phosphatidylethanolamine directly in the Schiff base linkage.

INTRODUCTION

Rhodopsin cannot be reduced by $NaBH_4$ unless the pigment is previously bleached. When an aqueous digitonin micellar suspension of bovine metarhodopsin$_{380}$ II is treated with $NaBH_4$ in the pH range 6.0–8.0, a reductive affixation of the chromophore occurs[1,2], the retinylidine group being covalently bound to the ε-amino group of a lysyl unit in the backbone protein[3,4]. In contrast to the behavior of illuminated rhodopsin, a quantitative yield of *N*-retinylphosphatidylethanolamine was obtained with methanol extracts of either heat-denatured, acid-buffered (pH 4.5) native rod outer segments or aqueous cetyltrimethylammonium bromide micellar suspensions of rhodopsin, that had been reduced with $NaBH_4$ (refs. 5, 6). Similar results were obtained with metarhodopsin$_{478}$ I, but extraction of the chromophore of metarhodopsin$_{380}$ II under the same experimental conditions resulted in a product in which the chromophore was bound to the ε-amino group of lysine[7].

The studies described in this report were undertaken to determine the nature of the product obtained when aqueous micelles of rhodopsin are reduced with $NaBH_4$ under conditions in which the rhodopsin is not bleached: incubation at 35°, in the dark.

Abbreviation: PHMB, *p*-hydroxymercuribenzoate

MATERIALS AND METHODS

Emulphogene BC-720, a polyoxyalkylene ethanol, was obtained from General Aniline and Film Co., New York, N. Y. Ultra-pure urea was purchased from Mann Research Laboratories, New York, N. Y. $NaBH_4$ was obtained from Ventron, Metal Hydrides Division, Beverly, Mass. Digitonin was purchased from Fisher Chemical Company, New York, N. Y. Silica gel G was obtained from Bio-Rad Laboratories, Richmond, Calif.

Spectra were recorded using a Gilford 2000 spectrophotometer (Gilford Instruments, Oberlin, Ohio). Fluorescence of the retinylidene derivative was detected using a Blak-ray UVL-22 long ultraviolet lamp (Ultra-Violet Products Incorporated, San Gabriel, Calif.).

Determination of sulfhydryl groups

Sulfhydryl groups were estimated spectrophotometrically with *p*-hydroxymercuribenzoate (PHMB)[8] as applied to rhodopsin[9].

Reaction with $NaBH_4$

Solutions containing approximately 80 nmoles of rhodopsin in either 2 % Emulphogene BC-720 in 0.10 M potassium phosphate buffer of pH 7.2, or in 2 % Emulphogene BC-720 in 0.33 M potassium phosphate prepared as previously described[9], were allowed to equilibrate for 5 min in a 35° shaking water bath in the dark. In addition, solutions containing approximately 80 nmoles of rhodopsin in Emulphogene BC-720 in 0.10 M potassium phosphate buffer of pH 7.2, containing 2.0 M urea were equilibrated for 5 min, at 3° in the dark.

Approximately 2 mg of dry $NaBH_4$ was added to each tube which was then capped with a bleeder valve, and the reaction allowed to proceed for 20 min. The absorbance was then measured at 498 nm using the Gilford spectrophotometer and the process was repeated until the absorbance at 498 nm decreased to 10–20 % of its original value. This took approximately 4 h for the samples in 0.1 M buffer, and less than 1 h for the samples in 0.33 M buffer, or in 0.1 M buffer and 2.0 M urea. The pH was never allowed to rise above 8.0, being adjusted to pH 7.0–7.2 with concentrated HCl, with rapid stirring, when necessary. This adjustment was not necessary when 0.33 M potassium phosphate buffer was used.

Spectra were recorded after centrifugation, when necessary at 30000 × *g* for 10 min, and corrected for the small dilution due to the addition of HCl. The precipitation noted was minor and due mainly to the effect of the increased salt concentration on the detergent. The precipitate exhibited almost no fluorescence. Controls to which no $NaBH_4$ was added were run simultaneously.

Extraction of reduced chromophore

After completion of the reaction, the pH was brought to 6.0 with concentrated HCl and the solutions incubated at 3° for 120 min in the dark to remove any excess $NaBH_4$. They were then examined for fluorescence of the retinylidene derivative under ultraviolet emission from a long ultraviolet lamp, bleached by exposure to light from three 100-W incandescent bulbs at a distance of 15 inches for 10 min at room temperature, and chilled at 0° for approximately 2 min. After two extractions with 6

vol. of a 2:1 chloroform:methanol mixture, followed by centrifugation at 1200 × *g* for 5 min, they were again examined for fluorescence. If fluorescence was found in the organic layers, the solvent was removed from it under a stream of nitrogen, followed by exposure to vacuum at 4° for 5 min. The dried reduced chromophore was immediately redissolved in 1.4 % digitonin in 0.10 M sodium phosphate buffer of pH 7.2 and the spectrum recorded, or immediately redissolved in chloroform and identified by thin-layer chromatography.

Thin-layer chromatography

The identity of the products extracted in organic solvents was established by thin-layer chromatography on Silica gel G, using appropriate solvent systems[6]. The spots were examined for fluorescence as above, and then sprayed with concentrated H_2SO_4 (ref. 10). With this spray all lipids, as well as other organic non-volatile compounds, stain as dark-brown or black spots on a white background.

RESULTS

Determination of sulfhydryl groups

Rhodopsin solubilized in Emulphogene in either 0.10 M potassium phosphate buffer of pH 7.2 or 0.33 M potassium phosphate buffer of pH 7.2, contained 0.9–1.02 sulfhydryl groups available for reaction with PHMB at 20° or 32°; 2.0–2.2 more sulfhydryl groups became reactive at 35° or in 2.0 M urea. No change in absorbance at 498 nm was observed during the course of the reaction. When the pigment was bleached by exposure to light, 2.0–2.3 more sulfhydryl groups became reactive.

Reaction with $NaBH_4$

Fig. 1 indicates the shifts in spectrum which occurred during reaction of rhodopsin solubilized in 2 %'Emulphogene in 0.1 M potassium phosphate buffer of pH 7.2 with $NaBH_4$. When the reaction was stopped, the absorption at 498 nm had decreased to 10–20 % of its original value, while absorption at 325 nm had increased concomitantly. The controls showed less than a 10 % decrease in absorbance at 498 nm and less than a 5 % increase in absorbance at 325 nm over the same period. The spectra contain no isosbestic point, suggesting the presence of intermediates with absorbing half-lives sufficient to show this effect. Similar spectra were recorded when the solutions were in 0.33 M potassium phosphate buffer of pH 7.2, or in 2.0 M urea and 0.1 M potassium phosphate buffer of pH 7.2, or in 2.0 M urea and 0.1 M potassium phosphate buffer of pH 7.2. The rates of reaction, however, were faster under either of these conditions than in 0.1 M phosphate buffer alone.

Extraction of reduced chromophore

When the reaction mixture contained 0.10 M potassium phosphate buffer of pH 7.2, all the reduced chromophore was extracted in the organic layer, the aqueous layer exhibiting no fluorescence. The reconstituted lower layer exhibited a spectral maximum at 325 nm, characteristic of *N*-retinylphosphatidylethanolamine. More than 70 % of the reduced chromophore was recovered, based upon an ε-valve of 50000 at 325 nm (ref. 3).

When the reaction mixtures contained either 0.33 M potassium phosphate

buffer of pH 7.2 or 2.0 M urea, all the reduced chromophore was found as a precipitate at the interface between the organic and aqueous phases, the organic layer exhibiting no fluorescence, implying binding of the chromophore to the protein.

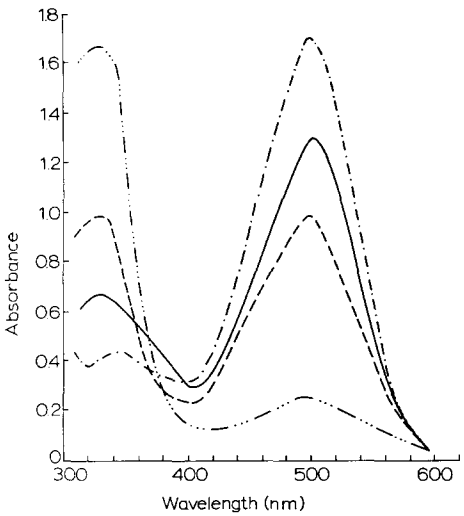

Fig. 1. Formation of $NaBH_4$ reduction product of rhodopsin with time. Rhodopsin, solubilized in Emulphogene BC-720 in 0.1 M phosphate buffer, was incubated at 35°, in the dark, with successive 2-mg additions of $NaBH_4$ every 20 min. Spectra were recorded initially (–·—·–), at 1 h (———), 2h (— — —) and 4 h (—· · ·—) after centrifugation at 30000 × *g* for 10 min when necessary.

That the increase in concentration of phosphate buffer or the addition of 2.0 M urea did not cause irreversible shifting of the chromophore from a binding site on the lipid to a binding site on the protein was indicated by the recovery of the reduced chromophore from the organic layer in samples which were allowed to incubate in 0.33 M potassium phosphate buffer at 35° or in 2.0 M urea in 0.10 M potassium phosphate buffer at 3°, for 1 h, in the dark, dialyzed against two changes of water at 3°, made to 0.10 M potassium phosphate buffer and reacted with $NaBH_4$ at 35° as above.

Thin-layer chromatography

Two spots were found in each of the systems examined, the major spot (approximately 70 % of the total) corresponding to *N*-retinylphosphatidylethanolamine and the minor spot to retinaldehyde; the retinaldehyde being non-fluorescent. The R_F values were the same as those previously reported, ±5 % (ref. 6).

DISCUSSION

At 35° or in 2.0 M urea at 3°, rhodopsin in aqueous micellar detergent suspensions will not bleach in the absence of light. There is, however, some change in the conformation of the protein, as indicated by the appearance of two sulfhydryl groups reactive toward PHMB and the ability of $NaBH_4$ to reduce the Schiff base linkage between the chromophore and its site of attachment.

It appears that the bond between the chromophore and the lipoprotein is very labile, and that under conditions of prolonged incubation retinylidene exchange occurs. These findings, together with other recent studies[11,12], indicate that phosphatidylethanolamine is not directly involved in a Schiff base linkage.

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