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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXVIII. CLASSIFICATION OF SULFHYDRYL GROUPS IN RHODOPSIN AND OTHER PHOTORECEPTOR MEMBRANE PROTEINS

W. J. DE GRIP*, S. L. BONTING and F. J. M. DAEMEN

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen (The Netherlands)

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SUMMARY

Reaction of isolated bovine rod outer segment membrane with radioactive *N*-ethylmaleimide, both in the presence and absence of 1 % dodecyl sulfate followed by dodecyl sulfate-polyacrylamide gel electrophoresis, shows that six sulfhydryl groups (96 % of total sulfhydryl in this membrane) are located on the rhodopsin molecule.

On the basis of their reactivity towards *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate in suspensions of outer segment membranes, the sulfhydryl groups of rhodopsin can be divided into three pairs. One pair is rapidly modified, both in light and darkness. This modification does not impair the recombination capacity of opsin with 11-*cis* retinaldehyde under regeneration of rhodopsin. A second pair is modified upon prolonged interaction with the *p*-chloromercuri-derivatives in darkness. Modification of this pair leaves the typical rhodopsin absorbance at 500 nm intact, but a proportional loss of recombination capacity does occur. The third pair is only modified after illumination and is probably located in the vicinity of the chromophoric center.

The difference between these results and those obtained by modification with dithiobis-(2-nitrobenzoic acid) or *N*-ethylmaleimide in suspension, where even upon prolonged exposure to light as well as in darkness only two sulfhydryl groups of rhodopsin are modified, is explained by the detergent-like character of the *p*-chloromercuri-derivatives.

INTRODUCTION

In a previous paper [1], we have shown that about six sulfhydryl groups are present per mole of rhodopsin in the bovine rod photoreceptor membrane. In mem-

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

* Present address: Biological Laboratories, Harvard University, Cambridge, Mass. 02138, U.S.A.

branes, suspended in aqueous media, two of these six groups are immediately accessible to the water-soluble sulfhydryl reagents 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) and *N*-ethylmaleimide, in darkness as well as upon illumination. Additional groups become accessible to these reagents upon treatment with detergents. The uncovering of additional sulfhydryl groups upon illumination of rhodopsin, solubilized in detergents [2–6], was shown to be a detergent effect.

Now we have investigated, by means of reaction with radioactive *N*-ethylmaleimide, which protein species in the membrane contribute to the sulfhydryl group population. In an effort to distinguish these sulfhydryl groups further, in addition to DTNB and *N*-ethylmaleimide the more aggressive sulfhydryl reagents *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate have been employed.

MATERIALS AND METHODS

Materials

p-Chloromercuribenzoate is obtained from Fluka (Buchs, Switzerland), *p*-chloromercuribenzene sulfonate and DTNB are obtained from Sigma (St. Louis, Mo. U.S.A.), Triton X-100 is a product of BDH (Poole, U.K.). *N*-ethylmaleimide and sodium dodecyl sulfate are obtained from Merck (Darmstadt, Germany). *N*-ethyl-[1-^{14}C]maleimide (spec. act. 11 Ci/mol) is obtained from NEN (Dreiecheinhain, Germany).

Isolation of rod photoreceptor membranes

Bovine rod photoreceptor membranes are isolated by means of the procedure previously described [7], during which all opsin present is converted into rhodopsin by treatment with 11-*cis* retinaldehyde. Sucrose density gradients with a density range of 1.08–1.15 (rather than 1.05–1.18) have been employed. After isolation from the gradient, the layer containing the rod outer segments is diluted with 0.5–1 vol. Tris/HCl buffer 0.16 M (pH 7.4), containing 1 mM $CaCl_2$, 4 mM $MgCl_2$ and 1 mM dithioerythritol. The rod outer segments are then sedimented (10 °C, 4000 × *g*, 15 min) and the sediment is stored overnight at 4 °C. Next it is twice washed with doubly distilled water, sedimented each time (0 °C, 45 000 × *g*, 30 min) and suspended in phosphate buffer (0.2 M, pH 7.0) to a final concentration of $8 \cdot 10^{-5}$M rhodopsin. The resulting suspension is immediately used for the modification experiments.

Modification of sulfhydryl groups

To the suspension of photoreceptor membranes in 0.2 M phosphate buffer (pH 7.0), a 10–100 fold molar excess of *N*-ethylmaleimide or DTNB or a 2–200 fold molar excess of *p*-chloromercuribenzoate or *p*-chloromercuribenzene sulfonate with respect to rhodopsin is added. The reagents are added as a freshly prepared 0.05–0.1 M solution in the same phosphate buffer. *p*-Chloromercuribenzoate is first dissolved in a small amount of 0.1 M NaOH and the solution is quickly brought to the desired volume by addition of 0.2 M phosphate buffer (pH 7.0). The reaction time is varied between 10 min and 24 h at 20 °C, after which the preparation is rapidly diluted with 20 vol. ice-cold phosphate buffer (0.2 M, pH 7.0) and centrifuged (0 °C, 80 000 × *g*, 10 min). The sediment is twice washed with doubly-distilled water and precipitated each time by centrifugation (0 °C, 80 000 × *g*, 20 min). If necessary, the

sediment is stored overnight at 4 °C. Finally, the sediment is suspended in the original volume of the same phosphate buffer, and the sulfhydryl- and rhodopsin contents and the recombination capacity of rhodopsin are determined. Occasionally, one half of the suspension is treated with 10 mM dithioerythritol in the same phosphate buffer (1 h at 20 °C) in order to remove all reacted reagent. The resulting preparation is subjected to the same analytical procedure.

Under the conditions of pH and temperature used in this study, *N*-ethylmaleimide, DTNB, as well as the chloromercuri-derivatives do not react with disulfide bridges.

Gel electrophoresis

Polyacrylamide gel electrophoresis is performed according to Osborn and Weber [8] or to Laemmli et al. [9] with gels varying in gel percentage between 9 and 13.5 % (5×90 mm). In the Laemmli procedure, stacking gels are not applied. The gels are subjected to pre-electrophoresis for 1.5 h and then loaded with 50 µl of a solution composed of Tris/HCl buffer (12 mM, pH 6.8), containing 2 % (w/w) sodium dodecyl sulfate, 0.01 % (w/w) bromophenol blue and 20 % (v/v) glycerol, in which 5–20 µl of photoreceptor membrane suspension is dissolved to a maximal rhodopsin concentration of 0.22 % (w/v). For molecular weight determinations the gels are standardized with a mixture of phosphorylase A (mol. wt 94 000), bovine serum albumin (68 000), phosphoenolpyruvate kinase (57 000), ovalbumin (43 000), α-chymotrypsinogen (25 700) and β-lactoglobulin (18 200). The gels are first run at 1 mA each for 30 min and then at 2–3 mA each. After completion of electrophoresis the gels are stained with Coomassie Blue R-250 according to Fairbanks et al. [10].

When ^{14}C-labeled membranes are analysed by electrophoresis, one gel is subjected to the staining and destaining procedures and the other one is cut in 2–3 mm slices. The slices are placed in counting vials and extracted for some hours at 37 °C, either with 900 µl Tris/HCl buffer (62 mM, pH 6.8) containing 2 % sodium dodecyl sulfate or with 3 ml concentrated formic acid. The formic acid is evaporated by a hot air stream. After addition of 10 ml Insta-Gel (Packard) the vials are counted in a Philips scintillation counter. The two extraction procedures give similar results. The extraction with dodecyl sulfate solution is somewhat less time consuming, but the presence of the buffer solution decreases the counting efficiency.

Analytical methods

The rhodopsin content of the samples is determined as previously described [7]. After addition of 25 µl suspension to 200 µl 0.2 M phosphate buffer (pH 7.0) containing 2 % Triton X-100 and 0.05 M hydroxylamine, the 500 nm absorbance (A_{500nm}) is determined before and after illumination. The recombination capacity of rhodopsin, i.e. the relative amount of rhodopsin regenerated upon addition of 11-*cis*-retinaldehyde to a photolyzed sample, is determined as previously described [7].

The sulfhydryl content of the samples is determined with DTNB (Ellman's reagent) as previously described [1], except that the reaction is performed at pH 7.0 instead of 8.0. This decreases the reaction rate slightly, but renders other conditions more suitable. Following modification with high concentrations of *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate, determination of residual sulfhydryl

groups poses a problem, especially with the more hydrophobic benzoate, since all excess reagent must be removed completely before the assay. Reliable results are possible up to a 30-fold molar excess (with respect to rhodopsin) of the benzoate and up to a 100–200-fold molar excess of the sulphonate.

RESULTS

Reaction with DTNB and N-ethylmaleimide

Photoreceptor membrane suspensions have been incubated with a 10–100-fold molar excess (based on rhodopsin) of DTNB and *N*-ethylmaleimide for 30 min to 24 h at 20 °C. The reaction appears to reach completion in 3–4 h with DTNB and in 6–8 h with *N*-ethylmaleimide, when in each case 2.1 ± 0.2 ($n = 4$) sulfhydryl groups are modified, expressed per mol of rhodopsin present. Increasing the excess of the reagent from 10 to 100-fold makes no difference in the number of sulfhydryl groups modified. Modification of these two sulfhydryl groups, whether carried out in darkness or in light, does neither seriously influence the recombination capacity, nor the rate of recombination of the visual pigment with 11-*cis* retinaldehyde (Fig. 1).

The distribution of the sulfhydryl groups over the various membrane components has been studied by reaction with *N*-ethyl-[1-^{14}C]maleimide, followed by separation of the membrane proteins by means of polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. A typical experiment, carried out in suspension (Fig. 2A), shows that about 85 % of the total radioactivity on the gels coincides with the rhodopsin band (mol. wt 37 000–38 000), while 5 % coincides with a weak band in the 70 000 mol. wt range. Another 5 % remains on top of the gel, and probably represents polymerized material. Thus, about 90 % of the radioactivity which has entered the gel is present in the rhodopsin band, equivalent to 1.8–1.9 sulfhydryl group. Assuming that the band of 70 000 dalton represents a rhodopsin dimer, as argued below, then 96 % of the total radioactivity, equivalent to 2.0 sulfhydryl

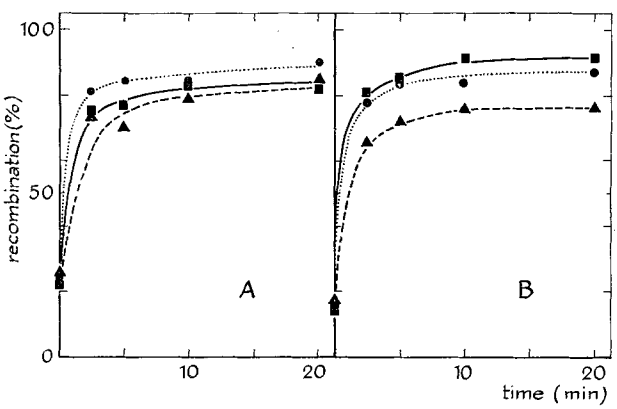

Fig. 1. Rate of recombination of opsin and 11-*cis* retinaldehyde of rod outer segment membranes with and without sulfhydryl group modification. A: modification in darkness (as rhodopsin), removal of excess reagent, illumination, incubation with 11-*cis* retinaldehyde. B: modification in the light (as opsin), removal of excess reagent, incubation with 11-*cis* retinaldehyde. Modification with DTNB: ▲- - -▲; modification with *N*-ethylmaleimide: ■- - -■; control: ●- - -●.

groups, is located on rhodopsin itself. Very weak bands of radioactivity, each representing < 1 % of the total activity, are observed with mobilities corresponding to mol. wts of 18 000, 28 000 and 100 000, respectively.

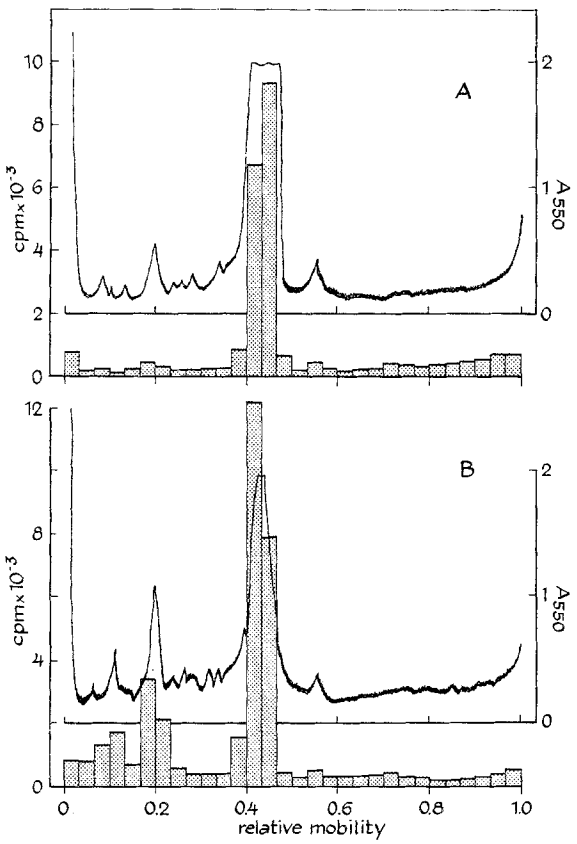

Fig. 2. Dodecyl sulphate-polyacrylamide gel electrophoresis of rod outer segment membranes, chemically modified with *N*-ethyl-[1-^{14}C]maleimide, either in suspension (A) or in 1 % dodecyl sulphate (B). Shaded bars: radioactivity of slices of 2.6 mm; recorded tracing: absorbance at 550 nm after Coomassie Blue staining. Gel percentage 10 %; loading 55 (A) and 35 (B) μg of protein, respectively. Separate gels are used for radioactivity measurement and scanning at 550 nm.

In order to assess the distribution of all sulfhydryl groups in photoreceptor membranes, including those not normally exposed to the aqueous environment, reaction with labeled *N*-ethylmaleimide is performed in 1 % dodecyl sulphate (Fig. 2B). Now, the major part of the radioactivity in the gel is distributed over three bands with mol. wts of 37 000 (71 % of the radioactivity), 72 000 (17 %) and 98 000 (8 %), presumably the monomer, dimer and trimer of rhodopsin, respectively.

The occurrence of dimers and trimers of rhodopsin on the gels, especially after modification in dodecyl sulfate, is explained by the absence of dithioerythritol during modification, washing and electrophoresis. This reagent, normally added to prevent association of protein monomers, can obviously not be used, when modifi-

cation and quantitative determination of sulfhydryl groups is desired. Therefore, 96 % of the sulfhydryl groups present in photoreceptor membranes, appear to be located on the rhodopsin molecule.

Reaction with p-chloromercuri derivatives

Application of a 2–3 fold molar excess of *p*-chloromercuribenzoate with respect to rhodopsin to a suspension of photoreceptor membranes yields similar results as obtained with DTNB and *N*-ethylmaleimide: 1.5–2 sulfhydryl groups are modified without any effect on the recombination capacity. Addition of a larger excess of reagent leads to modification of additional sulfhydryl groups, illuminated membranes being more susceptible than membranes kept in darkness. In the latter case, reaction with a 12-fold molar excess of *p*-chloromercuribenzoate leads at first to a rapid modification of two sulfhydryl groups, without any effect on spectral and recombination properties of rhodopsin (Fig. 3). This is followed by additional slow modification of 1–1.5 sulfhydryl group, concomitant with a decrease in the recombination capacity to 40 %. In illuminated membranes under similar circumstances we observe a rapid modification of all 6 sulfhydryl groups, leading to a total loss of recombination capacity (Fig. 3). All reacted *p*-chloromercuribenzoate can be removed by 1 h incubation with 10 mM dithioerythritol at 20 °C with partial reversal of the decrease in recombination capacity. The recombination capacity, decreased to 40 % upon reaction with *p*-chloromercuribenzoate in darkness, is increased to 70–80 %, while the complete loss of recombination capacity suffered by illuminated membranes is restored up to 40 %. This is only the case upon immediate treatment with dithioerythritol. When treatment is postponed for 24 h after reaction with *p*-chloromercuribenzoate, virtually no restoration of recombination capacity is observed.

The decrease in recombination capacity is dependent on the molar excess of *p*-chloromercuri-derivative. Membrane suspensions are treated with *p*-chloromercuribenzoate and its less hydrophobic analogue *p*-chloromercuribenzenesulfonate for 30 min at 20 °C. With increasing molar excess of both reagents the recombination capacity is further decreased (Fig. 4). Membranes kept in darkness are more resistant than illuminated membranes, with the sulfonate acting less effectively than the benzoate. When kept in darkness, rhodopsin remains spectrally intact at all reagent concentrations used.

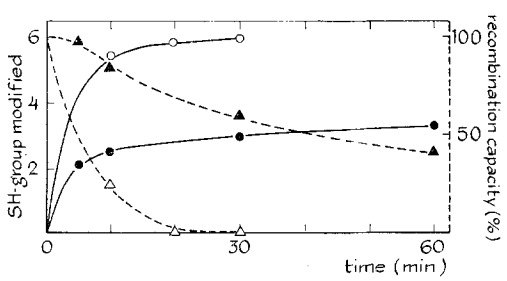

Fig. 3. Number of sulfhydryl groups modified per mol rhodopsin (circle, solid line) and recombination capacity (triangles, broken line) of outer segment membranes upon treatment with a 12-fold molar excess of *p*-chloromercuribenzoate at 20 °C (pH 7.0). Filled symbols: modification in darkness (as rhodopsin); open symbols: modification in the light (as opsin).

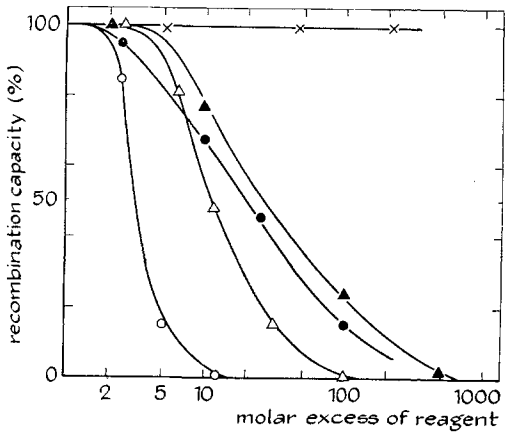

Fig. 4. Recombination capacity of rod outer segment membrane upon treatment with varying concentrations of *p*-chloromercuribenzoate (○, ●) and *p*-chloromercuribenzene sulphonate (△, ▲). Filled symbols: modification in darkness (as rhodopsin); open symbols: modification in the light (as opsin). Modification with DTNB or *N*-ethylmaleimide (×) has no influence on the recombination capacity, both in darkness and in the light. All incubations at 20 °C, pH 7.0, during 30 min. For further details: see text.

The relation between the number of modified sulfhydryl groups and the remaining recombination capacity is illustrated in Fig. 5. For membranes kept in darkness a linear relationship between these two parameters exists, running from 2.1 modified sulfhydryl groups without loss of recombination capacity to 4.2 modified groups with full loss of recombination capacity. For illuminated membranes, a similar relationship seems to exist down to about 20 % remaining recombination capacity; in this case the data suggest that substitution of sulfhydryl groups by thiomercuribenzoate has somewhat more impact on the recombination capacity

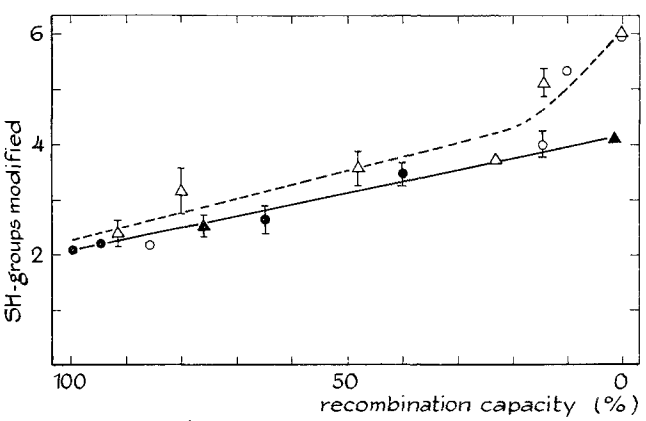

Fig. 5. Relationship between number of sulfhydryl groups modified in outer segment membranes by *p*-chloromercuribenzoate (circles) and *p*-chloromercuribenzene sulphonate (triangles) and the recombination capacity. Filled symbols: modification in darkness (as rhodopsin); open symbols: modification in the light (as opsin). Most points represent the average of 2–3 experiments (with S.E.). Modification conditions: 20 °C, pH 7.0, varying incubation times and reagent concentrations.

than substitution by thiomercuribenzene sulphonate. While in darkness two of the six sulfhydryl groups are never modified during reaction with either chloromercuri-derivative, all six can be rather easily modified in illuminated membranes, as mentioned before (Fig. 3).

DISCUSSION

Location of sulfhydryl groups

The experiments with labeled *N*-ethylmaleimide show that nearly all (96 %) of the sulfhydryl groups of photoreceptor membranes are located on the rhodopsin molecule, indicating a total number of six sulfhydryl groups per rhodopsin molecule. According to a cautious estimate, no more than 0.3 sulfhydryl group (per rhodopsin molecule) are located on other membrane proteins. One of these proteins is the enzyme retinol dehydrogenase (retinol: NAD^+ oxidoreductase EC 1.1.1.105), which is completely inactivated by reaction with DTNB or *N*-ethylmaleimide in suspension (de Grip et al., to be published). Since 96 % of the sulfhydryl groups belong to rhodopsin, all further experiments discussed here can be considered to refer to the rhodopsin sulfhydryl groups.

Effects of p-chloromercuri-derivatives

Our previous conclusion from modification with DTNB and *N*-ethylmaleimide [1] that only two of the six sulfhydryl groups of rhodopsin are normally exposed to the aqueous phase seems to be confirmed by the present experiments with both *p*-chloromercuri-derivatives. In the initial, rapid phase of modification with these two reagents, two sulfhydryl groups are reactive without loss of recombination capacity.

The action of *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate upon prolonged reaction very much resembles that of DTNB and *N*-ethylmaleimide in combination with detergents like Triton X-100 and cetyl trimethylammonium bromide. In both cases, 3–4 sulfhydryl groups are modified in darkness, while all sulfhydryl groups are modified in light. Recently, Robertson et al. [20], using radioactive *p*-chloromercuribenzoate, have demonstrated the same phenomenon in a more qualitative way. The more extensive modification by these mercury compounds is not surprising, since their structure shows the same polarity separation (amphipatic character) typical of detergents: a large hydrophobic mercuriphenyl tail combined with a relatively small charged group. Various observations appear to support this concept. First, the benzoate, which is the more hydrophobic of the two, owing to the much lower acidity of the carboxyl group as compared to the sulfonic acid group, is also the more effective agent in the sulfhydryl modification (Fig. 4). The denaturing side effects of these reagents are therefore probably due to aspecific, mainly hydrophobic interactions of excess reagent, resembling the action of detergents. Second, determination by atomic absorption photometry of the mercury content of well-washed photoreceptor membranes after treatment with high concentrations of the mercurials shows that at least 2–3 times more of these compounds are bound than would be expected from the number of modified sulfhydryl groups. Third, Carter [11] has recently reported that *p*-chloromercuribenzene sulfonate is able to extract up to 40 % of the protein from washed erythrocyte membranes.

We have observed that photoreceptor membranes are much less readily sedimented after *p*-chloromercuribenzene sulfonate treatment. Finally, combination of *p*-chloromercuribenzene sulfonate and digitonin slowly attacks the chromophoric center of rhodopsin [12], probably ultimately leading to complete denaturation. Therefore, the detergent-like character of the two mercuri-compounds may well explain the difference between the result obtained with *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate on the one hand, and DTNB and *N*-ethylmaleimide on the other hand, and also the reaction of additional sulfhydryl groups with *p*-chloromercuribenzoate upon illumination of the excised retina [13].

These findings illustrate the need to apply modifying agents with discretion, comparing the effects of different reagents and under differing reaction conditions (molar ratio, time of reaction, temperature). If this is not done, misleading results may be obtained. On the other hand, the application of this approach enables us to come to the classification of sulfhydryl groups in rhodopsin discussed below.

Classification of rhodopsin sulfhydryl groups

The modification studies with DTNB, *N*-ethylmaleimide, *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate suggest the presence in the rhodopsin molecule of at least three categories of two sulfhydryl groups each: type I, accessible under non-denaturing conditions (DTNB, *N*-ethylmaleimide); type II, accessible only under conditions of partial denaturation (the *p*-chloromercuri-derivatives, presence of the detergents Triton X-100 and cetyl trimethylammonium bromide); type III, accessible only upon illumination of a partly denatured membrane or in darkness upon complete denaturation (sodium dodecyl sulfate). Our classification cannot readily be compared with that of Zorn and Futterman [4] since these authors have studied rhodopsin preparations with a grossly altered protein structure through detergent solubilization and lipid extraction.

Type I sulfhydryl groups

Two sulfhydryl groups of rhodopsin are exposed to the aqueous environment and are readily accessible to sulfhydryl reagents under non-denaturing conditions. Their modification has no influence on the spectral properties, recombination capacity, recombination rate and the photolytic behaviour (unpublished results) of rhodopsin. The increased reactivity of one sulfhydryl group of rhodopsin upon illumination [1, 20], as measured with less reactive sulfhydryl reagents, suggests that these two sulfhydryl groups do not behave quite alike.

Recently, it has been suggested that the two sulfhydryl groups accessible in darkness might not be the same as the two accessible in illuminated membranes [21] i.e. illumination would cause a disulfide exchange, which is reversed upon reaction of opsin with 11-*cis* retinaldehyde, reflecting a possible role of sulfhydryl groups in the excitation mechanism. However, we have observed that *N*-ethylmaleimide or DTNB-modified opsin as well as opsin obtained from *N*-ethylmaleimide or DTNB-modified rhodopsin recombine with 11-*cis* retinaldehyde at the same rate as unmodified opsin (Fig. 1). This appears to exclude a disulfide exchange, since particularly *N*-ethylmaleylation of a sulfhydryl group would drastically decrease its disulfide exchange rate. A definite answer will have to await amino acid sequence studies involving sulfhydryl-labeled rhodopsin and opsin.

Type II sulfhydryl groups

This type comprises $4.2-2.1 = 2.1\pm0.3$ sulfhydryl groups, which are normally "buried" in the photoreceptor membrane, and are partially or completely exposed upon partial denaturation of the membrane. In darkness, such partial denaturation can be brought about by several reagents: *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate, detergents like cetyl trimethylammonium bromide, Triton X-100 and Emulphogene BC-720. This partial denaturation, which presumably involves changes in protein structure, is characterized by a change in several other parameters: loss of recombination capacity, decrease in thermal stability of rhodopsin, changes in photolytic behaviour of rhodopsin, changes in circular dichroism [14, 15] and increase in reactivity of amino groups [16]. However, the rhodopsin spectrum is retained under these circumstances, indicating that part of the rhodopsin molecule, containing the chromophoric center, is rather rigid and resistant towards denaturing agents. This is probably mainly due to the stabilizing presence of the chromophoric group, since upon removal of the latter, complete denaturation is observed under conditions which lead to partial denaturation only in membranes kept in darkness.

It should be noted that our observation that the recombination capacity of rhodopsin is abolished proportionally to the degree of modification of the type II sulfhydryl groups (Fig. 5), does not imply a direct participation of these groups in the recombination reaction. Secondary effects on the conformation of the protein, due to the mere presence of the mercuriphenyl group in this region of the molecule, may equally well be responsible.

Type III sulfhydryl groups

The third type consists of 1.8 ± 0.4 sulfhydryl groups, which are also normally "buried" in the photoreceptor membrane, but become exposed upon illumination under partially denaturating conditions or in darkness upon complete denaturation by sodium dodecyl sulfate. As long as the rhodopsin spectrum is preserved intact, these sulfhydryl groups do not react. This suggests that they are a structural part of the chromophoric center of rhodopsin, yet without being exposed upon illumination under normal conditions.

In one special case, solubilization of the membrane by the mild detergent digitonin, an additional two sulfhydryl groups are uncovered upon illumination without loss of recombination capacity [1, 2]. Here the two type III sulfhydryl groups are apparently uncovered, while the type II sulfhydryl groups remain buried*. This situation seems to represent a smaller change in protein structure than occurs in the partial denaturation described above, since regeneration is still possible and in darkness only type I sulfhydryl groups are accessible. Taking this into consideration, our present results obtained in suspension agree with the earlier data of Wald and Brown obtained in digitonin solution [2]. They found that (1) the less-reactive sulfhydryl reagents monoiodoacetic acid and monoiodoacetamide do not influence the recombination capacity, (2) modification of opsin with *p*-chloromercuribenzoate

* This view is supported by a recent publication by Zorn [22]. Modification of illuminated rhodopsin in digitonin with *N*-ethylmaleimide blocks 3–4 sulfhydryl groups without serious loss of recombination capacity. Subsequent reaction with *p*-chloromercuribenzoate modifies 2–3 additional sulfhydryl groups and leads to complete loss of recombination capacity.

abolishes its recombination capacity, an effect partly reversible upon addition of glutathione, (3) modification of rhodopsin with *p*-chloromercuribenzoate does not influence its typical absorbance spectrum, but in this case the recombination capacity was not determined.

The role of the type III sulfhydryl groups on the chromophoric center may be merely structural, but at present a possible role in the excitation mechanism cannot be excluded. The sulfhydryl groups of types II and III seem to be buried within the rhodopsin molecule itself rather than being masked by the lipid structure of the photoreceptor membrane. Upon removal of over 90 % of the phospholipids from rod outer segment membranes by means of phospholipase C treatment and hexane extraction [17] in a nitrogen atmosphere, both the exposed and the total number of sulfhydryl groups remain constant (unpublished observation from our laboratory).

Concluding remarks

The classification of sulfhydryl groups in rhodopsin presented here is based on overall numbers of sulfhydryl groups modified as related to functional parameters of rhodopsin. Direct analytical evidence that each of the three pairs indeed represents two well-defined sulfhydryl groups in the amino acid sequence of the protein would be desirable. The simplest method to obtain such information would be peptide mapping, but its application meets with difficulties in view of the predominantly hydrophobic nature of rhodopsin and of the necessity to use detergents in order to obtain sufficiently drastic proteolytic digestion (van Breugel et al., to be published). The recently published studies of the sulfhydryl groups in rhodopsin with fluorescent probe [18] and spin-labeled [19] sulfhydryl reagents suffer even more from a lack of knowledge of the location of these groups. A more definitive understanding of the functions of the sulfhydryl groups in rhodopsin must await determination of their location in the molecule.

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