Covalent Modification of Rhodopsin with Imidoesters: Evidence for Transmembrane Arrangement of Rhodopsin in Rod Outer Segment Disk Membranes†

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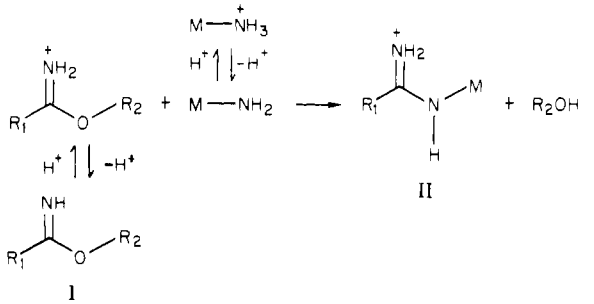
ABSTRACT: The transmembrane disposition of the visual pigment rhodopsin was studied by the covalent labeling of protein amino groups with membrane-permeable and -impermeable imidoesters. A new, highly reactive permeable reagent, 2-(methylsulfonyl)ethyl acetimidate (SAI) was developed for this purpose. The permeabilities of both this compound and the "impermeable" reagent isethionyl acetimidate (IAI) across the rod outer segment disk membrane were directly measured. Our results indicate that rhodopsin contains three classes of amino groups. One class (35–55% of the total) reacts rapidly with the membrane-impermeable reagent and is presumably exposed on the outside surface of the membrane. A second class (35–55% of the total) is located on the internal surface of the disk since its rate of reaction is dependent on the relative permeabilities of the labeling reagents. The remaining 10% of the rhodopsin amino groups are inaccessible to either type of imidate and are largely accounted for by the single lysine residue which specifically binds the chromophore retinal. These findings, taken together with evidence from freeze–fracture electron microscopy, imply that rhodopsin is a transmembrane protein.

The initial event in the process of visual excitation in the vertebrate rod is the absorption of photons by the protein rhodopsin. Most of the rhodopsin is tightly bound to flattened membrane vesicles called disks. The absorption of light by rhodopsin in the disk membrane results in a decrease in the sodium conductance of the ROS[1] plasma membrane (Korenbrot & Cone, 1972; Yoshikami & Hagins, 1973) which is physically separated from the disks. Therefore, it has been proposed that the disks contain a transmitter substance which carries the light signal to the plasma membrane (Baylor & Fuortes, 1970; Yoshikami & Hagins, 1971; Cone, 1973) and that rhodopsin functions as a light-activated channel or carrier which mediates communication between the intra- and extradiskal space. If this is the case, then it is likely that at least during the bleaching cycle rhodopsin comes into contact with both the internal and external aqueous compartments of the disk.

We have investigated the topological arrangement of rhodopsin within the disk membrane using membrane-permeable and -impermeable imidoesters to specifically convert membrane amino groups to acetamidine derivatives. The reaction between an imidoester (I) and membrane-bound amino group ($M-NH_2$) is diagrammed below. The amidine derivative (II) retains the positive charge associated with the unmodified amine near physiological pH. It has been shown that amidination is a mild treatment which in most cases preserves the biological activities of the modified substrates (Means & Feeney, 1972; Hunter & Ludwig, 1972). For example, amidination has little effect on red blood cell membrane associated acetylcholinesterase activity, as well as the rates of potassium leakage and facilitated diffusion of glucose through this membrane (Whiteley & Berg, 1974).



II

I

Reactions between imidates and primary amines exhibit bell-shaped pH–rate profiles (Hand & Jencks, 1962). A changeover in reaction mechanism occurs as the reaction pH passes through the pH–rate maximum. On the alkaline side of the maximum, attack of the free amine on a protonated imidate is rate determining (Hand & Jencks, 1962), and the reaction results in quantitative amidine formation. On the acidic side, however, breakdown of a tetrahedral intermediate containing elements of both amine and imidate is rate determining, and the reaction leads to a mixture of products. The consequences of carrying our amidination reactions at pH values below the pH–rate maximum are that (1) it is impossible to saturate accessible amino groups with a single treatment of reagent and (2) undesirable side reactions occur which include cross-linking of substrate amino groups by monofunctional imidates (Browne & Kent, 1975; Peters & Richards, 1977). [Such side reactions may account in part for the observation that sodium dodecyl sulfate ($NaDodSO_4$) gel patterns of red blood cell membranes amidinated at pH 8 with the monofunctional imidoester ethyl acetimidate (EAI) show high molecular weight protein aggregates not visible in gel patterns of unmodified membranes (Whiteley & Berg, 1974).] Amidination with previously available alkyl imidates which may be employed as membrane-permeable reagents must be carried out near pH 10 in order to ensure maximal derivatization and minimal formation of side products (Peters & Richards, 1977). At pH values greater than 8–8.5, ROS membrane vesicles become much more permeable to ions and

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[1] Abbreviations used: ROS, rod outer segment; CAI, choline acetimidate [[2-(acetimidoxy)ethyl]trimethylammonium chloride]; IAI, isethionyl acetimidate; SAI, 2-(methylsulfonyl)ethyl acetimidate; TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl.

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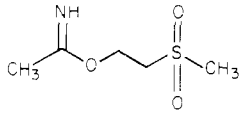
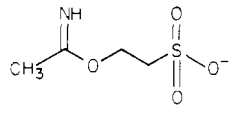
small molecules than at physiological pH (unpublished observations). Either alternative—incomplete saturation of amino groups and side reactions or leaky membrane vesicles—is particularly troublesome for experiments in which the differential labeling of membrane amino groups by permeable and impermeable imidates is used as probe of the transmembrane arrangement of proteins and lipids.

We have developed a new permeable imidoester, 2-(methylsulfonyl)ethyl acetimidate (SAI), for which the amidination reaction rate maximum has been shifted to significantly lower pH. Consequently, this reagent rapidly saturates all available amino groups near physiological pH and does not lead to cross-linking. Further, it is an excellent structural analogue of the membrane-impermeable imidoester isethionyl acetimidate (IAI) originally developed to label the surfaces of red blood cell membranes (Whiteley & Berg, 1974). The reaction rate maximum for IAI occurs at sufficiently low pH so that cross-linking reactions are not significant when amidination is carried out under mildly alkaline (pH <8) conditions.

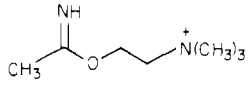


SAI



IAI

In addition, we have more recently prepared choline acetimidate [[2-(acetimidoxy)ethyl]trimethylammonium chloride, CAI], which is a positively charged structural analogue of IAI.



CAI

Amidination with this reagent, as with SAI, is devoid of the problems associated with reactions of the simpler alkyl imidates. In order to assess the possible roles of charge effects on reagent reactivities, we have compared the extent of labeling of rhodopsin amino groups with the two oppositely charged reagents.

Proper interpretation of amidination experiments requires knowledge of the permeability properties of the labeling reagents with respect to the ROS disk membrane. By determining the extent of reaction with lysine molecules entrapped within the disk lumen, we have found that SAI rapidly penetrates to the intradiskal space. IAI and CAI, while much less permeable that SAI, nevertheless exhibit measurable rates of leakage across the disk. Quantitation of the SAI and IAI labeling reactions as well as more limited results with CAI allows us to conclude that rhodopsin contains aqueous faces both on the internal side and on the external side of the disk membrane, and therefore it spans the breadth of the lipid bilayer.

Experimental Procedure

Membrane Preparations. Highly purified ROS disk membranes were prepared according to Raubach et al. (1974), modified by dialyzing the ROS membrane suspension against 200–800 volumes of cold (4 °C) pH 7.0 buffer containing 0.67 mM sodium phosphate, 0.1 mM EDTA, and 0.15 mM $CaCl_2$ for 36 h under an inert atmosphere maintained by gently bubbling argon through the medium. Precautions were taken to avoid oxidative damage to polyunsaturated fatty acids (Farnsworth & Dratz, 1976; Stone et al., 1979) and sulfhydryl groups (Schwartz et al., 1977).

Synthesis of Reagents. Isethionyl acetimidate (IAI) was prepared according to a modification of the procedure used by Whiteley & Berg (1974).

The synthesis of 2-(methylsulfonyl)ethyl acetimidate (SAI) was carried out as follows. 2-Mercaptoethanol was S-methylated according to the method of Booth et al. (1944). Dimethyl sulfate (100 mL) was added dropwise to a solution of 100 g of 2-mercaptoethanol in aqueous sodium hydroxide (58 g in 385 mL of H_2O). The temperature was maintained below 30 °C during the course of the reaction. The mixture was stirred overnight and then extracted with four 125-mL portions of chloroform. The extracts were pooled and the solvent was evaporated at reduced pressure. Vacuum distillation afforded 2-(methylthio)ethanol (bp 68–69.5 °C, 17 mmHg). The thioether was then oxidized to the corresponding sulfone with hydrogen peroxide (Numata et al., 1970). 2-(Methylthio)ethanol (10 g) was dissolved in 109 mL of glacial acetic acid. The solution was warmed to 70 °C in an oil bath, and 35.4 g of 30% hydrogen peroxide was added dropwise at a rate which maintained the temperature constant at 70 °C. After the addition was complete, the reaction mixture was heated for an additional 90 min. The resulting solution was reduced to a colorless oil on a rotary evaporator under vacuum, 50 mL of H_2O was then added, and the solution was reevaporated. Finally, the oil was mixed with 20 mL of benzene, and the solvent was distilled off to remove the last traces of water as an azeotrope. The residue was dissolved in 50 mL of chloroform and further dried by stirring with magnesium sulfate overnight. The chloroform solution was filtered and the chloroform was removed under reduced pressure. The product (2-hydroxyethyl methyl sulfone) was used without further purification: NMR (dimethyl-d_6 sulfoxide) δ 2.05 (s, $-OH^*$), 3.05 (s, $-SO_2CH_3^*$), 3.25 (t, $-SO_2CH_2^*$), 4.08 (t, $HCOCH_2^*-$). The sulfone was converted to the acetimidate by the Pinner synthesis (Pinner, 1892). Acetonitrile (50 mL) (freshly distilled out of magnesium sulfate) was acidified by bubbling with HCl gas. (HCl was generated by the dropwise addition of 50 g of sulfuric acid to 60 g of sodium chloride.) The gas was passed through a series of three drying tubes filled with Drierite before introduction into the acetonitrile. The liquid was cooled in an ice bath during acidification. Next, a solution of 3–4 g of 2-hydroxyethyl methyl sulfone in 10 mL of acetonitrile was added to the solution at a rate slow enough to maintain the temperature under 4 °C. The reaction was allowed to proceed overnight at this temperature. The imidate was precipitated from the reaction mixture by the addition of dry diethyl ether (prepared by distillation out of lithium aluminum hydride) and isolated by rapid filtration. The solid was washed with dry ether and stored in a vacuum desiccator. The product, 2-(methylsulfonyl)ethyl acetimidate, is a white crystalline solid with a melting point of 138–141 °C and which shows one titratable proton per 202 daltons with a pK of 6.0 (the equivalent weight was calculated on the basis of the sum of titratable imidate function and ammonia generated during titration by reagent hydrolysis): NMR (dimethyl-d_6 sulfoxide) δ 2.47 [s, $-C(=NH)-CH_3^*$], 3.12 (s, $-SO_2CH_3^*$), 3.73 (t, $-SO_2CH_2^*-$), 4.82 [t, $-C(=NH)-OCH_2^*-$]. Hydrolysis of the reagent resulted in a decrease in the intensity of the above peaks and the appearance of resonances at δ 2.08 [s, $-C(=O)-CH_3^*$], 3.05 (s, $-SO_2CH_3^*$), 3.5 (t, $-SO_2CH_2^*-$), and 4.38 [t, $-C(=O)-OCH_2^*-$].

Choline acetimidate [[2-(acetimidoxy)ethyl]trimethylammonium chloride, CAI] was prepared by condensing 14 g of choline chloride (Sigma Chemical Co.) with an acidified solution of 100 mL of acetonitrile. The procedure is analogous

to the one outlined above with the exception that the reaction was carried out at room temperature. CAI is a white crystalline solid which melts at 151–154 °C and which shows one titratable proton per 215 daltons (calculated as for SAI) with a pK of 5.2: NMR (D_2O) δ 2.56 [s, $-C(=NH)-CH_3$*], 3.32 [s, $-N^+(CH_3*)_3$], 4.03 [t, $(CH_3)_3N^+-CH_2*-$], ~5 [m, partially obscured by the downfield side of the HDO resonance, $-C(=NH)-OCH_2*-$]. Hydrolysis gives rise to resonances at δ 2.19 [s, $-C(=O)-CH_3$*], 3.26 [s, $-N^+(CH_3*)_3$], 3.79 [t, $(CH_3)_3N^+-CH_2*-$], and ~4.5 [m, partially obscured by the upfield side of the HDO resonance, $-C(=O)-OCH_2*-$].

Trapping of Tritiated Lysine by ROS Disk Membranes. A dialyzed disk preparation containing 10–20 mg of rhodopsin was suspended in 40 mL of pH 7.0 buffer containing 0.67 mM sodium phosphate, 0.1 mM EDTA, and 1.0 mM L-lysine monohydrochloride (0.67 mM PEL-7 buffer). The suspension was pelleted and resuspended in the same buffer to a membrane concentration corresponding to 5–10 mg of rhodopsin per mL of suspension. The membranes were bleached by continuous room light or by repeated flashes from a Honeywell Strobonar 700 flashlamp with an OG515 filter. The remaining procedures were carried out under ambient light levels. One microcurie of L-[4,5-^{3}H]lysine monohydrochloride (21.6 Ci/mol; Amersham/Searle) dissolved in water was added per mL, and the suspension was sealed under an inert atmosphere of argon and incubated for 3 h at 24 °C. The sample was made isotonic by the addition of an equal volume of 134 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, 0.2 mM $CaCl_2$, 1.0 mM L-lysine, and 1 μCi/mL tritiated lysine solution (to form a membrane suspension in 67 mM PECML-7 buffer containing 1 μCi/mL tritiated lysine) and was incubated for 3 h at 24 °C. The incubated suspension was taken up to a total volume of 40 mL in 67 mM PECML-7 buffer, pelleted, and resuspended in the above buffer to a concentration of 5–10 mg of rhodopsin per mL, and triplicate 65-μL samples were withdrawn into hematocrit tubes. The remainder of the sample was washed as before, once with the same buffer and three additional times with a buffer of the same composition but lacking 1.0 mM L-lysine (67 mM PECM-7 buffer). In some experiments the washed membrane suspension was dialyzed overnight against three 4-L changes of an appropriate buffer. Samples were withdrawn for analysis after each wash and after dialysis as described above.

The hematocrit tubes were placed in specially designed adapters, and the membranes were pelleted by centrifugation for 15 min at 16300g in a Sorvall HB-4 rotor. The lengths of the pellets and the supernatants were measured, and the hematocrit tubes were scored with a sharp file and broken at the pellet–supernatant interface. Both pellet and supernatant were dissolved in 1 mL of 10% Triton X-100 and mixed with 20 mL of scintillation fluid composed of 3 L of toluene, 2 L of 2-methoxyethanol, 15 g of 2,5-diphenyloxazole (Sigma), and 0.25 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (Sigma). The radioactivity of each sample was counted on a Beckman LS 200-B scintillation counter. Counting efficiency was determined by the method of internal standards. Membrane-bound radioactivity was expressed as the disintegrations per minute (dpm) retained by the pellet per unit of pellet height. The values for the disintegrations per minute inside the vesicles were obtained by correcting the total pellet disintegration per minute for the supernatant counts in the [^{14}C]inulin (New England Nuclear) accessible (extravesicular) space (Heller et al., 1971). Typically, 25–30% of the total pellet volume excluded [^{14}C]inulin.

Imidoester Labeling of ROS Disk Membranes and Ribonuclease A. Dialyzed membranes were suspended in 40 mL of pH 7.8 buffer of the same ionic composition as 67 mM PECM-7 (67 mM PECM-7.8 buffer). The suspension was pelleted and resuspended in 67 mM PECM-7.8 to a concentration of 2 mg of rhodopsin per mL. Imidoester was weighed out in an amount which corresponded to a 25-fold molar excess over membrane amino groups. [The number of amino groups per rhodopsin was calculated assuming 75 phospholipids per rhodopsin (Stubbs & Litman, 1978) containing 45% phosphatidylethanolamine and 15% phosphatidylserine (Miljanich et al., 1979) and 13 to 14 lysine residues per rhodopsin (Shields et al., 1967; Shichi & Lewis, 1969; Zorn & Futterman, 1971; De Grip et al., 1973; Plantner & Kean, 1976).] The solid was dissolved in 0.5 mL of 67 mM PECM-7.8, a precalculated volume of 1 N NaOH was quickly added to bring the pH to 7.8, and the solution was added to the membrane suspension to initiate the reaction. During the course of the reaction, the pH was maintained at 7.8 by a Radiometer automatic titrator, which added 1 N HCl to the reaction mixture as needed to neutralize the ammonia generated by the hydrolysis of excess reagent. A reaction sequence was completed by the repeated addition of imidoester 3 times at 5-min intervals. During the entire reaction sequence, the membrane suspension was flushed with argon. Five minutes after the last addition in a series, the membranes were washed free of excess reagent and reaction byproducts by centrifugation and were resuspended in 67 mM PECM-7.8 to the same concentration as before. Samples were removed to assess the extent of labeling of the amino groups of rhodopsin and entrapped lysine (if present). The remaining membranes were subjected to further labeling. All reaction sequences were carried out at room temperature. The extent of reaction was assayed by reacting unamidinated amino groups with trinitrobenzenesulfonic acid (TNBS; Nutritional Biochemicals) and determining the number of trinitrophenyl (TNP) derivatives colorimetrically (Raubach et al., 1974). Gel electrophoresis was performed according to a modification of the method of Fairbanks et al. (1971). For the labeling of ribonuclease A or ROS disk membranes dissolved in 3% Triton X-100, the same procedure was followed with the exception that excess reagent and reaction byproducts were removed between reaction sequences by dialysis against four 1-L changes of an appropriate buffer at 10-min intervals.

Quantitation of Labeling of Entrapped Lysine. A membrane suspension containing 2 mg of rhodopsin which had been preloaded with tritiated lysine and reacted with imidoester was washed with 67 mM PECM-7.8, pelleted, and resuspended in 1–2 mL of 0.1 M NaCl and 0.1 M sodium borate (pH 9.0). The suspension was extracted with an equal volume of chloroform/methanol (1:1) to remove lipids. The aqueous phase was separated and reacted with 0.2 mL of a 0.5 M TNBS solution for 30 min at 37 °C. The reaction mixture was lyophilized, and the solid was dissolved in 0.1 mL of doubly distilled water and applied to a 20 × 20 cm silica gel H TLC plate (EM Laboratories, Inc.). Any remaining solid was solubilized with 0.1 mL of acetone and layered over the same spot as the water extract. The plate was eluted with 1-butanol–acetic acid–water (80:20:20 v/v), 0.5-cm sections were scraped into scintillation vials, and the tritiated lysine was counted as described in the text.

Results and Discussion

Incorporation of Radioactive Lysine into Disk Membranes. Table I shows the amount of radioactivity retained by ROS disk membranes loaded with tritiated lysine after a series of washes with isotonic buffer, followed by either imidoester

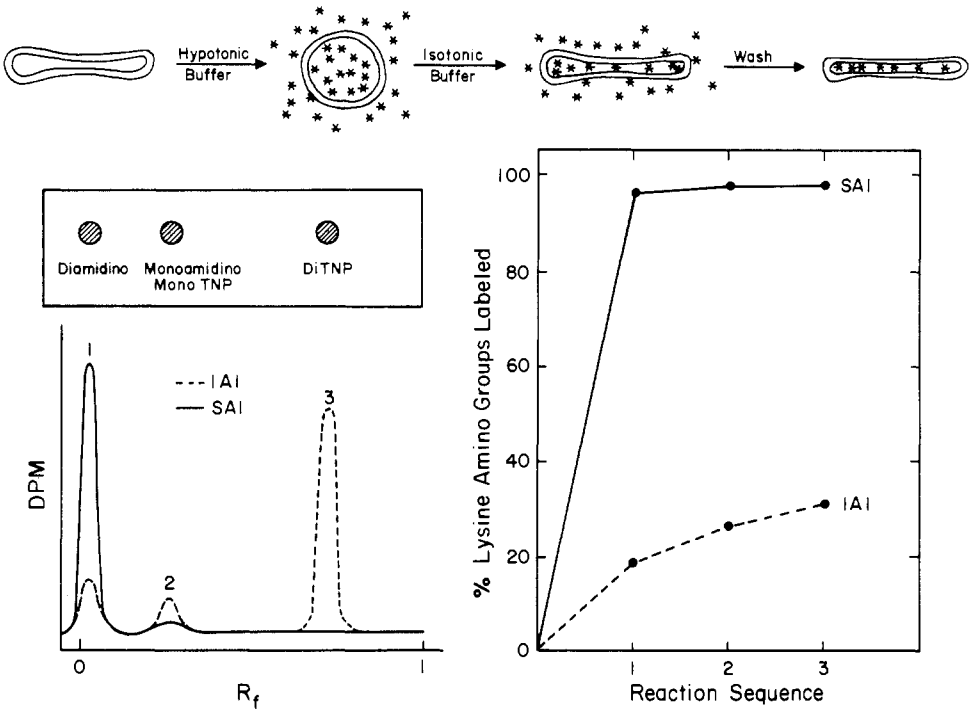


FIGURE 1: Imidoester labeling of tritiated lysine entrapped in ROS disk membranes. A diagram of the method of incorporation of radioactive lysine (depicted by asterisks) into disk membranes is shown at the top. On the left, the elution profile of a thin-layer chromatogram of trinitrophenylated extracts of imidoester-labeled [^{3}H]lysine-loaded disks is illustrated. A plot of the percent of the total lysine amino groups labeled as a function of the number of reaction sequences is shown on the right. Since each lysine molecule contains two amino groups, the percent of the total lysine amino groups labeled is calculated according to $[[2(\mathrm{dpm}_{\mathrm{peak}\,1}) + \mathrm{dpm}_{\mathrm{peak}\,2}]/2\sum_{i=1}^{n=3}\mathrm{dpm}_{\mathrm{peak}\,i}] \times 100$.

labeling or dialysis. During incubation of the disks with tritiated lysine under hypotonic conditions, the membranes incorporate radioactivity, which, after a resealing period in isotonic buffer, is washed away only very slowly by 67 mM PECML-7 and which is not displaced by nonradioactive lysine. About 90% of the radioactivity bound after five washes is retained when the disks are dialyzed for 12 h against 4-L changes of the wash buffer (67 mM PECML-7) at 4 °C. In contrast, the same fraction is lost when dialysis is carried out against hypotonic buffer (0.67 mM PEL-7). These results indicate that the radioactivity which becomes associated with the disks represents lysine molecules entrapped within membrane vesicles rather than bound at a membrane surface.

The intradiskal concentration of radioactivity can be determined from knowledge of the amount of radioactivity associated with a pellet of disk membranes, the total volume of the pellet, the amount of radioactivity per unit of volume in the supernatant, and the fraction of the pellet volume which excludes [^{14}C]inulin (25–30%). The average concentration of radioactive lysine in the internal volume of the disk membranes was ~80% of that in the resealing incubation medium. This indicates that most of the vesicles contain lysine and thus that the results of the labeling experiments are presumably characteristic of the entire disk population rather than a small unrepresentative sample of membrane vesicle.

The amount of radioactivity lost by IAI-labeled disk membranes after each reaction sequence is comparable to the amount expected to be washed away from unlabeled membranes which receive one buffer wash in lieu of each reaction sequence. Thus, labeling the membranes with IAI does not appreciably increase the permeability of the vesicles to the entrapped lysine.

Imidoester Labeling of Lysine Entrapped in ROS Disk Membranes. Three radioactivity-containing spots are found after thin-layer chromatography of trinitrophenylated aqueous extracts of imidoester-labeled ROS disk membranes (Figure 1). These migrate with R_f values of 0.03 (peak 1), 0.26 (peak 2), and 0.71 (peak 3) and correspond to the diamidino, monoamidino–mono-TNP, and di-TNP derivatives of lysine, respectively. Figure 1 shows the percent of the total lysine amino groups labeled by imidoester as a function of reaction sequence. It is evident that SAI is freely membrane permeable, labeling 97% of the entrapped lysine amino groups after a single reaction sequence. IAI labels 20% of the total lysine amino groups during the first reaction sequence and an additional 11% over the next two series. This indicates that although IAI is considerably less permeable than SAI, it nevertheless reacts with a fraction of the amino groups inside the vesicles with each successive treatment. In separate experiments it was found that CAI and IAI label lysine entrapped in ROS disks to the same extent within experimental

Table I: Amount of Radioactivity Retained by [^{3}H]Lysine-Loaded Disks after Various Treatments[a]

washes	tritium bound (dpm/in. of pellet height)[b]	treatment	% radioact. remaining[c]
1	17 200 ± 800	12-h dialysis against	
2	13 100 ± 500	67 mM PECML-7	89 ± 3
3	12 700 ± 200	0.67 mM PEL-7	10 ± 3
4	11 900 ± 300	no. of IAI sequences	
5	11 100 ± 200	1	82 ± 4
		2	72 ± 8
		3	76 ± 2

[a] Tritiated lysine was incorporated into ROS disk membranes as described in the text. The loaded disks were washed twice with 67 mM PECML-7 and 3 additional times with 67 mM PECM-7 buffer. After five washes the membranes were labeled with imidoester or dialyzed against an appropriate buffer. Samples were assayed for bound tritium after each wash, labeling sequence, and dialysis as described in the text. [b] Values are mean ± SD for six samples. [c] Values are mean ± SD for three samples.

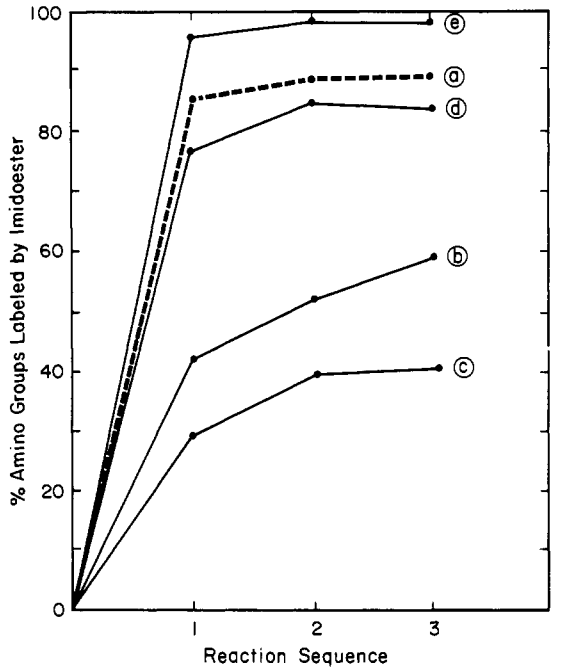


FIGURE 2: Imidoester labeling of rhodopsin and ribonuclease under various conditions. (a) Percent of the rhodopsin amino groups labeled by SAI in intact disks; (b) same as (a) but using IAI as the labeling reagent; (c) calculated percent of the total rhodopsin amino groups labeled by IAI on the external surface of the disk (see the text); (d) percent of the total rhodopsin amino groups labeled by IAI in disk membranes solubilized in 3% Triton X-100; (e) percent of the total ribonuclease A amino groups labeled by IAI, all as a function of the number of reaction sequences. (The conditions of labeling are as described under Experimental Procedure.)

error (data not shown), suggesting that the two charged reagents penetrate the membrane at similar rates.

Imidoester Labeling of Rhodopsin in Intact and Detergent-Solubilized ROS Disk Membranes. Figure 2 shows the percent of the total rhodopsin amino groups labeled by successive reaction sequences of imidoesters under various conditions. In the intact membrane, SAI reacts with 86% of the rhodopsin amino groups during the first reaction sequence; an additional 3% are labeled by the two subsequent series (curve a, Figure 2). Since a single SAI treatment saturates entrapped lysine amino groups (Figure 1) as well as all the phosphatidylserine and phosphatidylethanolamine of the disk membrane (G. P. Miljanich, P. P. Nemes, and E. A. Dratz, unpublished experiments), it follows that the unreacted rhodopsin amino groups (~10% of the total) are inaccessible to this reagent. In a preliminary effort to determine the nature of the unreactive amino groups, it was found that reductive affixation of 11-*cis*-retinal to the protein by borane dimethylamine (Hall & Bok, 1976) prior to amidination abolishes the TNBS reactivity of approximately one amino group per rhodopsin (data not shown). This suggests that a lysine residue not labeled by imidoester can be identified with the retinal binding site. Amino acid analysis data from different laboratories show that there are on the average 13 to 14 lysine residues per rhodopsin molecule (Shields et al., 1967; Shichi & Lewis, 1969; Zorn & Futterman, 1971; De Grip et al., 1973; Plantner & Kean, 1976). We derive approximately the same number from measurement of the 340-nm absorption of fully trinitrophenylated rhodopsin. [The molar extinction coefficient at 340 nm of trinitrophenylamino derivaties was taken to be 1.2×10^4, which is a reasonable estimate based on values reported in the literature (Satake et al., 1960; Goldfarb, 1966; Habeeb, 1966; Mokrasch, 1967; De Grip et al., 1973).] Therefore, the 10% of rhodopsin amino groups inert to SAI are largely accounted for by the single lysine residue which binds the chromophore.

The IAI labeling profile of ROS disk membranes dissolved in 3% Triton X-100 resembles that seen with SAI in the intact membrane (curve d, Figure 2). Furthermore, over 97% of the amino groups of the water-soluble protein ribonuclease A react with IAI during a single reaction sequence (curve e, Figure 2). These observations show that the intrinsic reactivity of this reagent is sufficient to label all available amino groups under the conditions of our experiments. However, a single treatment of disk membranes with IAI only labels approximately half as many rhodopsin amino groups as SAI does under similar conditions. In addition, a 17% increment in labeling is observed upon further IAI treatment (curve b, Figure 2). The biphasic nature of the IAI labeling profile could conceivably be due to the existence of two classes of external rhodopsin amino groups with intrinsically different reactivities toward IAI but the same reactivity toward SAI. Space-filling models of SAI and IAI show them to be of nearly identical size and shape; therefore, the two reagents are not likely to have different chemical reactivities for steric reasons. The presence of the negatively charged sulfonate moiety on IAI could confer a lower reactivity on this reagent toward amino groups located in negatively charged regions of rhodopsin due to electrostatic repulsion. However, recent labeling experiments show that CAI, which bears a positively charged quaternary ammonium group, exhibits a labeling profile virtually indistinguishable from that of IAI toward rhodopsin amino groups (data not shown). As discussed above, CAI and IAI exhibit similar permeabilites to the ROS disk membrane. Thus, the different behavior of SAI and IAI toward membrane amino groups is not due to simple differences in chemical reactivity.

The results of the experiments with entrapped lysine suggest that a portion of the IAI labeling is due to the penetration of this reagent to amino groups exposed on the internal face of the disk. Using the IAI labeling data, it is possible to calculate the fraction of the total rhodopsin amino groups on the external surface of the disk on the basis of a simple model detailed below. (The Appendix presents several alternative models for the analysis of the labeling data. The full range of models considered leads to identical qualitative conclusions and rather similar quantitative results.) The fraction of total rhodopsin amino groups labeled after the ith reaction sequence, f_L^i, may be expressed as

$$f_L^i = \chi_0^i f_0 + \chi_I^i f_I \quad (1)$$

where f_0 and f_I are the fractions of the total which are accessible to labeling reagent on the external and internal faces of the disk, respectively, and χ_0^i and χ_I^i are the respective fractions of the external and internal amino groups labeled after the ith reaction sequence. From curve a, Figure 2, $f_0 + f_I = 0.9$ (SAI-accessible amino groups), and f_L^i is given by curve b, Figure 2. χ_I^i is assumed to be equal to the fraction of entrapped lysine labeled by IAI after the ith reaction sequence (right-hand side of Figure 1). Substituting $f_I = 0.9 - f_0$ and solving eq 1 for f_0 gives

$$f_0 = \frac{f_L^i - 0.9\chi_I^i}{\chi_0^i - \chi_I^i} \quad (2)$$

When all external amino groups are saturated with reagent $\chi_0^i = 1$; thus, a plot of $(f_L^i - 0.9\chi_I^i)/(1 - \chi_I^i)$ against the number of reaction sequences increases to a constant value of f_0. Such a plot, derived from the experimental data, is shown

in curve c, Figure 2, and indicates that IAI labeling of externally exposed rhodopsin amino groups saturates at ~40% of the total by the second reaction sequence. It is possible that IAI has a different reactivity toward the amino groups of lysine free in solution and membrane amino groups. The fact that IAI saturated RNase amino groups with a single treatment of reagent while the amino groups of rhodopsin outside the membrane are calculated to be saturated only after two reaction sequences (Figure 2, curve e, and very similar conclusions from the other models in the Appendix) suggests that IAI is somewhat more reactive toward free amino groups than those which are membrane bound. If this were the case, it would have the effect of overestimating χ_1^i. Overestimation of this parameter leads to an underestimation of f_0, as discussed in the Appendix. The possible uncertainty in χ_1^i must be kept in mind when interpreting the results. However, overestimation of χ_1^i by as much as 50% (an overly pessimistic assessment) only decreases the value of f_0 calculated according to eq 2 by ~10%. Since other methods of calculating f_0 (presented in the Appendix) are less sensitive to this parameter, it follows that the uncertainty in χ_1^i does not have a large effect on the results.

We interpret the difference between the IAI reactivities shown in curves b and c (Figure 2) to be the result of a low but measurable leakage of this reagent through the membrane to amino groups exposed on the internal membrane surface. These same groups are rapidly labeled by the freely permeable reagent SAI. CAI and IAI have virtually the same disk membrane permeabilities and also show identical biphasic behavior toward rhodopsin. Since CAI and IAI are oppositely charged, electrostatic effects do not seem to make an important contribution to reagent reactivity toward rhodopsin. Therefore we conclude that the labeling behavior of rhodopsin amino groups in the intact disk membrane reflects the presence of a family of lysine residues exposed at the external surface and readily available to all three imidoester reagents, as well as a set which is presumably on the internal membrane surface since its reactivity is governed by the rate of permeation of imidoester into the intradiskal space.

The results of freeze–fracture experiments indicate that rhodopsin is asymmetrically disposed in the disk membrane since it is associated with the outer monolayer of the disk after fracture (Chen & Hubbell, 1973; Raubach et al., 1974; Corless et al., 1976). Freeze–fracture experiments also show that the membrane preparations we use for labeling are ≥95% right side out (Raubach et al., 1974; unpublished observations). The above information combined with the labeling data strongly suggest that rhodopsin is a transmembrane protein which exposes segments of its polypeptide chain to both the internal and external aqueous compartments of the disk.

This result is consistent with the findings of this and several other laboratories (Chen & Hubbell, 1973; Raubach et al., 1974; Jan & Revel, 1974; Schwartz, 1975; Schwartz & Dratz, 1976; Hubbell et al., 1977; Röhlich, 1976; Gaw et al., 1977; Dratz et al., 1979; Fung & Hubbell, 1978). Chen & Hubbell (1973) have observed that etching of freeze-fractured ROS disk membranes produced pitting of the otherwise smooth convex fracture faces. The pits were attributed to the sublimation of ice from holes produced when rhodopsin is pulled out of the internal monolayer. Röhlich (1976) has used ferritin-labeled concanavalin A to stain the carbohydrate moiety of rhodopsin in frog ROS membranes, and Adams et al. (1978) used a related technique on bovine preparations. Both groups found binding sites only on the internal surface of the disk. It has been shown that rhodopsin is attacked by a variety of proteases in intact, right-side-out frog and cattle disk membranes (Gaw et al., 1977). Combined with the localization of the carbohydrates to the internal membrane surface, this finding also implies that rhodopsin contains intra- and extradiskal domains. In addition, Jan & Revel (1974), using peroxidase-coupled antibodies to rhodopsin, reported antibody binding sites both at the external and at the internal surface of the disk. Recently, Fung & Hubbell (1978) have reported the results of lactoperoxidase-catalyzed radioiodination of proteolyzed rhodopsin in reconstituted membrane vesicles. Rhodopsin is randomly oriented in these membranes, and only one of the two orientations is attacked by papain. The fact that both intact rhodopsin and its proteolytic fragments are labeled by the impermeable lactoperoxidase system indicates that the protein is exposed to the aqueous medium at both apices and is therefore transmembrane in nature. Finally, X-ray diffraction data on ROS membranes obtained from intact retinas suggest that rhodopsin spans the lipid bilayer with a relatively uniform cross-sectional area through the hydrocarbon region (Schwartz, 1975; Schwartz & Dratz, 1976; Dratz et al., 1979).

In summary, a simple analysis of the data from our labeling experiments is consistent with 40% of the amino groups of rhodopsin being exposed on the external surface of the disk with an additional 50% accessible from the disk lumen. Other methods of data analysis presented in the Appendix yield values for the percent of externally exposed rhodopsin amino groups ranging from 35 to 55%. The detailed locations of the amino groups are not known; they may, for example, protrude into an aqueous compartment or line an aqueous channel. We are continuing to investigate the molecular topology of the disk membrane by examining the imidoester labeling of rhodopsin fragments obtained from proteolyzed disks in order to gain a better understanding of how the polypeptide is folded into the membrane.

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Appendix

The method of analysis of the IAI labeling data presented in the main body of this paper makes use of the assumption that the disk population is homogeneous with respect to its permeability to labeling reagent. However, results presented here as well as those of subsequent experiments (not reported) suggest that this assumption is oversimplified and that the disk population may consist of subpopulations of membranes which are heterogeneous with respect to reagent permeability. The subpopulations can be conveniently divided into the following three classes. (1) Vesicles not leaky (NL) to reagent are assumed to have been loaded with radioactive lysine at the original (incubation) specific activity and then fully resealed. NL vesicles are totally impermeable to IAI, and thus the internal rhodopsin and phospholipid amino groups of these membranes are not labeled by IAI at all. (2) Vesicles leaky (LK) to reagent are assumed to have been fully loaded with radioactive lysine but only partially resealed and contain lysine at or below the original specific activity. The extent of internal amino group labeling is determined by the degree of leakiness of these membranes to reagent. (3) Totally leaky (TL) vesicles do not reseal at all and therefore contain no readioactive lysine. These membranes are fully permeable to IAI, and, hence,

internal and external amino groups are labeled with approximately the same efficiency.

Four methods of data analysis based on the presence of various admixtures of these membrane classes in the total population are presented below.

(a) The membrane population consists of only leaky (LK) vesicles. This is the case already described in the text, and analysis leads to eq 1 for f_L^i, the fraction of rhodopsin amino groups labeled after the ith reaction sequence, and eq 2 for f_0, the fraction of rhodopsin amino groups located on the external side of the membrane. (The plot of eq 2 in Figure 2 and subsequent expressions for f_0 are derived with the assumption that external amino groups have been saturated with reagent; i.e., χ_0^i is taken to be equal to 1.)

(b) The membrane population contains leaky (LK) and not-leaky (NL) vesicles. In this case f_L^i can be expressed as

$$f_L^i = \chi_0^i f_0 + \chi_I^i f_{LK} f_I \tag{3}$$

where f_{LK} is the fraction of the total membrane population which consists of LK vesicles, and all other symbols are as previously defined. This leads to

$$f_0 = \frac{f_L^i - 0.9\chi_I^i f_{LK}}{1 - \chi_I^i f_{LK}} \tag{4}$$

(c) The membrane population consists of leaky (LK) and totally leaky (TL) vesicles. Since all of the amino groups of the totally leaky membranes are labeled with the same efficiency as externally exposed amino groups, f_L^i is given for this case by

$$f_L^i = \chi_0^i f_0 + (\chi_0^i f_{TL} + \chi_I^i f_{LK}) f_I \tag{5}$$

where f_{TL} is the fraction of the total membrane population which consists of TL vesicles. Since $f_{LK} + f_{TL} = 1$, it follows that

$$f_0 = \frac{f_L^i - 0.9[f_{TL} + \chi_I^i(1 - f_{TL})]}{(1 - f_{TL})(1 - \chi_I^i)} \tag{6}$$

(d) The membrane population contains all three classes of vesicles. In this case, the expression for f_L^i is the same as eq 5, but now $f_{LK} + f_{TL} + f_{NL} = 1$ (f_{NL} is the fraction of the total membrane population consisting of nonleaky vesicles) and

$$f_0 = \frac{f_L^i - 0.9[f_{TL} + (1 - f_{TL} - f_{NL})\chi_I^i]}{1 - f_{TL} - (1 - f_{TL} - f_{NL})\chi_I^i} \tag{7}$$

Data from recent experiments in which a greater number of reaction sequences were employed than shown in Figure 2 show that the labeling of internally trapped lysine plateaus (data not shown). This implies the existence of a population of NL-type vesicles; thus, it seems most appropriate to use eq 4 or 7 to analyze the labeling data.

In order to apply analyses b–d, we must obtain estimates for f_{TL}, f_{NL}, and f_{LK}. Since at most only two of these are independent variables ($f_{TL} + f_{NL} + f_{LK} = 1$), evaluation of two of these parameters uniquely determines the third. An upper limit for f_{TL} can be estimated from the amount of radioactive lysine retained by the disk membranes after washing. The radioactive lysine contained in the volume inside the disks at the start of labeling is 80% of the radioactive lysine content of an identical volume of incubation medium (see the text). Loss of lysine can occur as a result of the loss of all the lysine from some vesicles, loss of some lysine from all vesicles, or a combination of the above. The crucial point is that the fraction of the radioactivity lost from the disks on washing is an upper limit on the fraction of totally leaky vesicles. Thus, for the experiment described in the text, $f_{TL} \leq 0.2$.

An additional 24% of the radioactive lysine is lost from the disks during the course of labeling (Table I). This can be interpreted in several ways. (1) Equal fractions of unlabeled and amidinated lysine leak out of the membrane vesicles. This has no effect on the calculated value of f_0. (2) Unlabeled lysine leaks out of the disks in preference to labeled lysine. This would have the effect of overestimating χ_I^i (since the leak increases the apparent fraction of amidinated internally trapped lysine). However, the plateauing of the lysine labeling in recent experiments provides evidence against this possibility. (3) Labeled lysine leaks out in preference to unlabeled lysine. This has the opposite effect of (2) above, but it is unlikely due to the higher charge density and greater size of the amidinated material. (4) A fraction of the disks lose all associated radioactivity during labeling, hence increasing f_{TL}. Therefore loss of radioactive lysine from the disks during labeling can be taken into account in the calculation of f_0 by making an appropriate adjustment in f_{TL} each time the number of reaction sequences, i, is incremented.

The saturation of internal lysine labeling allows one to calculate an upper limit to the fraction of not-leaky vesicles. If internal lysine labeling saturates at P percent amino groups labeled, the upper limit for the fraction of not-leaky vesicles is $(100 - P)/100$ [i.e., $f_{NL} \leq (100 - P)/100$]. Even though lysine labeling has not plateaued completely in the experiment reported herein, $100 - P$ may be estimated to be ~65% by smooth extrapolation.

Since the internal amino groups of NL vesicles are not labeled by IAI at all, $0.9 - f_L^i$(saturation) is equal to the product of the fraction of not-leaky vesicles and the fraction of rhodopsin amino groups on the internal side of the membranes, i.e.

$$0.9 - f_L^i(\text{saturation}) = f_I f_{NL} \tag{8}$$

As will be seen, calculated values of f_0 range from 0.35 to 0.55, and since $f_I = 0.9 - f_0$, f_I varies complementarily between the same limits. A range of values may be calculated for f_{NL} by inserting appropriate values for f_L^i(saturation) and f_I into eq 8.

It is instructive to examine the effects of the magnitude of f_{TL}, f_{NL}, and χ_I^i on the calculated value of f_0. f_0 is obtained by applying a subtractive correction to the total observed rhodopsin labeling to take into account internal amino groups labeled due to leakage of reagent to the internal disk surface. An increase in f_{TL} or χ_I^i increases this corrective term and hence decreases f_0, while an increase in f_{NL} has the opposite effect. Thus, for a given set of experimentally determined parameters (f_{TL}, f_{NL}, f_L^i, and χ_I^i), analysis b would give the highest value of f_0 and analysis c would give the lowest value of f_0, methods a and d giving intermediate results. With recently available data, the range of values using analyses a–d is $0.35 \leq f_0 \leq 0.55$. Applying the most appropriate analyses, b and d, yields $0.4 \leq f_0 \leq 0.55$. Therefore, although some uncertainty exists as to the quantitative distribution of rhodopsin amino groups, the conclusion that the protein spans the disk membrane is supported by all our methods of data analysis.

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