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LOCATION OF TWO SULFHYDRYL GROUPS IN THE RHODOPSIN MOLECULE BY USE OF THE SPIN LABEL TECHNIQUE

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Summary

Sulfhydryl groups of membrane-bound rhodopsin are studied with the spin label technique by using five maleimide derivative probes of different lengths. Two sulfhydryl groups are titrated per molecule of rhodopsin. These groups are located in protected but probably different environments, less then 12 Å away from the aqueous phase. A distance of about 37 Å is measured between the two groups. These results are consistent with a model in which the two groups would be located close by the external surface of the protein but embedded within the membrane layer, the strong immobilization of the label molecules resulting from phospholipid-protein interactions.

Introduction

More than twenty years ago, Wald and Brown [1] reported that two moles of sulfhydryl groups are titratable per mole of rhodopsin. These authors worked with rhodopsin solutions obtained by treating dark adapted rod outer segment membranes with digitonin. In 1973, de Grip et al. [2] found the same result in the case of membrane bound rhodopsin. This latter investigation offers a tempting interpretation on the problem which concerns the number of sulfhydryl groups becoming accessible to titrating agents after illumination [1,2—4]: when rhodopsin is embedded in the disk membrane, illumination does not further increase the number of titratable sulfhydryl groups but once rhodopsin is solubilized in detergents, additional sulfhydryl groups are "released" upon light exposure. In addition, de Grip et al. [5] proposed to classify the rhodopsin sulfhydryl groups into three pairs on the basis of their reactivity towards sulfhydryl reagents. They conclude however that in order to understand the functional role of these groups, more knowledge is needed about their location in the molecule.

The present paper reports a study which concerns sulfhydryl group properties of membrane bound rhodopsin in the dark adapted stage. The spin label technique is used to measure the number of sulfhydryl groups accessible to spin labeled maleimide derivatives per rhodopsin molecule. Probes of different lengths are used as "molecular dipsticks" in order to estimate the average depth at which the sulfhydryl groups are located with respect to the aqueous phase. Finally, the dipolar interaction existing between the bound label molecules is measured and from this data the distance separating the paramagnetic centers is estimated.

Materials and Methods

Rod outer segment membranes were isolated from cattle retinas as described by de Grip et al. [6]. Usually 30—35 retinas were treated simultaneously in dim red light. The isolated membranes were resuspended in 6 ml of 0.066 M phosphate buffer (pH 7.2). 1 ml was withdrawn for rhodopsin concentration deter-

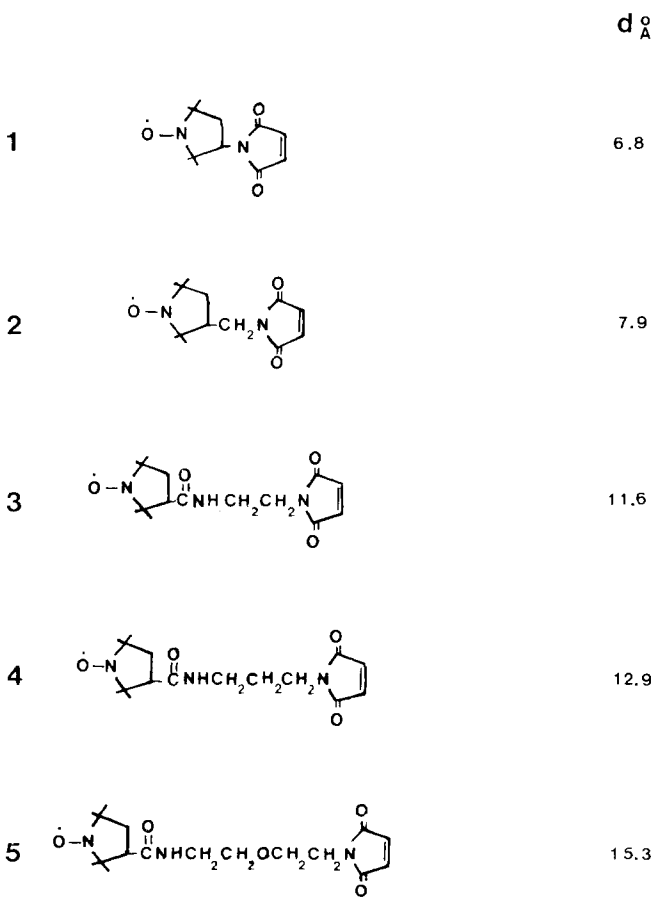

Fig. 1. Chemical structures of the maleimide spin labels. The lengths of the molecules were computed from molecular models.

mination: 0.2 ml aliquots were dissolved in 0.8 ml Emulphogene solution (2% w/w). After centrifugation (15 000 $\times g$, 10 min) the absorption spectrum of the solution was measured (A_{280}/A_{500} : 2.5, ΔA_{500} before and after bleaching: 0.3/retina).

The 5 ml remaining of suspension were employed for spin labeling. Five maleimide derivative spin labels were used (Syva, Palo Alto, U.S.A.). Their chemical structures are shown in Fig. 1 as well as the corresponding lengths of the molecules as evaluated from molecular models (Büchi, Switzerland). Labels were dissolved in phosphate buffer at a concentration of 5 mM and to one ml of each label solution, one ml of membrane suspension was added. The five samples were incubated overnight at 4° C. Thereafter, the samples were washed five times in buffer (30 ml, 25 000 $\times g$, 10 min) in order to eliminate any unbound label. This was verified by recording the ESR spectra of the supernatants. The final pellets were then resuspended in 0.5 ml of buffer. The suspensions were introduced in special flat quartz cells (Varian) and the ESR spectra recorded at room temperature with a Varian E3 spectrometer.

In order to measure the spin label concentration, the ESR spectra were integrated following the Wyard method [7]. Spectra corresponding to label solutions of well known concentrations were used for calibration.

For measuring the distance between the free radicals bound on the rhodopsin molecules, the method proposed by Kokorin et al. [8] was followed. The ESR spectra were recorded at −196° C at low microwave power (1 mW). The ratio of the total spectrum amplitude (d) to the amplitude of the central component (d') served as a working parameter. This parameter (d/d') depends upon the dipolar interaction existing between the radicals. Therefore it relates directly to the mean distance separating the interacting spins [8]. A reference curve giving the evolution of this parameter versus the mean distance between the radicals was obtained from label solutions of different concentrations. The parameter d/d' was measured in the case of the membrane suspension and the distance separating the label molecules was evaluated from the reference curve.

Results

The ESR spectra of the rod outer segment membranes labeled with the five different probes are shown in Fig. 2. Because of the extensive washing procedure, these spectra correspond to label molecules which are covalently bound on membrane proteins. Since rhodopsin amounts to 85% of the proteins present [5,6], the labeling procedure concerns mainly the visual pigment. Moreover, when the suspension is previously treated with an SH reagent such as *N*-ethylmaleimide prior to label addition, no label fixation occurs. It can therefore be concluded that the observed spectra relate mainly to label molecules attached to sulfhydryl groups of the rhodopsin molecules [9].

We reported previously [9] that illumination of a membrane suspension labeled with probe 1 does not produce any modification of the spectrum. The conclusion can be extended here to the other probes. Light does not induce any configuration change detectable at the label fixation sites.

Before analyzing the spectral features, a point concerning their intensities should be first emphasized. The spectra surfaces are directly proportional to the

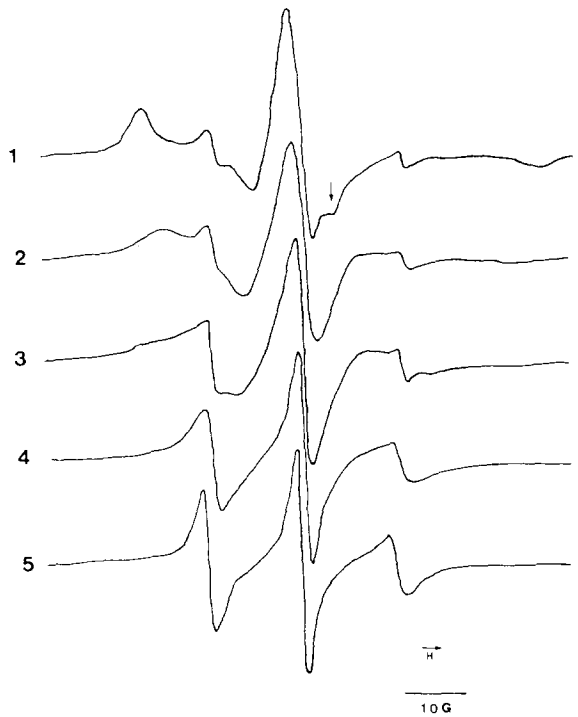

Fig. 2. ESR spectra of rod outer segment membrane suspensions labeled with the different probes. Each spectrum relates to the corresponding label. Microwave power: 4 mW. Modulation amplitude: 1 G. Temperature: 25°C. Recording gains: $1.25 \cdot 10^5$ (1), $8.0 \cdot 10^4$ (2), 10^5 (3), $6.2 \cdot 10^4$ (4), $5.0 \cdot 10^4$ (5).

number of spins detected. Each spectrum was integrated using the Wyard algorithm [7]. The results are given in the second column of the table. These values can be converted into spin concentrations by calibrating the data from measurements of the surfaces of reference spectra corresponding to label solutions of well known concentrations (third column). Since, on the other hand, rhodopsin concentration is measured from the difference in optical density at 500 nm, before and after bleaching, the number of label molecules bound per rhodopsin can be calculated (fourth column). It follows that about two sulfhydryl groups per rhodopsin are accessible to each type of label. This result is in agreement with those obtained by de Grip et al. [2] by using direct biochemical techniques.

The spectra shown in Fig. 2 are different because the lengths of the label molecules vary. The distance between the reacting double bond of the maleimide group and the free radical on the N-O bond increases from 6.8 to 15.3 Å. When the distance is minimum (spectrum 1), the label molecules are essentially in strongly immobilized positions [9]. With labels 2 and 3, the splitting between the outermost lines gradually decreases although the spectral features reveal that the motions of the labels are still hindered. On the other hand, the spectra 4 and 5 correspond to isotropic motions of the probes. Consequently the correlation times can be calculated [10]. As expected, the tumbling rate of the fourth label is slower ($\tau_c = 2.5 \cdot 10^{-9}$ s) than the corresponding one of the fifth

($\tau_c = 1.4 \cdot 10^{-9}$ s). The isotropic hyperfine splitting is equal to 15.7 G. Since the same value is observed with aqueous solutions of the labels, it indicates that the probes 4 and 5 are located in an aqueous environment.

At present, it does not seem possible to clearly differentiate from the spectral analysis one fixation site from the other. However, the presence of a hump in the central region of the first spectrum (see the arrow in the figure) reveals, by analogy with computer simulated spectra, that this spectrum represents an envelope over more than one component. It suggests that the two SH groups are located in different environments in the protein structure, one being more protected than the other. Quantitatively, one can estimate that none of them is located deeper than 12 Å away from the aqueous phase since the spectrum corresponding to label 4 reveals a motion which is entirely isotropic.

Besides the strongly immobilized components, narrow lines are also apparent in spectra 1—3. In spectra 4 and 5, these lines are superimposed on the main component. These sharp peaks correspond to a "weakly immobilized" component [9]. Its intensity was found variable from one preparation to another and it seems to depend upon the purity of the material. The origin of this component is at least twofold: label fixation on denatured rhodopsin molecules and on other membrane proteins. These lines appear indeed more intense when the membrane extraction procedure is carried out at room temperature [9]. Moreover, upon treatment with detergents, its intensity is drastically enhanced [11]. On the other hand, these narrow lines can be selectively reduced by treating the spin labeled membranes with diluted solutions of sodium dodecyl sulfate (Virmaux, N. and Delmelle, M., unpublished). At concentrations lower than 0.1%, this detergent solubilizes preferentially opsin as well as other membrane proteins leaving the rhodopsin molecules apparently unaffected [12].

Whatever the precise origin of this weakly immobilized component may be, it can be easily computed that, on the average, its surface amounts to less than 5% of the total spectrum intensity. Hence, its presence does not interfere significantly with our conclusions.

Since only two label molecules are bound per molecule of rhodopsin, one can easily measure the mean distance between the two sulfhydryl groups [8]. Fig. 3 represents two low temperature ESR spectra which correspond respectively to concentrated ($2.3 \cdot 10^{-1}$ M) and diluted ($5.7 \cdot 10^{-3}$ M) spin label solutions. In the former spectrum, strong dipolar interactions perturb the signal feature. The d/d' parameter measures this pertubation. The evolution of d/d'

TABLE 1

NUMBER OF SPIN-LABELED MALEIMIDE DERIVATIVES FIXED PER RHODOPSIN MOLECULE

Spin label	Results of integration (arbitrary units)	Spin concentration per cavity ($\times 10^{15}$)	Number of label molecules per rhodopsin
1	3710	3.78	1.94
2	3565	3.64	1.87
3	2880	2.94	1.51
4	2947	3.01	1.54
5	3291	3.36	1.72

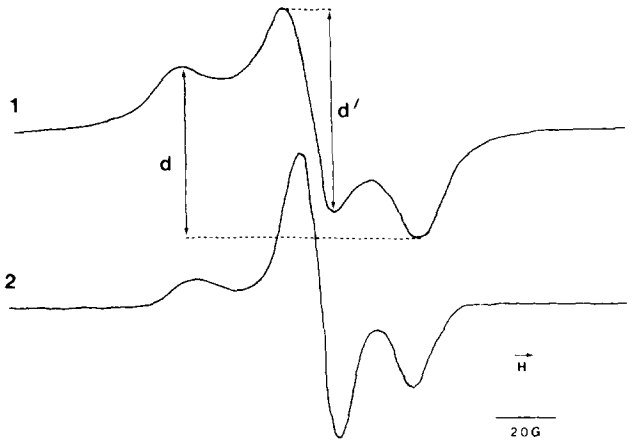

Fig. 3. ESR spectra of spin label 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl, in frozen water/glycerol solution (50/50, v/v). Concentrations: 0.23 M (1), 0.0057 M (2). Microwave power: 1 mW. Modulation amplitude: 1 G. Temperature: —196°C. Recording gains: $1.25 \cdot 10^2$ (1), 10^4 (2).

versus the mean distance between the interacting spins is shown in Fig. 4 and this graph is used as a calibration curve. The d/d' value was found equal to 0.39 ± 0.01 in the case of the rod outer segment membranes. It can be deduced from the graph that this value corresponds to a distance of about 37 Å between the two SH groups. Since the d/d' value does not increase significantly from label 1 to 5, the two probes attached on each rhodopsin molecule must be more or less parallel to each other.

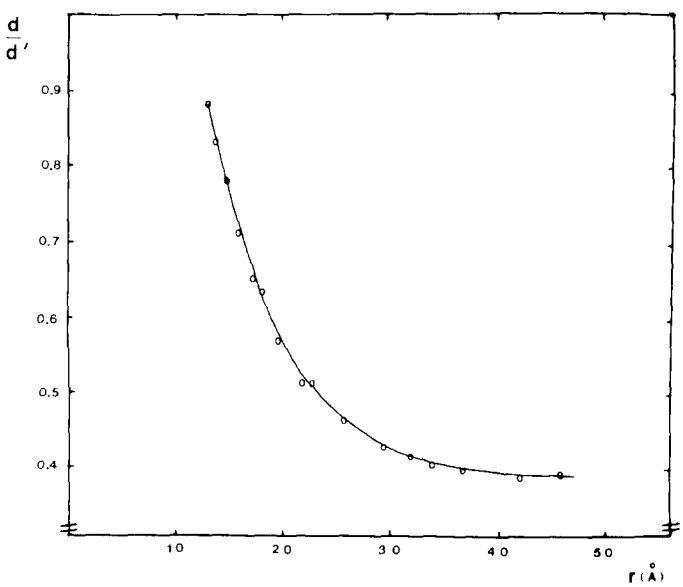

Fig. 4. Dependence of the parameter d/d' versus the mean distance r between the interacting spins. The distance r was computed on the basis of the following expression [8]: $r_{Å} = 0.7\, C_M^{-1/3}$ where C_M represents the number of paramagnetic center per cm^3.

The distance measurement described above implies that spin-spin interactions occur only between the two labels bound on the same molecule of rhodopsin: i.e. the distance between the two SH groups must be small as compared to the one between adjacent rhodopsin molecules. In view of our results and taking into account the close packing of the rhodopsin molecules in the membrane, such a hypothesis becomes questionable. If intermolecular interactions occur, the reference curve must be modified: to a given value of d/d' would correspond a larger mean distance between the interacting radicals [8]. Such correction cannot modify drastically our conclusions since an upper limit for the distance between the SH groups is fixed by the diameter of the rhodopsin molecule.

Discussion

The visual pigment rhodopsin is a membrane protein and its insolubility in water limits the application of many biochemical or biophysical techniques. This difficulty can be partly overcome by extracting rhodopsin from the membrane with surface active agents such as detergents. In recent years however, along with the major effort undertaken in order to understand the structure and the function of biological membranes, studies of the rhodopsin properties in detergent solutions became more questionable. Differences were indeed observed in the behavior of solubilized rhodopsin as compared to that of the membrane-bound protein [13]. This concerns, among other properties, the accessibility of the functional groups [2]. This variability results probably from modifications occurring in the rhodopsin structure under detergent influence [11]. However, in membrane suspensions as well as in digitonin extracts kept in darkness, only two SH groups are titrated per molecule of rhodopsin [1,2,14]. The maleimide derivatives used in this study are known to react with sulfhydryl groups [15,16] and to be devoid of the detergent-like character of the *p*-chloromercuri derivatives [5]. First our results confirm the previous findings regarding the number of sites present. Furthermore, the ESR technique which provides a method for probing the environment of some specific reactive groups [17–19] allows to reach, in the case of rhodopsin, additional information. The two accessible SH groups are not located on the external surface of the protein otherwise the ESR spectra would reveal an isotropic motion whatever the label used. In fact, label molecules must be about 12 Å long in order to give rise to this kind of spectrum. In a more qualitative way, a similar conclusion can be deduced from a previous observation made by Fujimori [20] who showed that in digitonin solutions, the rhodopsin SH groups react only with high concentrations of spin labeled iodoacetamide and that in such instances, the ESR spectrum is moderately immobilized. In those experiments however, the combined influence of the detergent and the denaturating effect of the label should be taken into account.

Concerning the distance separating the two SH groups, the value measured which could be underestimated is however fairly large since the diameter of the rhodopsin molecule as determined from X rays studies [21] is 42 Å. At this point of the discussion, three relevant observations should be mentioned:

(1) Treatment with papain of a labeled membrane suspension does not signif-

icantly modify the ESR spectrum (Virmaux, N. and Delmelle, M., unpublished) although this proteolytic enzyme removes about 36% of the peptide chains of the rhodopsin molecule [22]. Hence, the SH groups are probably not located in the portion of the protein protruding in the aqueous phase.

(2) Alternatively, detergent treatments perturb the lipid-protein interactions but modify also drastically the ESR spectrum [11]. Moreover, Zorn and Futterman have shown [4] that removal of most of the phospholipids associated with the protein results in an increase in the reactivity of the sulfhydryl groups.

(3) Finally, it is worth mentioning that Barratt et al. [23] have observed that a spin label bound on the external surface of a protein can exhibit a large freedom of motion in an aqueous solution but can become strongly immobilized when the protein is complexed with hydrophobic molecules.

In conclusion, the following model can be proposed concerning the two SH groups of membrane bound rhodopsin: they could be located close by the external surface of the protein but within the membrane layer and not further down than 12 Å with respect to the membrane surface. They are situated in different environments [5,14]. The strong immobilization observed with label 1 could result from the interactions with the lipids coating the protein. In those conditions, the absence of any light influence on the ESR spectrum implies that the SH environments are not significantly perturbed upon illumination. Hence, light does not lead to a reorganization of the whole protein structure.

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