Biochimica et Biophysica Acta, 325 (1973) 315–322

BBA 46635

BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS. XXIII. SULFHYDRYL GROUPS AND RHODOPSIN PHOTOLYSIS

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(Received June 6th, 1973)

SUMMARY

1. The number of exposed sulfhydryl groups in cattle rod photoreceptor membranes has been determined in suspension and after solubilization in various detergents both before and after illumination.

2. In suspensions, two sulfhydryl groups are modified per mole of rhodopsin, both by Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide, while no extra SH groups are uncovered upon illumination. Neither reagent affects the spectral integrity of rhodopsin at 500 nm and the recombination capacity is retained upon modification of both rhodopsin and opsin.

3. However, in detergents (digitonin, Triton X-100 and cetyltrimethylammonium bromide (CTAB)) 2–3 additional sulfhydryl groups appear upon illumination, in agreement with earlier reports.

4. A total number of six sulfhydryl groups and two disulfide bridges are found in rod photoreceptor membranes, expressed per mole of rhodopsin.

5. DTNB reacts somewhat faster with membrane suspensions after than before illumination. The less reactive sulfhydryl modifying agents *O*-methylisourea and methyl-*p*-nitrobenzene sulfonate show a similar behavior.

6. It is concluded that illumination of rhodopsin *in vivo* will not uncover additional SH groups, although the reactivity of one exposed SH group may increase somewhat. These findings also exclude a role of SH groups in the covalent binding of the chromophore.

INTRODUCTION

In 1952, Wald and Brown[1] presented evidence that in dark-adapted bovine rod outer-segment membranes, solubilized in digitonin, about 2 moles of sulfhydryl groups per mole of rhodopsin are accessible to Ag^+ upon amperometric titration, and that upon illumination two additional sulfhydryl groups become titratable. This was interpreted as indicating that a conformational change of the opsin molecule takes place during photolysis. This concept was incorporated into a schematic model for the transition of rhodopsin to opsin[2].

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); CTAB, cetyltrimethylammonium bromide.

Similar observations were reported by Ostroy *et al.*[3], except that they detected up to four sulfhydryl groups reacting with Ag^+ in darkness, while illumination "released" two to four more residues. Illumination of membranes solubilized in Triton X-100 is also reported to unmask two additional SH groups, as determined by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) or *p*-hydroxymercuribenzoic acid[4]. The results of the latter study may, however, have been affected by air oxidation, since in darkness only one SH group was detected. Earlier determinations in cetyltrimethylammonium bromide (CTAB)[5] probably suffer from the same effect.

Wald and Brown[1] further showed that upon reaction of rhodopsin with the very active sulfhydryl reagent *p*-chloromercuribenzoic acid the absorbance spectrum remains intact, but that the capacity of opsin to recombine with the chromophoric group, 11-*cis*-retinaldehyde, is gradually lost. The less active reagents iodoacetic acid and iodoacetamide had no effect on the recombination capacity.

We decided to reinvestigate the sulfhydryl group behavior of photoreceptor membranes in view of the following two points:

(1) All investigations to date have been done on outer-segment membranes solubilized by detergents. Incorporation of rhodopsin into detergent micelles is known to have a destabilising effect on the rhodopsin structure. It results in changes in the circular dichroic spectrum[6], a decrease in thermal stability[7] and a partial or complete loss in the recombination capacity. These effects increase in the order digitonin $\ll$ Triton X-100 $<$ CTAB. The reactivity of SH groups before and after illumination may, therefore, be due, at least in part, to a detergent effect. Hence, we used photoreceptor membranes in suspension as well as solubilized by various detergents.

(2) All bovine retinas, either obtained from local slaughterhouses and dark-adapted immediately after slaughtering or obtained commercially (G. Hormel and Co., Austin, Minn., U.S.A.), contain appreciable amounts of opsin[8,9]. This will cause an overestimation of the number of sulfhydryl groups relative to the amount of rhodopsin. Therefore, in this study opsin-free photoreceptor membranes were used throughout.

MATERIALS AND METHODS

Materials

5,5′-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB) and sodium dodecyl sulfate were obtained from Sigma, St. Louis, Mo, U.S.A.; *p*-chloromercuribenzoic acid and digitonin from Fluka A.G., Buchs, Switzerland; cetyltrimethylammonium bromide (CTAB) was obtained from Eastman, Rochester, N.Y., U.S.A.; *N*-ethylmaleimide from Merck, A.G., Darmstadt, Germany; *O*-methylisourea hydrogensulfate from Merck-Schuchardt, München, Germany; methyl-*p*-nitrobenzene sulfonate from Pierce, Rockford, Ill., U.S.A. and Triton X-100 from the British Drug Houses Ltd, Poole, Great Britain. Vinylquinoline was prepared as described by Krull *et al.*[10].

Methods

Isolation and characterization of rod outer-segment membranes. Bovine rod outer-segment membranes are isolated as reported previously[8]. Between two sucrose density gradient centrifugations the outer segments are treated with 11-*cis*-retinaldehyde in

order to convert all opsin to rhodopsin. The isolated membranes are frozen or lyophilized and stored at −70 °C. As judged from electronmicrographs and succinate dehydrogenase assays, they are nearly pure and contain no detectable mitochondrial contamination. Rhodopsin content and recombination capacity, determined in the usual way[8], average 8.1 nmoles per mg lyophilisate and 90% (of the original 500-nm absorbance), respectively.

Assay of sulfhydryl groups. Sulfhydryl groups are assayed with DTNB in nitrogen atmosphere at 20 °C[11]. Outer-segment membranes, in aqueous suspension or as lyophilisate, are added to 0.15 M Tris–HCl, pH 8.0, either with or without 1% (w/v) detergent, to a final concentration of 5–6 μM rhodopsin. The rhodopsin concentration is determined from its absorbance at 500 nm. A 20–40-fold excess of DTNB (4 mM in 30 mM acetate buffer, pH 5.3) is rapidly added. The absorbance increase at 412 nm is followed until it has reached its maximal value (30–45 min in detergent, 4–5 h in suspension). The absorbance increase is corrected by means of appropriate controls (a reagent blank without membranes and a membrane blank without DTNB). The amount of sulfhydryl groups is calculated by using a value of 13600 for ε_{412}[11]. In view of a recent report of a deviating value[12], we have redetermined ε_{412} and found the original value to be correct. When the total number of free sulfhydryl groups has to be determined, the assay is performed in 1% sodium dodecyl sulfate.

The reaction with DTNB in detergent solutions is followed on a Zeiss PMQ III spectrophotometer. The suspensions are measured on a Rapid T3 spectrophotometer (Kieler Howaldt Werke, Kiel, Germany), which permits, through placement of the cuvets immediately in front of the photomultiplier system, the determination of absorption spectra of suspensions containing up to 5 mg/ml membrane material.

Modification of sulfhydryl groups. Modification with DTNB is performed under similar conditions as in the assay, except that up to 10-fold higher concentrations of the reactants are used. The other modifying agents used are, together with the reaction conditions: *N*-ethylmaleimide[13] (20-fold excess: 0.2 M phosphate buffer, pH 7.0, 20 °C, 30 min), *O*-methylisourea[14] (up to 100-fold excess, 0.2 M phosphate buffer, pH 7.0, 4 and 20 °C) and methyl-*p*-nitrobenzene sulfonate[15] (up to 100-fold excess, 0.2 M Tris–HCl buffer, pH 8.2, 35 °C). In all cases a membrane concentration equivalent to 5–10 mg/ml lyophilisate is used.

The number of sulfhydryl groups modified by any of these reagents is calculated from the total number of remaining free sulfhydryl groups, assayed with DTNB in sodium dodecyl sulfate solution. For this purpose the modifying agents are removed by washing the suspensions twice with 0.2 M phosphate buffer (pH 7.0) prior to determination.

When the assay or modification of the membrane sulfhydryl groups is performed in the presence of detergents or at elevated temperature, air should be excluded in order to prevent oxidation of SH groups. In Triton X-100, CTAB, and sodium dodecyl sulfate, and to a less extent in digitonin, rapid oxidation of SH groups by air is observed even at 4 °C, bleached samples being even more susceptible than unbleached ones.

Determination of disulfide groups. Disulfide groups are determined in three ways: (1) By performic acid oxidation of all cysteine and cystine groups to cysteinic acid[16], which is determined by amino acid analysis as described previously[21]. (2) By reduction of cystine groups to cysteine with dithiothreitol and determination of all free cysteine

groups with DTNB in 1% sodium dodecyl sulfate in the precense of arsenite as described by Zahler and Cleland[17]. This method gives rather divergent results since the difference in reactivity of the protein sulfhydryl groups and the arsenate-complexed dithiothreitol is not large enough. (3) By previous reduction with mercaptoethanol and reaction of the sulfhydryl compounds with a slight excess of vinylquinoline as described by Krull *et al.*[10]. The reactions are performed in Tris buffer, pH 7.5[10], containing 1% sodium dodecyl sulfate. Excess reagent is removed by gel filtration (Sephadex G-100) after which the number of protein sulfhydryl groups is calculated from the absorbance at 318 nm, corrected by the absorbance of a similarly treated blank preparation, using a value for the ε_{318} of 10000 (ref. 10).

RESULTS

Table I shows the sulfhydryl groups, which are accessible to DTNB in dark-adapted and illuminated outer-segment membranes under various conditions. It makes no difference in any of these cases, whether illumination takes place before or after addition of DTNB.

TABLE I

NUMBER OF EXPOSED SULFHYDRYL GROUPS, CALCULATED PER MOLE OF RHODOPSIN, PRESENT IN BOVINE ROD OUTER-SEGMENT MEMBRANES, UNDER VARIOUS CONDITIONS, AS DETERMINED BY DTNB

The number of determinations is given in parentheses. No difference is observed between frozen and lyophilized rod outer-segment membrane preparations. The reaction is followed during 45–60 min; after this time interval the reaction is over 99% complete in detergent solution. In suspension, however, the reaction is then for only 90–95% complete, and reaches its maximal extent in 4–5 h. The real number of exposed SH groups in suspension will, therefore, be close to two.

	Suspension (5)	*Digitonin* (4)	*Triton X-100* (5)	*CTAB* (2)	*Sodium dodecyl sulfate* * (5)
In darkness	1.8±0.1	2.0±0.1	3.0±0.1	3.6±0.3	5.9±0.2
After illumination	1.8±0.2	3.9±0.2	6.0±0.2	5.8±0.3	5.9±0.2

* Rhodopsin is not stable in sodium dodecyl sulfate solution at 20 °C and is in darkness already immediately thermally bleached.

In suspensions the unbleached samples yield about two accessible SH groups per mole rhodopsin, but no further increase is observed upon illumination. With *N*-ethylmaleimide the same results are obtained as with DTNB: about two SH groups before and after illumination (Table II). On the other hand, in digitonin solution two SH groups per rhodopsin are observed in unbleached samples, and an additional two become accessible upon illumination (Table I). In Triton X-100, CTAB and sodium dodecyl sulfate solutions the number of SH groups modified in darkness increases to 3.0, 3.6 and 5.9, respectively, in light to about 6 in all three cases.

Modification of suspensions and of the solutions in digitonin, Triton X-100 and CTAB with DTNB in darkness leaves the absorbance spectrum of rhodopsin un-

TABLE II

EFFECT OF MODIFICATION OF SUSPENDED ROD OUTER-SEGMENT MEMBRANES WITH DTNB OR *N*-ETHYLMALEIMIDE ON SOME MEMBRANE PROPERTIES

	In darkness			*After illumination*	
	SH groups modified*	*ΔA_{500nm}*** (%)	*Recombination capacity* (%)	*SH groups* modified*	*Recombination capacity* (%)
DTNB	1.8 ± 0.1 (5)	100	95 ± 3 (2)	1.8 ± 0.2 (5)	98 ± 4 (2)
N-ethylmaleimide	1.6 ± 0.3 (2)	100	102 ± 5 (2)	1.7 ± 0.2 (2)	104 ± 6 (2)

* Per mole of rhodopsin.
** The value of untreated controls is taken as 100%.

changed. The recombination capacity remains unchanged in the case of membrane suspensions, both before and after illumination (Table II). In digitonin solution the latter parameter remains unchanged in darkness, but upon modification with DTNB after illumination the recombination capacity decreases to less than 30% of the control. In Triton X-100 and CTAB the recombination capacity is already abolished by the detergent alone[7,23].

Under complete denaturing conditions (1% sodium dodecyl sulfate) we detect six SH groups per mole of rhodopsin originally present (Table I). The same number is exposed in Triton X-100 and CTAB after illumination, although in this case the reaction is somewhat slower than in sodium dodecyl sulfate. The presence of 8 M urea accelerates the reaction but without uncovering additional SH groups.

These experiments indicate that six free SH groups are present per rhodopsin molecule in photoreceptor membranes. After performic acid oxidation, acid hydrolysis and amino acid analysis we detect 10–11 cysteinic acid residues per rhodopsin molecule. Total sulfhydryl determination after reduction of disulfide groups with excess mercaptoethanol and reaction with DTNB[17] or vinylquinoline[10] yields 8–10 sulfhydryl groups. Thus it appears that, in addition to six free SH groups, about two S–S bridges are present in the photoreceptor membrane preparation per rhodopsin molecule.

Reaction with DTNB appears to proceed somewhat faster in illuminated membranes. Therefore, we have investigated the possibility that illumination changes the reactivity of the two SH groups exposed in suspension. For this purpose we have used two reagents with low activity: *O*-methylisourea, which is reported to methylate the very reactive SH group in papain at neutral pH (ref. 14) and methyl-*p*-nitrobenzene sulfonate, which methylates sulfhydryl groups at higher pH (>8.5) and elevated temperature (40 °C)[15]. The number of unmodified SH groups is again determined with DTNB. With both reagents we find some evidence for increasing reactivity of one SH group after illumination. Membranes, kept in darkness, do not show any reaction with *O*-methylisourea even after prolonged reaction times (7 days, 20 °C). Illuminated membranes, however, react relatively fast (1 day, 4 °C) up to a certain degree (0.6–1 mole/mole rhodopsin), after which no further reaction is observed. Methyl-*p*-nitrobenzene sulfonate is able to methylate about one SH group in darkness after long

reaction times (6–8 h, pH 8.2, 35 °C). In illuminated membranes one SH group is fairly rapidly (1–2 h) modified, the other one reacting again much more slowly. Some relevant data are collected in Table III. In all cases absorbance spectra and recombination capacity remain intact.

TABLE III

NUMBER OF SULFHYDRYL GROUPS AVAILABLE FOR MODIFICATION, CALCULATED PER MOLE OF RHODOPSIN PRESENT, IN BOVINE PHOTORECEPTOR MEMBRANES BEFORE AND AFTER ILLUMINATION

Determination with reagents of different reactivity.

Reagent	*Before illumination*		*After illumination*	
	Number of SH groups modified	*Reaction time*	*Number of SH groups modified*	*Reaction time*
DTNB	2	4–5 h	2	3–4 h
O-Methylisourea	0	7 days	≈ 1	1 day
Methyl-*p*-nitrobenzene sulfonate	≈ 1	6–8 h	≈ 2	6–8 h

DISCUSSION

Our experiments clearly demonstrate that the use of more aggressive detergents (digitonin ≪ Triton X-100 < CTAB < sodium dodecyl sulfate) is paralleled by a more extensive modification of sulfhydryl groups in rod photoreceptor membranes with DTNB (Table I). This is most likely explained by an increased accessibility of SH groups in the same order, as is also apparent from the increased sensitivity to air oxidation. A progressive disintegration, both of the membrane structure (protein–lipid interaction) and of the protein structure (partial unfolding of polypeptide chains) may cause this phenomenon. Similarly it is clear from the same table, that in detergents which do not denature rhodopsin spectrally, opsin is more susceptible to chemical modification than rhodopsin. However, this increased availability of SH groups upon illumination is not seen in membrane suspensions, both with DTNB and *N*-ethylmaleimide as reagent (Table II). Thus, the unmasking of SH groups upon illumination in detergent solution is due to the higher susceptibility to detergent action of opsin as compared to rhodopsin, rather than to a light-induced conformational change itself. Since membrane suspensions more closely approximate the natural environment of rhodopsin, it seems very likely that illumination *in vivo* will not uncover sulfhydryl groups either.

We confirm earlier reports[1,3,4] that opsin is more susceptible than rhodopsin to chemical modification in detergent solution. This shows that binding of the chromophore has a stabilizing effect on the opsin structure. Strong detergents like Triton X-100 and CTAB influence the structure of rhodopsin to a certain extent leading to exposure of one or two additional sulfhydryl groups, but the absorbance spectrum remains intact. Under the same conditions the structure of opsin is much more affected since all four additional SH groups become exposed. This suggests that different

regions of the opsin backbone are tightly held together by the chromophore thereby structuring a part of the molecule in a detergent-resistant conformation, which conditions the typical rhodopsin spectrum.

Upon complete denaturation and suitable chemical degradation we find a total of six sulfhydryl groups and two disulfide bridges per molecule rhodopsin in bovine rod photoreceptor membranes, both before and after illumination. Since about 15% of the membrane protein is non-rhodopsin protein, we cannot exclude that some of the groups may not be localized in the rhodopsin molecule. Settling this matter would require analysis of pure rhodopsin. Data on more or less purified rhodopsin are, however, not consistent[4,5,19].

While our results indicate that illumination of rhodopsin in membrane suspension and presumably also *in vivo* does not uncover additional sulfhydryl groups, light may increase to some extent the reactivity of one sulfhydryl group (Table III). This may be due either to an opening of the structure around this residue, so that it becomes more accessible, or to the uncovering of a strongly basic group in the neighbourhood of this SH group. The liberated retinaldehyde-binding ε-amino lysine group[18] might play such a role. The observation that no additional SH groups appear upon illumination seems to rule out the hypothesis that a sulfhydryl residue is involved in the covalent binding of the chromophore[1,5]. This is also supported by the Raman scattering data of Rimai *et al.*[20], which confirm that in intact photoreceptor membranes, the chromophoric group is linked *via* a protonated aldimine band ($-C=\overset{H}{N^{+}}$). Relevant in this connection may also be the absence of cysteine residues in a bacterial analogue of rhodopsin, which has retinaldehyde as chromophore and shows an absorbance maximum at 560 nm[22]. Since, moreover, no direct evidence favoring this hypothesis has ever been reported, it must be considered untenable.

ACKNOWLEDGEMENTS

This investigation was supported in part by the Netherlands Organization for the Advancement of Basic Research (Z.W.O.), through the Foundation for Chemical Research in the Netherlands (S.O.N.).

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