DARK BLEACHING OF RHODOPSIN BY ORGANIC MERCURIAL

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1. Introduction

Previous work [1–3] has shown that *p*-mercuribenzoate at concentrations of $1-7 \times 10^{-4}$ M does not markedly effect the 498 nm absorption maximum of bovine rhodopsin. This implies that either SH-groups are not involved in the interaction between the chromophore (11-*cis*-retinal) and the protein (opsin) or, if they are, that they are in regions inaccessible to the mercurial. Other workers have shown that rhodopsin in digitonin solution is stable in the presence of urea in concentrations of up to 8 M [3, 4], but it is bleached in 2–5 M urea when solubilized with cetyltrimethyl ammonium bromide [5]. Guanidine hydrochloride (a stronger protein denaturant than urea) has been shown to bleach rhodopsin in the dark in digitonin solution [4]. This work was undertaken to determine the effect of varying concentrations of p-mercuriphenylsulfonate (PMPS) on rhodopsin. It will be shown that higher concentrations of PMPS, which is more soluble than *p*-mercuribenzoate, can considerably bleach rhodopsin in digitonin solution in a unique way, and that this bleaching is enhanced in the presence of urea.

2. Materials and methods

Rhodopsin was prepared from frozen bovine retinas (G. Hormel Company, Austin, Minn.) by the method of Shichi et al. [6] with several modifications. After the initial grinding, 1/2 teaspoon of alumina per 10 retinas was added to the mortar, and the mixture was reground to a uniform consistency. This mixture was treated as described, with the following exceptions. Treatment with alum was for 20 min at 4°C. Lyophilization, petroleum ether extraction and column purification were omitted. Digitonin (Sigma) extraction was as described [7], but done overnight at 4°C. PMPS (Sigma), urea (Baker) and guanidine hydrochloride (Eastman) were used as obtained. All absorption spectra were taken using a Cary 14 spectrophotometer.

3. Results

When rhodopsin was treated with 2.5×10^{-2} M PMPS, dark bleaching of the pigment occurred gradually. As fig. 1a shows, the absorbance at 500 nm was considerably reduced after 8 hr, with a concomitant increase in absorbance at 375 nm. The increase at 375 nm lagged behind the decrease at 500 nm, with little or no increase occurring during the first hour (induction period), even though there was a substantial decrease at 500 nm during this time. This initial decrease in absorbance was accompanied by a slight shift in absorption maximum from 498 nm to around 495 nm, apparently similar to a rapid spectral shift observed by Hubbard [4] with guanidine. The bleaching was not reversed by adding either 2-mercaptoethanol or glutathione. With 6 M guanidine, dark bleaching was more rapid than with 2.5×10^{-2} M PMPS, and no induction period was observed in the absorbance increase at 375 nm.

Reducing the concentration of PMPS to $10^{-3}-10^{-2}$ M decreased the rate of bleaching. Fig. 2a shows that the logarithm of the pseudo first order rate constant for bleaching increased linearly with the logarithm of the PMPS concentration. The slope of the line was 1.0;

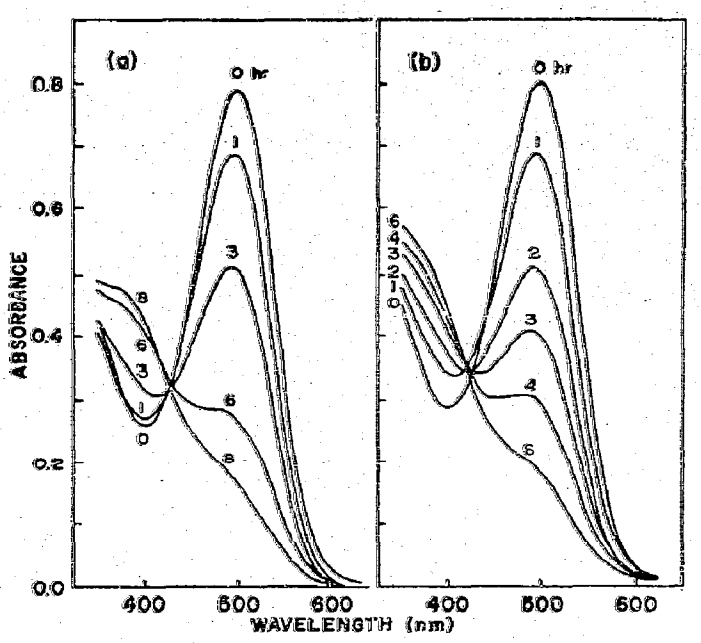

Fig. 1. Dark bleaching of rhodopsin in 1.4% digitonin phosphate buffer (pH 6.8) by PMPS in the absence and presence of 2 M urea: (a) 2.5×10^{-2} M PMPS; (b) 10^{-3} M PMPS + 2 M urea.

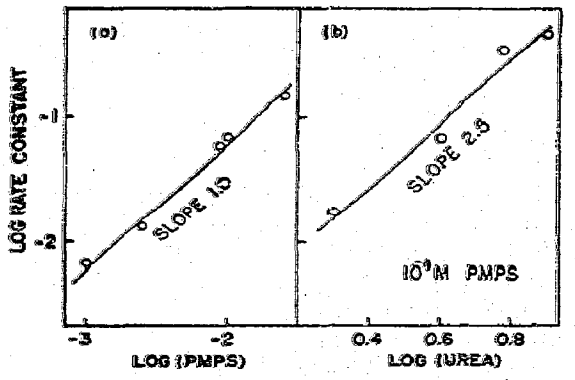

Fig. 2. Log–log plots of the bleaching rate constant against the PMPS concentration ($10^{-3} - 2.5 \times 10^{-2}$ M) (a) and against the urea concentration (2–8 M) in the presence of 10^{-4} M PMPS (b).

i.e., the rate constant was directly proportional to the PMPS concentration. This indicates that the bleaching is the result of the bimolecular interaction between rhodopsin and PMPS. It appears that one chromophore molecule is bleached when one specific SH-group of rhodopsin is blocked by PMPS.

Rhodopsin in digitonin solution was stable in the presence of 2 M urea, and only bleached slightly with

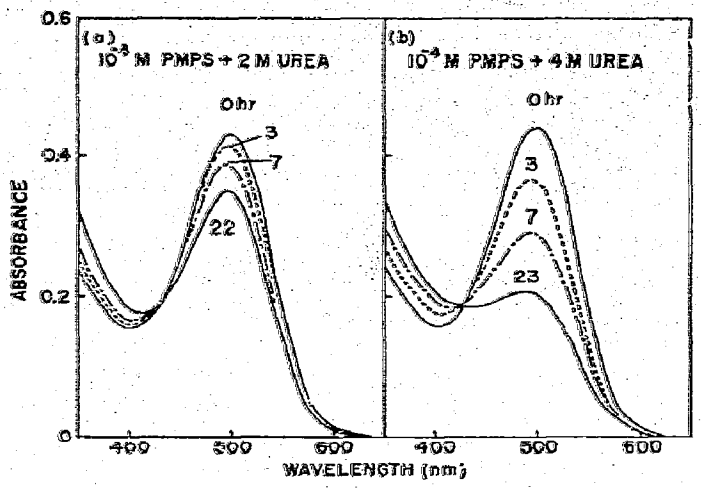

Fig. 3. Dark bleaching of rhodopsin by 10^{-4} M PMPS in the presence of 2 M urea (a) and 4 M urea (b).

10^{-3} M PMPS (less than 5% after 6 hr). When these reagents were combined in the above concentrations, however, dark bleaching of rhodopsin occurred at a rate greater than that caused by 2.5×10^{-2} M PMPS (fig. 1b), showing that urea is able to enhance the effect of PMPS. During the first hour, a lag was observed in the decrease in absorbance at 500 nm, when plotted against time, indicating that the enhancement effect of urea is being developed. With 10^{-4} M PMPS, the bleaching was very slight. In the presence of 2–4 M urea, however, the bleaching was accelerated as shown in fig. 3. Fig. 2b shows a log–log plot of the rate constant for bleaching against the urea concentration, giving a straight line of slope 2.5. This indicates that the bleaching reaction is of the 2.5th order with respect to urea. The similar reaction order of 2.4 was found by Giroch and Rabinowitch [5] in the bleaching of rhodopsin by urea alone in 5% cetyltrimethyl ammonium bromide. The data obtained by Hubbard [4] for the guanidine-promoted denaturation of rhodopsin are consistent with approximately 6th-order kinetics.

4. Discussion

Current investigations indicate the presence of three and possibly four different types of SH-groups in rhodopsin. Type I groups are reactive in native unbleached rhodopsin. While some workers [1, 8] did not demonstrate the existence of this type, others [2, 7, 9–11]

showed that it contains one to four SH-groups. When these SH-groups were labeled with fluorescent iodoacetamide and disulfide derivatives, the absorption maximum of rhodopsin was not altered [11]. Two type II groups become reactive after delipidation [10] or treatment with 2 M urea [2]. One to four type III groups become reactive after photobleaching [1, 2, 8–10]. These groups may be located in the vicinity of the chromophore-protein Schiff base linkage [12], and may even be involved in the linkage itself forming a substituted aldimine bond [8, 13]. Finally, two type IV groups become reactive after the bleached rhodopsin is denatured in 8 M urea [10].

Assuming the molar absorption coefficient of rhodopsin in digitonin solution as 40 000 M^{-1} cm^{-1} at 500 nm [14], the concentration of rhodopsin used in this study is estimated as $1-2 \times 10^{-5}$ M. It follows that 10^{-4} M PMPS is stoichiometrically comparable to 5–10 SH-groups per rhodopsin molecule. This concentration of mercurial does not appreciably perturb the absorption maximum of rhodopsin in digitonin solution. Increasing the concentration of PMPS to 10^{-2} M or greater appears to increase penetration of the mercurial inward to type II or III SH-groups, causing modification of the chromophore region. This is observed as a drop in absorbance at 500 nm without a concurrent rise occurring at 375 nm during the early stage of reaction. Subsequent further modifications of the protein lead to the ultimate release of free 11-*cis* retinal absorbing at 375–380 nm. With lower concentrations of PMPS ($1-2.5 \times 10^{-3}$ M) the extensive modification does not fully occur.

High concentrations of urea will denature many proteins by causing a weakening of hydrogen bonds, resulting in a loss of tertiary structure. Even though urea renders type II SH-groups reactive [2], the stability of the absorption peak of rhodopsin in digitonin solution is not affected. As the non-bleaching partial denaturation of rhodopsin by urea proceeds, the chromophore area becomes more susceptible to attack by PMPS. This is analogous to the situation observed by Zorn [2] where 2 M urea allows the chromophore–protein bond to be reduced by borohydride, a process usually requiring concomitant photobleaching of rhodopsin [15, 16].

The finding reported here suggest that one specific SH-group, rather well protected, plays some role in maintaining the special integrity of rhodopsin. It may either participate in or help stabilize the chromophore–protein interaction responsible for the characteristic 500 nm absorption.

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