CHARGE ASYMMETRY OF THE PURPLE MEMBRANE MEASURED BY URANYL QUENCHING OF DANSYL FLUORESCENCE

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ABSTRACT   Purple membrane was covalently labeled with 5-(dimethylamino) naphthalene-1-sulfonyl hydrazine (dansyl hydrazine) by carbodiimide coupling to the cytoplasmic surface (carboxyl-terminal tail: 0.7 mol/mol bacteriorhodopsin) or by periodate oxidation and dimethylaminoborane reduction at the extracellular surface (glycolipids: 1 mol/mol). In 2 mM acetate buffer, pH 5.6, micromolar concentrations of UO_2^{2+} were found to quench the dansyl groups on the cytoplasmic surface (maximum = 26%), while little quenching was observed at the extracellular surface (maximum = 4%). Uranyl ion quenched dansyl hydrazine in free solution at much higher concentrations. Uranyl also bound tightly to unmodified purple membrane, (apparent dissociation constant = 0.8 μM) as measured by a centrifugation assay. The maximum stoichiometry was 10 mol/mol of bacteriorhodopsin, which is close to the amount of phospholipid phosphorus in purple membrane. The results were analyzed on the assumptions that UO_2^{2+} binds in a 1:1 complex with phospholipid phosphate and that the dansyl distribution and quenching mechanisms are the same at both surfaces. This indicates a 9:1 ratio of phosphate between the cytoplasmic and extracellular surfaces. Thus, the surface change density of the cytoplasmic side of the membrane is more negative than -0.010 charges/Å^2.

INTRODUCTION

The purple membrane from *Halobacterium halobium*, a simple light-energy transducer, occurs as differentiated regions of the bacterial membrane, and can be isolated as ~0.5 μm diam sheets (for a recent review see Stoeckenius and Bogomolni, 1982).

Many studies of isolated purple membranes indicate a high degree of charge asymmetry. Purple membrane sheets preferentially orient on polylysine-coated glass (Fischer et al., 1978) and they orient in an electric field (Shinar et al., 1977; Keszthelyi, 1980). The membrane binds cationic ferritin asymmetrically (Neugebauer et al., 1978). The proton pump activity of the purple membrane is influenced by salt concentration in a manner that suggests an asymmetric surface charge (Renthal, 1981; Kushmitz and Hess, 1981). However, no quantitative data are available on the charge asymmetry of the purple membrane.

Uranyl ion has a high affinity for phosphate groups, which are the major negative charges at the purple membrane surface. Moreover, uranyl readily undergoes exchange quenching with aromatic molecules (Matsushima and Sukaraba, 1971). We have utilized these properties, along with specific fluorescent labeling reactions (Renthal et al., 1983; Henderson et al., 1978) to estimate the number of phosphate groups exposed at the two surfaces of the purple membrane.

MATERIALS

Purple membrane was purified from *H. halobium* S9 by the method of Oesterhelt and Stoeckenius (1974). Dansyl hydrazine was obtained from Molecular Probes (Junction City, OR) or Sigma Chemical Co. (St. Louis, MO). Uranyl acetate was obtained from Polysciences Inc. (Warrington, PA). Dibenzoyl methane and borane dimethylamine were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

METHODS

Binding of UO_2^{2+} to the Purple Membrane

The total binding of UO_2^{2+} to purple membrane was measured by a centrifugation assay. Samples (1.5 ml) of purple membrane (1 μM bacteriorhodopsin in 2 mM acetate buffer, pH 5.6) were equilibrated briefly with various concentrations of uranyl acetate (0–50 μM) and then centrifuged at 20°C for 30 min at 40,000 g. Aliquots of 1.0 ml of supernatant were withdrawn from each sample and freeze-dried. The residue of each was dissolved in 0.4 ml water and mixed with 0.9 ml ethanol and 0.5 ml dibenzoyl methane solution (1% dibenzoyl methane in 95% ethanol). The absorbance of each sample was measured at 415 nm on a Cary 14 spectrophotometer (Cary Instruments, Monrovia, CA). A calibration curve of uranyl acetate samples without purple membrane was run with each experiment.

Fluorescent Labeling of Purple Membrane

Cytoplasmic Side. Dansyl hydrazine was coupled to carboxyl residues on the cytoplasmic side of purple membrane by a carbodiimide reaction, as previously described (Renthal et al., 1983).

Extracellular Side. Dansyl hydrazine was coupled to oxidized glycolipid on the extracellular side of purple membrane by a modification of the procedure described by Henderson et al. (1978) for attaching biotin hydrazide to glycolipid. Purple membrane (50 nmol) was suspended in 5 ml of 10 mM NaIO$_4$ in 0.1 M acetate buffer, pH 5.0, 0°C, for 1 h, in the dark. The oxidized membrane was centrifuged (30 min at 40,000 g, 5°C). The pellet was twice resuspended in 0.05 M NaCl and centrifuged. The washed pellet was resuspended in 4 ml of 0.1 M acetate buffer, pH 4.0, and 0.1 ml dansyl hydrazine (10 mM in ethanol) was added. The fluorescent labeling reaction was incubated for 1 h at 37°C. After storage overnight at 4°C, the hydrazone linkage was reduced by addition of 40 μl of 10% aqueous borane dimethylamine. The reduction proceeded for 1 h in the dark at room temperature, followed by centrifugation and resuspension as described above. The washed pellet was resuspended in 1.0 ml Tris buffer, 50 mM, pH 8.0, and incubated 1 h at 37°C. The membranes were again centrifuged and resuspended in 0.05 M NaCl and stored at 4°C. A slow release of dansyl label was observed for several weeks, but it did not interfere with the quenching measurements.

[³H]Dansyl hydrazine was prepared as previously described (Renthal et al., 1983) and was allowed to reacted with periodate oxidized purple membrane according to the above procedure.

Quenching of Dansyl Fluorescence by UO$_2^{2+}$

Samples of fluorescent-labeled purple membrane (3.0 ml, 1 μM bacteriorhodopsin, 2 mM acetate buffer, pH 5.6) were titrated with 10–30 μl aliquots of 0.1–1.0 mM uranyl acetate. After each addition, the fluorescence emission spectra were scanned on a Farrand Optical Co. (Valhalla, NY) Mark I ratio recording spectrofluorometer (excitation, 320 nm; bandwidth, 10 nm excitation, 2.5 nm emission). Similar titrations were measured in the absence of purple membrane.

Phosphate Analysis

Total organic phosphate of purple membrane was measured by ashing samples with with 10% Mg(NO$_3$)$_2$ in ethanol followed by HCl hydrolysis and the colorimetric method of Chen et al. (1956).

RESULTS

Uranyl Binding to Purple Membrane

In the presence of 2 mM acetate, pH 5.6, UO$_2^{2+}$ binds tightly and selectively to purple membrane (Fig. 1). The amount of bound UO$_2^{2+}$ leveled off at 10 mol/mol bacteriorhodopsin and remained at that stoichiometry up to a 50:1 ratio of total UO$_2^{2+}$ to bacteriorhodopsin. The binding assay was not sufficiently sensitive to reveal information about the binding mechanism. However, the binding did not appear to be a simple accumulation of UO$_2^{2+}$ in the diffuse double layer. Added NaCl or CaCl$_2$ at concentrations that diminish the surface potential (Renthal, 1981) have essentially no effect on the UO$_2^{2+}$ binding. Furthermore, we found no difference in UO$_2^{2+}$ binding with either 2 or 10 mM acetate buffer, conditions that change the complexation state of UO$_2^{2+}$ from predominantly free UO$_2^{2+}$ to predominantly (UO$_2$OAc)$^+$. Thus, the binding must be a complexation with ligands at the membrane surface. Assuming there are 10 independent noninteract-
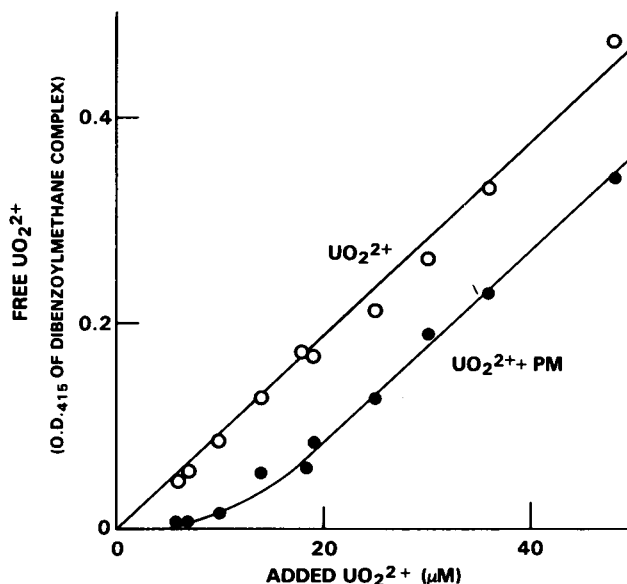

FIGURE 1   Binding of UO$_2^{2+}$ to purple membrane. (●) Samples of purple membrane (1 μM) were suspended in solutions of varying concentrations uranyl acetate (2mM acetate buffer, pH 5.6), 23°C, and then centrifuged. The UO$_2^{2+}$ concentration of the supernatants was measured as the dibenzoylmethane complex (absorbance at 415 nm). (o) Control experiments in which purple membrane was omitted.

ing sites per bacteriorhodopsin, the dissocation constant is <1 μM. Organic phosphate analysis of purple membrane showed 10 ± 1 mol phosphate/mol bacteriorhodopsin, in good agreement with the 8 mol phospholipid phosphate/mol bacteriorhodopsin reported by Kates et al. (1982).

Fluorescent Labeling of the Two Faces of the Purple Membrane

We previously reported a method of covalently coupling dansyl hydrazine to the carboxyl-terminal tail of bacteriorhodopsin (0.7 mol dansyl/mol bacteriorhodopsin) (Renthal et al., 1983), which is known to be on the cytoplasmic side of the purple membrane (Gerber et al., 1977; Ovchinnikov et al., 1977). The extracellular side of the membrane was labeled with the same fluorescent dye by a different series of reactions: (a) periodate oxidation of glycolipid, (b) formation of a fluorescent hydrazone, and (c) reduction to the more stable hydrazine. Use of [³H]dansyl hydrazine showed that ~1 mol of dansyl hydrazine was incorporated per mole of bacteriorhodopsin.

We do not see any evidence of a major change in the conformation of bacteriorhodopsin after the carbodiimide reaction (Renthal et al., 1983), or any large alteration in the overall surface charge of purple membrane (Renthal et al., 1979). After periodate oxidation, dansylation and borohydride reduction under conditions similar to those described in Methods, we extracted the purple membrane polar lipids and chromatographed them on silica layers.

The only differences observed in the lipid composition were expected changes in the mobilities of the periodate-sensitive glycolipid and phosphatidyl glycerol, and the appearance of several dansyl-containing spots. The amounts of the phospholipids were unaltered.

Uranyl Quenching of Dansyl Hydrazine Fluorescence

The fluorescence of dansyl hydrazine in 2 mM acetate buffer, pH 5.6, was quenched by small concentrations of added uranyl ion (Fig. 2). The linear Stern-Volmer plot suggests an exchange mechanism. The slope ($\sim 10^{11}$ $M^{-1}s^{-1}$, assuming the dansyl lifetime is ~ 10 ns) exceeded that expected for a simple diffusion-limited collisional mechanism, suggesting the presence of a ground-state complex between uranyl and dansyl hydrazine.

Uranyl Quenching of Fluorescence from Dansyl Purple Membrane

When UO_2^{2+} was added to purple membrane labeled with dansyl hydrazine on the cytoplasmic side (Fig. 3, upper curve) a maximum of 26% quenching was observed. By contrast, almost no quenching was observed when UO_2^{2+} was added to purple membrane labeled with dansyl hydrazine on the extracellular side (Fig. 3, lower curve).

DISCUSSION

Using specific fluorescent labeling and quenching reactions, we are able to distinguish between charged groups on the two faces of the purple membrane. Chemical modification reactions specific for each membrane surface provided a means of covalent fluorescent labeling of either the extracellular or cytoplasmic side. Heavy metal complexation at the membrane surface should quench the fluorescence of each side in proporation to the number and distribution of metal ligand sites on that membrane surface.

Nature of the UO_2^{2+} Binding Sites on Purple Membranes

In principle, fluorescence quenching of a membrane-bound fluorophore could be accomplished by collision with cations added to bulk solution (for example Cs^+). One might expect the quenching to be greater on the more negatively charged membrane surface, due to accumulation of the heavy cation in the diffuse double layer. However, this turns out to be impractical. At the Cs^+ concentrations required for quenching dansyl fluorescence (above 0.1 M), much of the double layer has been diminished due to charge screening (Renthal, 1981). Alterna-
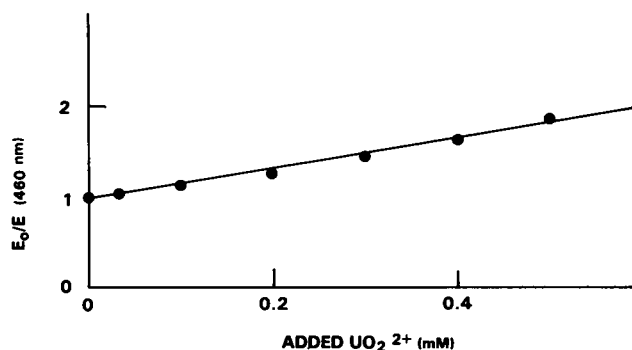

FIGURE 2 Quenching of dansyl hydrazine fluorescence by UO_2^{2+}. A solution of dansyl hydrazine (1 μM) in 2 mM acetate buffer, pH 5.6, was titrated with aliquots of uranyl acetate. The fluorescence spectra were than measured from 400 to 600 nm. The ratio of dansyl hydrazine emission at 460 nm, in the absence of added UO_2^{2+} (E_o) to emission in the presence of various concentrations of UO_2^{2+} (E) is plotted.

tively, one could choose a quencher that binds tightly at fixed positions on the membrane surface. UO_2^{2+} is ideal for this purpose. We found that it binds tightly to purple membrane (Fig. 1), and it quenches dansyl fluorescence effectively in free solution (Fig. 2).

The purple membrane surface contains three different functional groups that are known to form complex ions with UO_2^{2+}: phosphate (dissociation constant for UO_2 $H_2PO_4^+$ K = 3.6×10^{-4} M [Thamer, 1957]), carboxyl (K = 4.2×10^{-3} M for $UO_2CH_3CO_2^+$ [Ahrland, 1951a]), and sulfate (K = 1.8×10^{-2} M for $UO_2HSO_4^+$ [Ahrland, 1951b]).

We found an apparent dissociation constant (Fig. 1) of $< 10^{-6}$ M for UO_2^{2+} binding to purple membrane, considerably stronger binding than for isolated complex ions. The dissociation constant of UO_2^{2+} from phospholipid bilayers has been previously reported to be $< 10^{-5}$ M (Levine et al., 1973; Schullery and Miller, 1977). The higher affinity of UO_2^{2+} for phospholipid phosphate in lipid bilayers could be due to coordination of additional glycerol oxygens. Alternatively, cooperative effects might be involved, including interactions with protein carboxyl groups in intact membranes.

The UO_2^{2+} binding stoichiometry (Fig. 1) of 10 mol/mol bacteriorhodopsin is close to the amount of organic phosphate we found in the purple membrane and also consistent with the phospholipid composition of purple membrane reported by Kates et al. (1982), although the agreement could be merely a coincidence. Considering the preference of UO_2^{2+} for complexing phosphate over carboxyl or sulfate groups, as well as the UO_2^{2+} binding stoichiometry, and the phosphate content of purple membrane, it seems likely that the UO_2^{2+} binding sites on the purple membrane are primarily phospholipid phosphate groups, with one UO_2^{2+} per phosphate. A 1:1 stoichiometry of UO_2^{2+}/phosphate has been inferred previously from studies of UO_2^{2+} binding to phospholipid multilayers (Schullery and Miller, 1977).

Fluorescence Quenching Mechanism

We found that UO_2^{2+} quenches dansyl fluorescence both in free solution and at the purple membrane surface. A Stern-Volmer plot of UO_2^{2+} quenching of free dansyl hydrazine fluorescence gives a straight line (Fig. 2). However, the slope indicates a quenching rate that is an order of magnitude greater than expected for collisional quenching. This result suggests the possibility of a ground-state complex between dansyl hydrazine and UO_2^{2+}. A dissociation constant in the mM range would give a linear Stern-Volmer plot over the UO_2^{2+} concentration range shown in Fig. 2 (assuming that complex formation does not alter the absorbance of dansyl, and that the dissociation constant of the dansyl hydrazine-UO_2^{2+} complex is the same both in the ground and excited states of dansyl hydrazine).

The quenching of dansyl hydrazine that is covalently bound at the cytoplasmic membrane surface clearly occurs in a different manner, since 26% quenching is observed with ~5 μM UO_2^{2+} (Fig. 3). The same extent of quenching of free dansyl hydrazine required >40 times more UO_2^{2+}.

Quenching at the membrane surface occurs over the same UO_2^{2+} concentration range as UO_2^{2+} binding to unlabeled purple membrane. This indicates that the quenching occurs when UO_2^{2+} complexes with surface ligands other than dansyl hydrazine itself. In other words, the quenching at the surface does not necessarily occur by direct complex formation between dansyl and UO_2^{2+}. This conclusion is supported by the large difference in quenching on the two surfaces, despite the similarity in fluorescent labeling stoichiometry. The three most likely quenching mechanisms are: (a) exchange, (b) dipole-dipole resonance, and (c) photochemical reaction.

We estimate that the distance for 50% dipole-dipole energy transfer between dansyl and uranyl is ~11Å. This is approximately the distance found by Meares et al. (1981) for dominance of exchange interactions between lanthanides and energy acceptors. The diameter of the uranyl ion is somewhat larger than lanthanides (Rabinowitch and Belford, 1964). Exchange interactions could extend over a greater distance than for lanthanides. Thus, we cannot yet distinguish between these two mechanisms.

In any case, it is obvious that appreciable uranyl quenching of dansyl fluorescence could be expected to occur even if the dansyl groups were 15 Å from a uranyl binding site. Because the radius of bacteriorhodopsin measures between ~12 and 17 Å in the plane of the membrane (Henderson and Unwin, 1975) any dansyl-labeled protein side chain at the cytoplasmic surface could be quenched by UO_2^{2+} binding to lipid phosphate groups. However, the dansyl label on the extracellular side glycolipid might present a difficulty. What if all of the glycolipids were spacially segregated from the phospholipid on the extracellular surface, for example, in the central "doughnut-hole" lipid domain? This turns out to be unlikely. There must be five glycolipids per unit cell on the extracellular side of the

membrane (Kates et al., 1982; Henderson et al., 1978), but there is only room for three in the central domain (Hayward and Stroud, 1981).

Quenching could also occur by uranyl-mediated photooxidation of dansyl. The excitation of dansyl fluorescence unavoidably also excites uranyl, which absorbs at the same wavelength. Uranyl ion is known to undergo many photochemical reactions (Burrows and Kemp, 1974). With this mechanism, the quenching should increase as a function of excitation time. Because the observed fluorescence was stable with time, a photochemical mechanism is ruled out.

Regardless of whether the quenching mechanism involves exchange or dipole resonance, the observed dansyl fluorescence quantum yield, Φ, at a particular uranyl concentration, will be related to the surface concentration of uranyl, S, according to the following equation:

$$\Phi_o/\Phi = 1 + K\tau S \tag{1}$$

where Φ_o is the quantum yield in the absence of uranyl, K is a constant that depends on the quenching mechanism, and τ is the dansyl excited state lifetime in the absence of uranyl. When the membrane surface is saturated with uranyl, S will be the surface concentration of uranyl binding sites. All sites on the surface are filled when the quenching reaches a maximum value, $(\Phi_o/\Phi)_E$ or $(\Phi_o/\Phi)_C$, where subscript E refers to the extracellular side and subscript C refers to the cytoplasmic side. Thus, the ratio of uranyl sites on the cytoplasmic side to the extracellular side, S_C/S_E, is given by the following equation:

$$[(\Phi_o/\Phi)_C - 1]/[(\Phi_o/\Phi)_E - 1] = S_C/S_E. \tag{2}$$

This equation assumes that the quenching mechanisms, the dansyl lifetimes, and the distribution of dansyl sites are the same on both surfaces. The first two assumptions seem reasonable based on our knowledge of the membrane structure. The third assumption cannot be evaluated without knowing whether the glycolipids are clustered or randomly distributed. If they are clustered, the maximum observed surface concentration could be ~2.5 times less than the amount expected for the same number of uranyl sites randomly distributed (Kates et al., 1982).

Surface Charge Density

The results in Fig. 3 show that the maximum uranyl quenching of dansyl groups on the cytoplasmic surface was 26%, while the maximum quenching of dansyl groups on the extracellular surface was 4%. When Eq. 2 was used, the results indicated that the ratio of uranyl sites at the two surfaces, S_C/S_E was 8.4. If the uranyl binding was exclusively to phosphate groups, this ratio implies that there are nine phosphate groups per bacteriorhodopsin on the cytoplasmic side and only one per bacteriorhodopsin on the extracellular side. We can use this result to estimate a
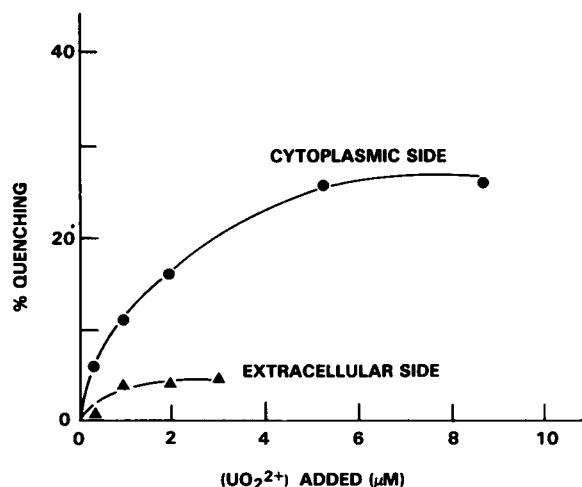
FIGURE 3 Quenching of dansyl hydrazine fluorescence by UO_2^{2+} at the purple membrane surface. Purple membrane was covalently labeled with dansyl hydrazine either at the carboxyl-terminal tail (●) (cytoplasmic surface), or glycolipid (▲) (extracellular surface). Samples of labeled membrane were titrated with UO_2^{2+} as in Fig. 2. The percent quenching of dansyl fluorescence was measured at 460 nm.

minimum value of the surface charge density of the two sides of the purple membrane at neutral pH. Most of the purple membrane phospholipid is phosphatidyl glycerol phosphate. At neutral pH, each disubstituted phosphate will have a -1 charge, while each monosubstituted phosphate will have a -1.5 charge. Thus, nine phosphate groups would have about -11 charges per bacteriorhodopsin (based on the results of Kates et al., 1982, showing 85% of the phosphate as phosphatidylglycerol phosphate). The extracellular side would have about -1 charge due to phosphate and -2 charges due to sulfate, or -3 charges per bacteriorhodopsin. Therefore, the surface charge density of the purple membrane at neutral pH is at least -0.010 charges/$Å^2$ on the cytoplasmic side and -0.003 charges/$Å^2$ on the extracellular side. Because bacteriorhodopsin has a number of negatively charged side chains exposed at the cytoplasmic surface, the actual surface change density is probably even more negative than -0.010 charges/$Å^2$. The surface charge density estimated by this method is compatible with the values previously used to fit to the Gouy-Chapman theory measurements of the effect of salt on light-induced changes in proton binding (-0.013 [Renthal, 1981]; -0.005 to -0.010, assuming all charge on one side [Kuschmitz and Hess, 1981]).

To further develop the method of surface charge measurement described here, it will be necessary to establish more firmly the nature of the uranyl binding sites and the fluorescence quenching mechanism. The technique of fluorescence quenching by bound uranyl may have some interesting applications to structural studies, since positions of uranyl ions can be readily located by electron microscopy and x-ray diffraction.

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