Cytochrome b_5 Induced Flip-Flop of Phospholipids in Sonicated Vesicles†

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ABSTRACT: Cytochrome b_5 induced flip-flop of phosphatidylethanolamine (PE) in sonicated vesicles prepared from a 9:1 mixture of phosphatidylcholine (PC) to phosphatidylethanolamine was determined as follows. First, vesicles having a nonequilibrium distribution of PE across the bilayer were prepared by amidinating the external amino groups with isethionyl acetimidate. Amidinated cytochrome b_5 was then added, and after the protein was completely bound, the rate of appearance of fresh PE on the outer surface was determined by removing aliquots at timed intervals and titrating the external amino groups with trinitrobenzenesulfonic acid. The results show an initial rapid phase of flip-flop (especially in the presence of salt) followed by a very slow phase, at 25 °C. Similar results were obtained when cytochrome b_5 was introduced into the amidinated vesicles by spontaneous transfer from PC donor vesicles. These results indicate that the accumulation of the transferable ("loose") form of cytochrome b_5 on the outer surface of a vesicle causes a transient, global destabilization of the bilayer that is relieved by lipid flip-flop. We speculate that this mechanism may be a significant driving force for the transfer of amphipathic molecules across membranes.

Transfer of phospholipids from one side of a bilayer to the other (flip-flop) takes place during many cellular processes, such as membrane biogenesis and turnover. However, the mechanism of this transfer is not understood. Early studies with liposomes showed that the half-times of flip-flop in unperturbed bilayers range from days to weeks (Thompson & Huang, 1978); in contrast, flip-flop in some biological membranes takes place in minutes or even seconds (Thompson & Huang, 1978; Crain & Zilversmit, 1980; Rothman & Kennedy, 1977; Langley & Kennedy, 1979). Several mechanisms can be proposed to account for the rapid flip-flop rate in biological membranes, as illustrated in Figure 1. One suggestion (mechanism I) is that membrane proteins have a *permanent* destabilizing effect on lipid bilayers which causes the membrane to be continuously "leaky", so to speak, to the transbilayer crossing of phospholipids. This idea has been tested in a few studies using liposomes with incorporated integral membrane proteins (Van der Steen et al., 1981, 1983; Barsukov et al., 1982; Dicorleto & Zilversmit, 1979; Nordlund et al., 1982).

It has also been suggested that *transient* flip-flop may result from temporary transbilayer packing imbalances caused by accumulation of excess protein (or lipid) on one side of the membrane. Such accumulations are likely to occur during membrane biogenesis and turnover. In most expressions of this model, illustrated by mechanism II in Figure 1, the bilayer is viewed as two coupled elastic sheets so that the induced packing stress is more or less evenly distributed throughout the system. This global destabilization of the bilayer is subsequently relieved by lipid flip-flop. We also suggest that transient *localized* disruption of the bilayer can take place during or shortly after protein binding, as illustrated in mechanism III. In the example shown here, the membrane binding segment of a protein has a different conformation in the aqueous phase than it does when stably incorporated in its final membrane conformation. Nevertheless, this putative aqueous conformation still has lipid binding sites because it is impossible to bury all the hydrophobic residues. When this form of the protein binds to the vesicle, it disrupts the vesicle at the site of interaction because the lipids cannot form a stable bilayer around this conformation. Destabilization causes flip-flop, which continues until the protein assumes its stable membrane conformation. Although this example is rather elaborate, the point is that complete insertion of a protein may not take place in one step; insertion may occur via one or more intermediates, any of which can transiently destabilize the bilayer at the site of interaction.

In the present investigation, we have been able to test these mechanisms by adding the integral membrane protein cytochrome b_5 to preformed vesicles having a chemically produced gradient of phosphatidylethanolamine (PE)[1] across the bilayer.

Cytochrome b_5 is a bipartite integral membrane protein consisting of a 12000-dalton water-soluble catalytic fragment at the amino-terminus and a 4000-dalton amphipathic tail at the C-terminus (Strittmatter & Dailey, 1982). In vivo, cytochrome b_5 inserts posttranslationally into intracellular membranes (Okada et al., 1982; Bendzko et al., 1982) and does not require protein receptors for insertion (Anderson et al., 1983). When this protein is added to preformed vesicles (other than those of dimyristoylphosphatidylcholine), it binds in a so-called "loose" configuration characterized by the rapid exchange of the protein from one vesicle to another (Roseman et al., 1977; Leto et al., 1980; Enoch et al., 1979) and by the susceptibility of the C-terminus to cleavage with carboxypeptidase Y (Enoch et al., 1979). Photolabeling studies by Takagaki et al. (1983a) suggest that in this configuration the tail extends to about the midplane of the bilayer and then loops back so that the C-terminus is on the same side of the membrane as the catalytic fragment.

Cytochrome b_5 can also be incorporated in a nontransferable or "tight" configuration by a variety of means such as cosonication of the protein with the lipid and detergent dialysis (Enoch et al., 1979). Photolabeling studies by Takagaki et

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[1] Abbreviations: PC, 1-palmitoyl-2-oleoylphosphatidylcholine; PE, transphosphatidylated phosphatidylethanolamine; IAI, isethionyl acetimidate hydrochloride; TNBS, trinitrobenzenesulfonic acid; DOC, sodium deoxycholate; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

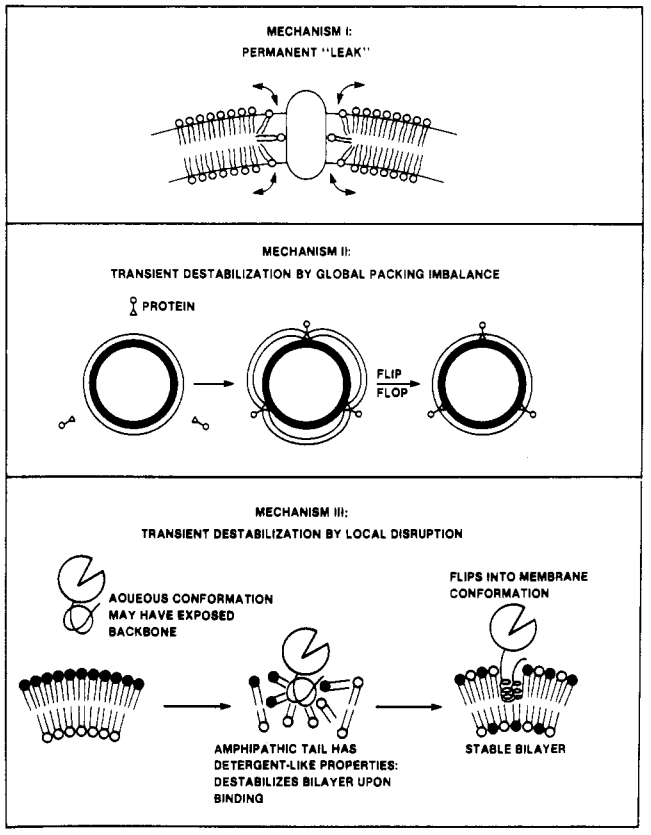

FIGURE 1: Three possible mechanisms of protein-induced phospholipid flip-flop. See text for details.

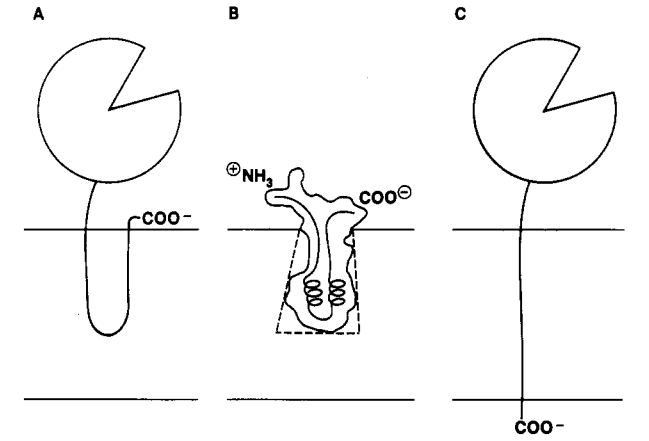

FIGURE 2: Proposed configuration of cytochrome b_5 in the "loose" and "tight" binding forms. See text for details.

al. (1983b) indicate that in the tight configuration, the tail spans the bilayer; highly schematic representations of the loose and tight configurations as determined by photolabeling are shown in Figure 2A,C.

However, before these studies were published, Strittmatter & Dailey (1982) proposed a detailed conformational model for the tight configuration, shown in rough outline in Figure 2B, which corresponds very well to the loose configuration proposed by the photolabeling group. Although this controversy has not yet been settled, we feel that the photolabeling results are more convincing at this point, and we shall assume that the model in Figure 2B best represents the loose configuration of the amphipathic tail.

Our results presented here show that a relatively small amount of cytochrome b_5 in the loose configuration can destabilize a vesicle and catalyze flip-flop in a manner consistent with mechanism II. We speculate that the loop-back hairpin conformation of the tail is very effective in "warping" the outer monolayer of a bilayer. The implication of this result is that accumulation of protein on one side of a membrane during membrane biogenesis may provide a driving force for spontaneous transfer of amphipathic molecules (including polypeptides) across the membrane.

A preliminary report of this work has been presented (Greenhut & Roseman, 1983).

Experimental Procedures

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (PC) and transphosphatidylated phosphatidylethanolamine (PE) were purchased from Avanti Biochemicals and used without further purification. Transphosphatidylated phosphatidylethanolamine (from egg phosphatidylcholine) was used in these studies because it does not oxidize as readily as egg phosphatidylethanolamine.

Isethionyl acetimidate hydrochloride (IAI), ethyl acetimidate, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma. Before use, IAI was washed several times with tetrahydrofuran and then dried overnight in vacuo over NaOH and paraffin.

Preparation of Small Vesicles. A solution containing 200–300 μmol of lipid in chloroform was evaporated to near dryness in a rotary evaporator and the lipid resuspended in a few milliliters of benzene. Benzene and residual solvents were removed by lyophilization overnight. The dried lipid was then resuspended in 11 mL of 0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4, transferred to a 15-mL Corex centrifuge tube, and subjected to sonication using a W375 Heat Systems sonicator fitted with a 0.5-in. probe. Sonication was carried out at 0 °C, in a nitrogen atmosphere, using the 50% duty cycle. After the solution reached constant clarity, the titanium was removed, and the small vesicles were obtained by the method of Barenholz et al. (1977). Lipid phosphorus was determined by the Bartlett (1959) procedure.

Preparation of Large Single-Walled Vesicles. Large single-walled vesicles, approximately 1000 Å in diameter, were prepared by the method of Enoch & Strittmatter (1979), with modifications. Sodium deoxycholate (DOC) was added to sonicated PC vesicles in 0.01 M $NaHCO_3$/0.1 M NaCl/10^{-4} M EDTA, pH 7.4, in a ratio of 1 mol of detergent per 2 mol of phospholipid. The concentration of PC never exceeded 0.01 M. The turbid solution was passed through a Sephadex G-25 gel filtration column equilibrated with the same buffer, and the void volume material was collected. To obtain large vesicles in low salt, the vesicles were first concentrated by ultrafiltration (Amicon, XM50 membrane) and then desalted by gel filtration on Sephadex G-50 or Sepharose 2B-CL with 0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4, as the eluting buffer. In our hands, attempts to produce large vesicles directly in low salt were unsuccessful. If the initial buffer contained 0.05 M NaCl or less, the vesicles produced were too small and extremely heterogeneous in size. Large vesicles produced in 0.1 M NaCl, and subsequently desalted, eluted in a sharp band in the void volume from the Sepharose 2B-CL column.

Amidination of PC/PE Vesicles. Amidination of the external amino groups with IAI was carried out with minor modifications according to the method published earlier (Roseman et al., 1975). The modifications, which include higher pH, lower temperature, and less IAI, produce vesicles with a consistently lower ratio of external to total PE than does the original procedure. A solution of sonicated PC/PE vesicles, containing 100–150 μmol of total lipid and a 9:1 ratio of PC to PE, was brought to 0.5 M Na_2CO_3, pH ~10, by addition

of 2 M Na_2CO_3, pH 10. A separate solution of IAI was rapidly prepared by adding 100 mg of IAI to 2–2.5 mL of 0.5 M Na_2CO_3, pH 10, and quickly readjusting the pH back to 10 with 2 drops of 10 N NaOH. This solution was immediately added to the vesicles, the tube flushed with argon, and the reaction allowed to incubate for 15 min at 10 °C. After this incubation period, an additional IAI solution was added and the reaction allowed to proceed for an additional 15 min. The pH was then adjusted to 8.0 by dropwise addition of 1 N HCl, and the mixture was concentrated to approximately 2 mL by using an Amicon ultrafiltration cell with an XM50 membrane. The vesicle mixture was passed through a Sephadex G-50 column (1.5 × 24 cm) in order to remove excess reagent and reaction byproducts, using 0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4, as the eluting buffer. The vesicles, eluting in the void volume, were monitored by their turbidity at 300 nm and were used immediately. The concentration and gel filtration steps were carried out at 4 °C.

Determination of the Ratio of the Phosphatidylethanolamine on the Outer Vesicle Surface to the Total Phosphatidylethanolamine Content by Reaction with TNBS. This was carried out according to the methods described earlier (Roseman et al., 1975) with modifications. The "standard" TNBS assay utilizes a high concentration of bicarbonate to maintain the pH. In order to study the effect of salt concentration on lipid flip-flop, we also devised an assay using Bicine as a buffer, which enabled us to measure flip-flop with salt concentrations as low as 0.01 M.

(*A*) *Determination of Phosphatidylethanolamine on the Outer Vesicle Surface.* An aliquot of vesicle solution containing a maximum (total) of 0.1 μmol of reactive amino groups was diluted with buffer to a final volume of 0.3 mL; 0.1 mL of 0.8 M $NaHCO_3$ or 0.04 M Bicine, pH 8.5, was added and the mixture vortexed. Ten microliters of 1.5% TNBS in water was added, the tubes were vortexed again, and the mixture was allowed to incubate in the dark for 10–60 min at room temperature. The reaction was terminated by rapid addition and mixing of 0.6 mL of 1.06% Triton X-100/1.5 N HCl. The absorbance was read immediately, for reasons given below, at 410 nm.

(*B*) *Determination of Total Phosphatidylethanolamine Content.* The same volume of vesicle solution was used for the total PE determination as was used for the external PE determination. After the volume was brought to 0.3 mL with buffer, the vesicles were disrupted by addition of 0.1 mL of 1.6% Triton X-100/0.8 M $NaHCO_3$ or 0.04 M Bicine, pH 8.5. Ten microliters of TNBS reagent was added, the tubes were vortexed, and the reaction mixture was allowed to incubate in the dark at room temperature for 5 min in the bicarbonate assay and 15 min in the Bicine assay. At the end of this period, an additional 0.2 mL of the 1.6% Triton X-100 solution was added, and the reaction was allowed to incubate for an additional 5 min in the bicarbonate assay and 15 min in the Bicine assay. The reaction was terminated by the addition of 0.4 mL of 0.4% Triton X-100/1.5 N HCl, and the samples were read immediately at 410 nm.

Separate blanks were run for the outside PE and total PE determinations because the order of addition of reactants affects the final blank reading; this is due to a different rate of TNBS hydrolysis (to picric acid) in the presence of lipid vesicles as compared to Triton X-100 micelles. Each sample and blank was done in triplicate.

The mole ratios of cytochrome b_5 to phospholipid in our flip-flop studies were 1:833, 1:500, and 1:250. At the highest ratio of protein to lipid, acidification of the sample caused a hazy precipitate to start forming after about 10 min of standing. This could be followed by an increase in the apparent absorbance at 410 nm. Readings could be safely taken within 5 min after acidification but were usually taken immediately.

As indicated by Nordlund et al. (1982), the error in these measurements is large because of the high background absorbance from cytochrome b_5 at 410 nm. In our hands, the error in determining the external PE:total PE ratio with amidinated vesicles is ±0.05.

Purification of Cytochrome b_5. Cytochrome b_5 was isolated from steer liver according to the method of Strittmatter et al. (1978), with two modifications. First, where the procedure calls for gel chromatography with Sephadex G-75, we use Sephadex G-150 instead, since we find that this modification more effectively removes the brownish high molecular weight impurities. In the purified preparations, the ratio of the absorbance at 413 nm to that at 280 nm is 2.7, which is equal to the best values reported in the literature (Dufourcq et al., 1976; Spatz & Strittmatter, 1971). Only one band is visible on SDS–polyacrylamide slab gels when 1 μg of protein is applied, but if the gel is overloaded with 10–15 μg of protein, several faint bands at higher molecular weight are visible.

Second, we always run the final Sephadex G-25 gel filtration step twice to ensure complete removal of detergent. Purification with tritiated DOC has shown that the residual DOC is only 1 mol per 100 mol of cytochrome b_5 (Dufourcq et al., 1976).

Amidination of Cytochrome b_5. Amidinated cytochrome b_5 was used in our flip-flop studies because this derivative does not react with TNBS. Cytochrome b_5 was amidinated according to the general procedure used by Dailey & Strittmatter (1979) to prepare amidinated catalytic fragments. Seventeen milligrams of cytochrome b_5 in 0.02 M Tris–acetate/2×10^{-4} M EDTA was concentrated to a volume of 2 mL in an Amicon ultrafiltration cell containing a PM10 membrane. The Tris buffer was replaced with 0.5 M sodium borate, pH 9.1, by gel filtration over Sephadex G-25 (1.5 × 23 cm column, run at 23 °C). A solution containing 13 mg of ethyl acetimidate hydrochloride in 0.5 mL of water was neutralized with 2 drops of 0.1 N NaOH and added to the cytochrome b_5 at room temperature. Three subsequent additions of freshly prepared reagent solution were made at 1-h intervals. One hour after the last addition, the solution was concentrated to 2 mL as before and passed through the same size Sephadex G-25 column, equilibrated with 0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4. The amidinated protein showed no reaction with TNBS. It was stored at −20 °C at concentrations of 0.1–0.2 mM. Amidinated catalytic fragments of cytochrome b_5 were prepared according to the method of Dailey & Strittmatter (1979) without significant modifications.

Analysis of the amidinated native protein by SDS–polyacrylamide gel electrophoresis revealed the presence of a newly generated contaminant having a molecular weight corresponding to an amidinated cytochrome b_5 dimer. Since cross-linking of purified, molecularly dispersed catalytic fragments does not take place, the cross-linking of the native cytochrome b_5 probably results from the high effective concentration of this form of the protein within water-soluble aggregates. To determine whether this contaminant would cause artifacts in the flip-flop experiments, dimer-free and dimer-enriched preparations of amidinated cytochrome b_5 had to be prepared. This was accomplished by subjecting highly dilute solutions of the amidinated protein to gel filtration in the absence of salt. As described by Calabro et al. (1976), these conditions facilitate dissociation of the protein into a

mixture of octamers and monomers. With the amidinated preparation, all the dimer eluted with the octamer fraction, giving a fraction that appeared to be about 50% dimer by weight (or 1 mol of dimer per 2 mol of monomer). The monomer fraction, however, could not be used without additional purification for the following reason. We have discovered that bovine cytochrome b_5 preparations undergo proteolysis in the hydrophobic tail region (in the absence of lipid or detergent) when the protein is subjected to gel filtration under dissociating conditions. The molecular weight of the degraded protein is greater than that of the catalytic fragment of trypsin-treated cytochrome b_5, indicating that a significant portion of the hydrophobic tail is retained. Nevertheless, the degraded protein does not bind to lipid vesicles and does not reaggregate upon addition of salt. We took advantage of the second observation to separate degraded from native protein. When the monomer preparation was subjected to gel filtration on Sephadex G-200 in the presence of 0.1 M salt, native cytochrome b_5 eluted as an aggregate in the void volume fraction, whereas the degraded protein was well included. This procedure also served to produce extremely pure cytochrome b_5, free of the high molecular weight contaminants, since these had eluted in the void volume of the original gel filtration step. No low molecular weight impurities were observed even in partially purified preparations.

Binding of Amidinated Cytochrome b_5 to Vesicles. Amidinated cytochrome b_5–vesicle complexes were formed by incubating the protein with vesicles (under argon) in low salt, 0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4, at 25, 30, or 37 °C for a minimum of 20 min. These conditions ensure complete binding of the protein (Leto & Holloway, 1979). Details of incubation conditions are given in the figure legends.

The binding of amidinated cytochrome b_5 to vesicles was confirmed by gel filtration with Sepharose 2B-CL, which distinguishes vesicle-bound from unbound protein (Rogers & Strittmatter, 1975). Since there are no residues in the membrane binding segment of bovine cytochrome b_5 that react with IAI, we would not expect amidination to affect the conformation of the tail or its lipid binding properties. Previously, Dailey & Strittmatter (1980) have shown that amidinated cytochrome b_5 binds to vesicles containing stearoyl-CoA desaturase.

Fusion of vesicles was looked for by gel filtration using Sepharose 2B-CL and by increases in 90° light scattering. A Perkin-Elmer 650-40 spectrofluorometer was used for the light-scattering measurements.

Results

To measure flip-flop, we started with sonicated vesicles having a 9:1 ratio of PC to PE and then blocked the external amino groups by amidination. This creates a gradient of PE across the bilayer. If flip-flop can occur in both directions, there will be a net flux of PE down its concentration gradient, from the inside to the outside of the vesicle. The rate of appearance of fresh PE on the outside was determined by withdrawing aliquots at timed intervals and titrating the external amino groups with TNBS. This general procedure has been described previously (Roseman et al., 1975). In those studies, it was shown that the half-time for PE flip-flop in PC/PE vesicles is at least 80 days at room temperature.

To determine the effect of cytochrome b_5 on PE flip-flop, the protein was added to amidinated vesicles for a period of time that ensures complete binding. Subsequently, aliquots were withdrawn at timed intervals, and the appearance of external amino groups was determined as before. We emphasize that TNBS measurements were not made until all the protein was bound. Therefore, we did not have to be concerned with the possibility that the vesicles may have become temporarily highly leaky to the reagent during the protein insertion process.

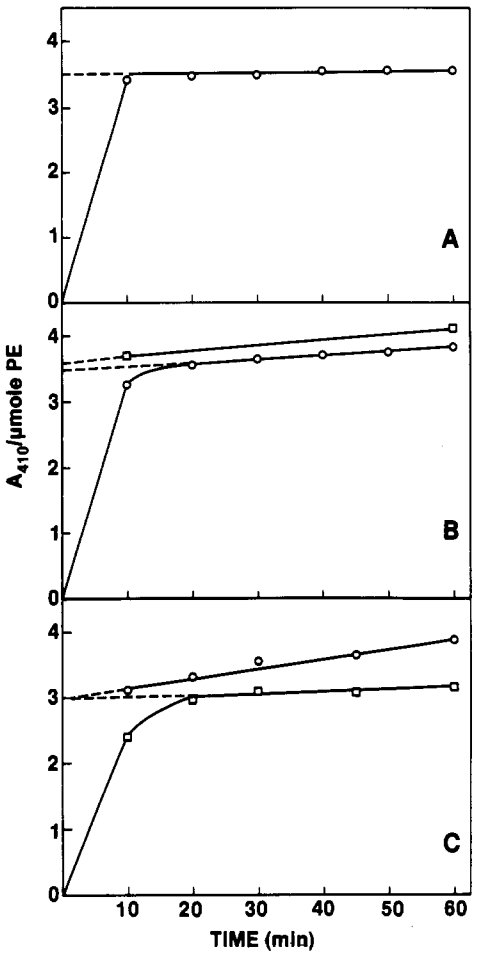

FIGURE 3: Trinitrophenylation of the external amino groups of (nonamidinated) PC/PE vesicles in the presence and absence of cytochrome b_5. (A) Standard bicarbonate assay with vesicles of PC/PE (7:3); (B) Bicine assay (○) and for comparison the bicarbonate assay (□) with vesicles of PC/PE (9:1); (C) Bicine assay (□) and bicarbonate assay (○) with vesicles of PC/PE (9:1) containing about 10 amidinated cytochromes b_5 per vesicle. Amidinated cytochrome b_5 (140 nmol) and vesicles (35 μmol in lipid phosphorus) were preincubated in a total volume of 2.55 mL of 0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4, for 20 min at 37 °C before aliquots were withdrawn.

Most of our studies were carried out by using a "standard" TNBS assay, as described under Experimental Procedures, which requires reacting the vesicles with reagent in 0.2 M $NaHCO_3$. We have also devised an assay which utilizes Bicine rather than bicarbonate as a buffer (Experimental Procedures). The rationale for using this modified assay will be discussed later.

Validation of the TNBS Assay for Lipid Amino Groups in the Presence of Cytochrome b_5. The TNBS assay in the presence of cytochrome b_5 was initially worked out by using nonamidinated vesicles composed of 9:1 PC/PE. The kinetics of trinitrophenylating these vesicles in the presence and absence of cytochrome b_5 are shown in Figure 3. With the standard bicarbonate assay, reaction of the external amino groups is virtually complete in 10 min (Figure 3A), whereas with the Bicine assay, 20–30 min are required for complete reaction (Figure 3B). The same external PE to total PE ratio is obtained with both assays. With many vesicle preparations, the reaction continues at a slow rate after the end point has been

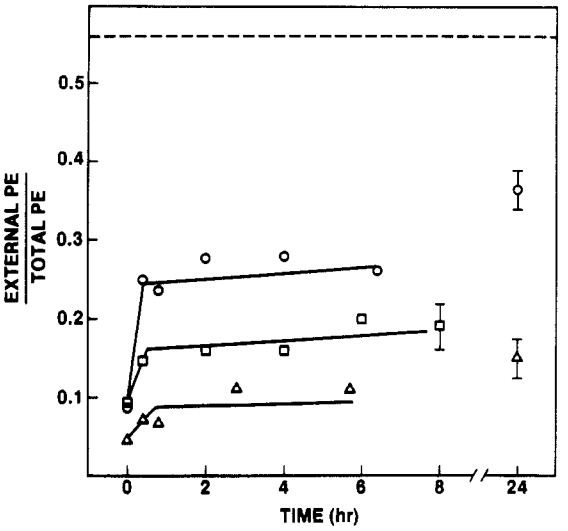

FIGURE 4: Effect of cytochrome b_5 on PE flip-flop in amidinated vesicles as determined by the standard bicarbonate assay. As described under Experimental Procedures, amidinated vesicles were always prepared from vesicles having a 9:1 ratio of PC to PE. Amidinated cytochrome b_5 and amidinated vesicles were preincubated for 20 min at 25 °C. The ratios of cytochrome b_5 to lipid in the three incubation mixtures are 1:250 (○), 1:500 (□), and 1:833 (△). Details of the incubation mixture are as follows: (○) 346 nmol of cytochrome b_5 and 86.4 μmol of lipid phosphorus, total volume 11.5 mL; (□) 172 nmol of cytochrome b_5 and 86 μmol of lipid phosphorus, total volume 10.1 mL; (△) 78 nmol of cytochrome b_5 and 65.2 μmol of lipid phosphorus, total volume 6.87 mL. For clarity of presentation, error bars are indicated on three points only. The dashed line shows the ratio of external PE to total PE before amidination, and therefore also represents the approximate end point if complete reequilibration of PE across the bilayer could take place. The zero time point shows the external PE/total PE ratio before protein was added.

reached, which probably results from leakage of reagent into the vesicles. Leakage is corrected for by extrapolating the linear slow phase back to zero time and taking the intercept as the true measure of external PE. When cytochrome b_5 is added to the vesicles (Figure 3C), the leakage rate is substantial in the bicarbonate assay and slow but detectable in the Bicine assay. After correcting for the leaks, we find that the PE surface ratio obtained in both assays is the same and, importantly, that the protein does not alter the surface ratio of the original vesicle. This last result confirms the observations of Nordlund et al. (1982).

Flip-Flop Experiments. Most of our studies were done at 25 °C in low salt (0.01 M $NaHCO_3/10^{-4}$ M EDTA, pH 7.4) and with a low ratio of protein to lipid. Under these conditions, Holloway and co-workers have shown that binding of cytochrome b_5 to vesicles is complete within a few minutes of mixing (Leto & Holloway, 1979) and that fusion of vesicles does not take place (Holloway & Katz, 1975; Roseman et al., 1977).[2] Furthermore, the mechanism of interaction has been studied (Leto & Holloway, 1979): the water-soluble octamer of cytochrome b_5 first dissociates, probably to monomer, which then interacts at nearly a diffusion-controlled rate with the vesicles.

When cytochrome b_5 was added to amidinated vesicles in low salt, and the appearance of external PE was determined by the standard TNBS assay, an unexpected result was obtained, as shown in Figure 4. By the time the first measurement could be made, considerable flip-flop appeared to have already taken place. This was followed by a much slower phase of flip-flop, barely detectable at 25 °C. No flip-flop of PE in vesicles without protein was observed, confirming our earlier observations (Roseman et al., 1975). Several control experiments were performed to determine whether this burst of flip-flop was due to contaminants or other factors. First, "ultrapure" protein was obtained via monomers as described under Experimental Procedures. When this was added to the amidinated vesicles, the flip-flop burst was actually somewhat larger than that observed with the original preparation. The contaminated octamer fraction, obtained as a byproduct of this purification, caused the same amount of flip-flop as the unfractionated preparation. These results indicate that flip-flop is not due to a protein contaminant.

Even though the level of contaminating detergent was negligible, we tested the effect of DOC and Triton X-100 on amidinated vesicles by adding these detergents in amounts equal in weight to that of the added cytochrome b_5 amphipathic tails. No significant burst of flip-flop was observed. Addition of the purified (amidinated) catalytic fragment of cytochrome b_5 caused no burst of flip-flop either. Flip-flop was not caused by vesicle fusion, as determined by gel filtration of the protein–lipid complex and by the observation that there was no increase in 90° light scattering. Since the control experiments were all negative, the results shown in Figure 4 suggest that cytochrome b_5 induces a substantial transient destabilization of the bilayer via mechanisms II and/or III that is relieved by subsequent rapid flip-flop. The slow phase, which appears almost as a plateau, presumably represents flip-flop in the stabilized vesicle. Consequently, mechanism I seems unimportant under these conditions. Although we could not tell from this experiment how fast the rapid phase of flip-flop is (since the event was over before we could make a measurement), we first assumed that it took place during the initial 20-min period when cytochrome b_5 was preincubated with the vesicles. However, we subsequently considered the possibility that rapid flip-flop may have been caused by the conditions of the TNBS assay itself: since the cytochrome b_5 and vesicles were contained in 0.01 M salt but the standard TNBS assay was carried out in high salt, the flip-flop burst could have been due to a salt shock. This seemed remote because we have previously shown that vesicles without protein do not undergo salt-induced flip-flop (see also the legend of Figure 5). In addition, transfer from low to high salt is not in the direction that would cause osmotic swelling and lysis. Nevertheless, a control experiment was designed so that the TNBS assay could be carried out at the same low salt concentration as the cytochrome b_5–vesicle incubation mixture. For this purpose, the Bicine assay was devised, as described earlier.

When cytochrome b_5 was added to amidinated vesicles in low salt and flip-flop determined by assaying aliquots in the low-salt Bicine assay, the results were strikingly different from those obtained with the standard TNBS assay. As shown in Figure 5, the flip-flop burst was greatly slowed and took place on a time scale accessible to the TNBS assay. Although it may not be evident from this figure, prolonged incubation showed that the same plateau is approached by the Bicine assay as is obtained rapidly by the bicarbonate assay. Since the low-salt Bicine assay should accurately depict the events taking place in the low-salt incubation mixture, these results show that transfer of an aliquot from the low-salt incubation mixture to the high-salt assay causes the external PE to total PE ratio of the vesicles in that aliquot to jump from a value on the Bicine curve up to the plateau level. Since the time

[2] We have recently found that cytochrome b_5 preparations that are not sufficiently purified cause fusion of a small fraction of the vesicles to which they are added. Our modified purification (Experimental Procedures) eliminates this problem.

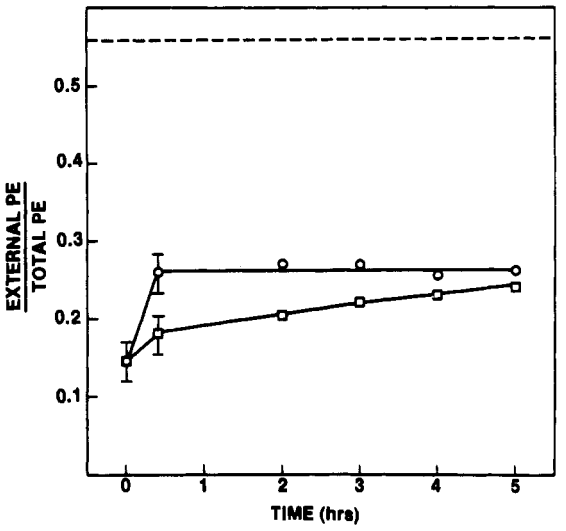

FIGURE 5: Effect of cytochrome b_5 on PE flip-flop in amidinated vesicles as determined by the low-salt Bicine assay. The ratio of cytochrome b_5 to phospholipid was 1:250, and the preincubation conditions were the same as those described in the legend of Figure 4. At timed intervals, aliquots were withdrawn and the external amino groups determined simultaneously by the bicine assay (□) and bicarbonate assay (○). Note that the external PE/total PE ratio of amidinated vesicles without cytochrome b_5 (zero-time point) is found to be the same for both assays. This shows that salt change does not induce flip-flop in vesicles without protein.

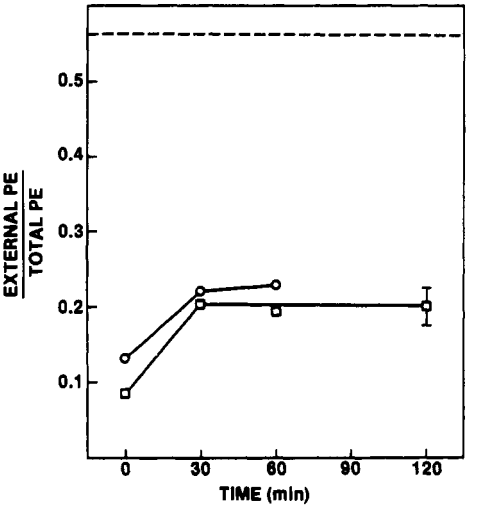

FIGURE 6: Net transfer of cytochrome b_5 from large PC vesicles to amidinated vesicles. Amidinated cytochrome b_5 and 1000-Å PC vesicles (1 mol of protein/250 mol of phospholipid, total phospholipid concentration of 4.6–5.0 mM) were preincubated for 30 min at 30 or 37 °C to ensure complete binding of the protein. A solution of amidinated vesicles was then added (about 10–15 mM in lipid phosphorus) to bring the donor:acceptor lipid ratio to 1:1 and the mixture was incubated at 30 °C. This temperature was chosen to ensure efficient transfer of cytochrome b_5 between donor and acceptor vesicles; according to Enoch et al. (1979), the half-time should be about 5 min. After 30 min, aliquots were withdrawn and assayed for external PE/total PE by the standard bicarbonate assay. The two curves show two separate experiments.

lag between mixing the aliquot with the bicarbonate buffer and subsequent addition of TNBS is usually no more than 30–60 s, the rapid jump to the plateau must take place on a time scale of seconds. In order to make sure that vesicles are not inordinately leaky just after transfer to bicarbonate, we did a control experiment in which we allowed the vesicles to incubate in the bicarbonate buffer for 20 min before addition of TNBS. The results were identical with those obtained with the usual protocol. Furthermore, the change in salt concentration does not cause fusion since no detectable increase in 90° light scattering was observed.

Therefore, as stated earlier, it appears that addition of cytochrome b_5 to vesicles in low salt causes a transient destabilization of the bilayer according to mechanisms II and/or III. In mechanism III, relief of the destabilization takes place by the protein assuming its ultimate membrane conformation, whereas in mechanism II, relief takes place by lipid flip-flop. According to our results, the rate of relief by either mechanism would have to be extremely sensitive to salt. We are currently carrying out an extensive series of experiments to determine the effect of salt and salt gradients on cytochrome b_5 induced flip-flop. Preliminary results indicate that flip-flop can be substantially enhanced by 0.1 M NaCl even when the salt concentration is the same across the bilayer. We do not yet know if this enhancement is as great as that caused by a salt gradient.

To distinguish between mechanisms II and III, we performed experiments in which the cytochrome b_5 was introduced into amidinated vesicles via transfer from donor vesicles of PC. The rationale is as follows: With our vesicles, cytochrome b_5 is bound in the loose configuration and exchanges between vesicles with a half-time of about 5 min at 30 °C (Enoch et al., 1979). This exchange takes place by transfer of protein through the aqueous phase rather than by vesicle collision (Leto et al., 1980), indicating that vesicles are always in dynamic equilibrium with free protein. Consequently, cytochrome b_5 is continually dissociating and rebinding to vesicles even during the slow phase of flip-flop. Suppose mechanism III is correct, and each time the protein dissociates from the vesicle it assumes the aqueous conformation shown in mechanism III. If so, each time the protein rebinds during dynamic equilibrium it should effectively catalyze flip-flop by local disruption of the bilayer. However, we observe that flip-flop is very slow after the burst phase, suggesting that the form of the protein in dynamic equilibrium with the vesicles does not catalyze flip-flop according to mechanism III. Mechanism II, on the other hand, predicts that it is the *net accumulation* of the protein on the vesicle that causes the burst phase. Therefore, *net transfer* of protein from donor vesicles to amidinated vesicles should cause a burst of flip-flop in the acceptors even though this form of the protein does not catalyze flip-flop during dynamic equilibrium.

In order to test this prediction, we initially carried out experiments in which sonicated vesicles of PC served as donors of cytochrome b_5 and amidinated vesicles as acceptors. When donor vesicles containing 10 cytochromes b_5 per vesicle were incubated with an equal number of amidinated vesicles, a flip-flop burst was observed that had a magnitude roughly corresponding to that observed when 5 cytochrome b_5 were directly added to the amidinated vesicles. However, as shown in Figure 4, addition of five cytochromes b_5 per vesicle produces a flip-flop burst that is just beyond the range of experimental error. More convincing results were obtained by using 1000-Å PC vesicles as donors because, as Enoch et al. (1979) have shown and we have confirmed, the equilibrium distribution of cytochrome b_5 between large vesicles and sonicated vesicles lies in favor of the small vesicles by a factor of about 4:1. Consequently, large vesicles are much more effective donors, and it is possible to introduce about seven to eight cytochromes b_5 into the acceptors. Transfer of cytochrome b_5 to the acceptor vesicles resulted in a definite flip-flop burst, as shown in Figure 6. These results are consistent with our prediction that the net accumulation of protein on one side of the bilayer is required for transient destabilization: mechanism II is favored over mechanism III.

This result is important for other reasons. As stated earlier, one of our major concerns was that the flip-flop burst obtained by adding protein directly to amidinated vesicles is due to contaminants in the purified protein preparation, or due to some other unique feature of the water-soluble form of purified cytochrome b_5. For example, it is possible that the conformation of the hydrophobic tail in the purified protein is kinetically trapped in an unusual conformation that disrupts the bilayer upon initial interaction. It is also possible that interaction with aggregates of cytochrome b_5 causes disruption of the bilayer. Even though the kinetic analysis by Leto & Holloway (1979) indicates that dissociation of the octamer must take place before binding can occur, their results do not rule out the possibility that intermediate aggregates, such as dimers and tetramers, bind directly to the vesicles. The fact that flip-flop occurs by introducing cytochrome b_5 into amidinated vesicles via transfer from other vesicles tends to rule out these possibilities. Furthermore, trace contaminants in the purified preparation that may cause flip-flop should be diluted out in the donor vesicles, or even stably bound and unavailable for transfer. Therefore, the possibility that contaminants in the purified preparation are responsible for flip-flop is further reduced.

Discussion

Our results suggest that accumulation of cytochrome b_5 in the outer monolayer of a vesicle causes a packing stress that is relieved by rapid lipid flip-flop, as illustrated in mechanism II. The other mechanisms for bilayer destabilization seem less important with this protein under our conditions. If our interpretation is correct, we must account for the observation that the rapid phase of flip-flop seen with amidinated vesicles occurs with net transfer of PE from the inner monolayer to the outer; intuitively, one might expect that relief of the externally induced packing stress would involve migration of phospholipid only from the outer monolayer to the inner. However, it seems just as reasonable to assume that in a stressed vesicle the kinetic barrier to flip-flop in both directions is temporarily lowered and the packing stress is then relieved by having a greater flux of lipid going in one direction than in the other until a more stable vesicle is obtained. Unfortunately, since we are unable to monitor the flip-flop of PC and amidinated PE with our assay, we cannot verify this explanation nor ascertain the total number of lipids that actually participate in the flip-flop event.

The role that salt plays in accelerating the fast phase of lipid flip-flop seems problematical without additional studies. Salt may further destabilize the strained vesicle or else stabilize the flip-flop intermediate. Since we do not know whether flip-flop takes place by the solubility–diffusion of single phospholipid molecules or involves more complex structures such as inverted micelles, speculation is difficult. There is even the possibility that flip-flop requires the close encounter of two or more cytochrome b_5 molecules in order to generate a region of bilayer instability. Salt could screen the negative charge on the proteins and facilitate these encounters. Further experiments to answer these questions are in progress.

Nevertheless, it is clear that cytochrome b_5 in the loose configuration has a large destabilizing effect on a vesicle (as indicated by the fact that the flip-flop rate of PE is temporarily accelerated by orders of magnitude). A surprisingly small amount of protein initiates this event: when 10 cytochrome b_5 molecules are added to a sonicated vesicle, insertion of the hydrophobic tail increases the mass of the outer monolayer by only about 3%. Such a small increase in mass seems like a minor perturbation. However, according to the theoretical treatments of Carnie et al. (1979), the destabilizing effect of a "guest" molecule on a "host" vesicle depends largely on geometric factors. For example, if the guest amphiphile is highly tapered, as is lysolecithin, a minimal amount of bilayer destabilization will occur when this molecule is added to preformed vesicles. Accordingly, De Kruijff et al. (1977) found that egg PC vesicles, when incubated with lysolecithin, were able to incorporate at least 13% of the lysolecithin into the outer monolayer without inducing flip-flop. On the other hand, if the guest molecule is a phospholipid that forms larger vesicles than does the host phospholipid, the guest will have a large destabilizing effect on the acceptor vesicle because it will try to increase the curvature of the outer monolayer. We may extend this idea to the extreme: what if the guest molecule tends to induce negative curvature in the outer monolayer? This may be the case with cytochrome b_5 if it binds in the conformation shown in Figure 2B. The possible effect on a vesicle (greatly exaggerated) is illustrated in mechanism II. Although we have not attempted a quantitative treatment, it is clear that introducing even the smallest region of negative curvature into a circular structure greatly distorts the shape. This can be demonstrated by joining the ends of a flexible wire with a "v"-shaped connector, thereby producing a heart-shaped curve known as a cardioid. A sonicated vesicle probably cannot be bent into this shape without having part of the bilayer assume a radius of curvature that is below the limit for vesicle stability.

With larger vesicles (or biological membranes), we may find that the packing stress is initially relieved by membrane deformation rather than flip-flop. With addition of more protein, however, the membrane should become as strained as a sonicated vesicle so that flip-flop would become the only mechanism available for relieving the destabilization. We tried a few experiments to determine this, using vesicles prepared by detergent dialysis from deoxycholate. Unfortunately, the results were ambiguous because these vesicles were very leaky to the amidinating reagent and TNBS. We are currently making large vesicles by other means.

It will also be necessary to see whether cytochrome b_5 induces flip-flop in vesicles with a different lipid composition, since Van der Steen et al. (1981) have shown that glycophorin catalyzes flip-flop in vesicles of dioleoylphosphatidylcholine but not in vesicles composed of whole lipid extracts of erythrocytes (Van der Steen et al., 1983).

We also feel that our studies may have important implications concerning the mechanism by which proteins are inserted into membranes. In vivo, cytochrome b_5 spontaneously inserts, posttranslationally, into intracellular membranes (Okada et al., 1982; Bendzko et al., 1982), and no protein receptor appears to be required for the initial binding (Anderson et al., 1983). Therefore, our method of incorporation—adding the protein to preformed vesicles—is a reasonable simulation of the initial binding events in vivo. Since endogenous cytochrome b_5 in microsomes is found to be in the tight configuration (Enoch et al., 1979; Poensgen & Ullrich, 1980), it seems likely that in vivo the loose configuration is really an intermediate structure that can flip into the tight configuration if the bilayer is sufficiently destabilized. The kinetic barrier separating these configurations probably results from the difficulty in transferring the two to three negatively charged residues at the C-terminus across the bilayer. Although it is now recognized that the polar residues of many proteins can spontaneously cross membranes (Wickner, 1980), the mechanism is completely unknown. One of the most detailed models, proposed by Engleman & Stietz,

(1981), suggests that the driving force for transmembrane insertions can arise from "helical hairpin" intermediate structures, similar to that proposed for the loose conformation of cytochrome b_5. The basic idea is that a sufficiently hydrophobic stretch of helix can provide the thermodynamic driving force for the insertion and crossing of a polar section of helix. Implicit in this and similar models is the idea that the bilayer remains structurally intact as the protein is inserted. The problem of insertion is therefore reduced to a solubility problem of transferring polar residues through a continuous film of hydrocarbon solvent.

However, our results show that cytochrome b_5 causes a large transient destabilization, perhaps breakdown, of the bilayer. The fact that the polar head groups of PE can rapidly cross the bilayer for a time shows that the barrier properties of the membrane have been altered. If polar head groups of lipids can cross the bilayer, why not the polar residues of the protein? Although with our vesicles this destabilization does not lead to transbilayer insertion of the protein, spontaneous formation of the tight configuration does take place when cytochrome b_5 is added to vesicles of dimyristoyllecithin, and also to vesicles of egg lecithin containing the integral membrane protein stearoyl-CoA desaturase (Enoch et al., 1979). The effect of cytochrome b_5 induced flip-flop in these systems has not been studied. Perhaps more than one destabilizing factor is required for transmembrane insertion; we are currently studying this in a systematic way. However, the main point is that models designed to explain spontaneous protein insertion should regard the bilayer as a structure that can be broken down, and not just as a two-dimensional solvent.

The results presented in this study may appear to conflict with those of Barsukov et al., (1982) and Nordlund et al. (1982), since both groups concluded that cytochrome b_5 does not cause flip-flop in lipid vesicles. However, there is no conflict. Barsukov et al. incorporated cytochrome b_5 into vesicles by detergent dialysis rather than by adding the protein to preformed vesicles. Therefore, they started their flip-flop measurements with stable protein–vesicle complexes and with cytochrome b_5 (probably) in the tight configuration. The negligible flip-flop that they observe may correspond to the slow phase that we observe after the initial packing stress is relieved.

In the studies carried out by Nordlund et al., they started with vesicles having an equilibrium distribution of PC and PE across the bilayer. Therefore, the rapid burst of PE flip-flop could not be detected by the TNBS method because no net change in PE distribution across the bilayer could have taken place. As indicated under Results, we verified their observations with nonamidinated vesicles. The crucial feature of our experimental setup is that we are starting with vesicles having a considerable nonequilibrium distribution of one lipid species across the bilayer.

Nevertheless, it is also possible that cytochrome b_5 induced flip-flop depends critically on vesicle composition, radius of curvature, ionic strength, and temperature. We are systematically exploring these possibilities.

Registry No. PC, 6753-55-5; IAI, 94732-92-0; cytochrome b_5, 9035-39-6.

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Primary Photochemical Event in Bacteriorhodopsin: Study with Artificial Pigments†

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ABSTRACT: Artificial bacteriorhodopsin (bR) pigments based on synthetic retinal analogues with selectively blocked single and double bonds are prepared and submitted to pulsed laser photolysis. Similar experiments are carried out with short-chain aromatic analogues. It is concluded that only the C_{13}=C_{14} double bond can be isomerized in the primary photoprocess. It is shown also that this process is accompanied by separation of the Schiff base from its protein counterion. The effective dielectric constant at the binding site and the nature of the Schiff base counterion play an important role in determining the absorption maximum of bR.

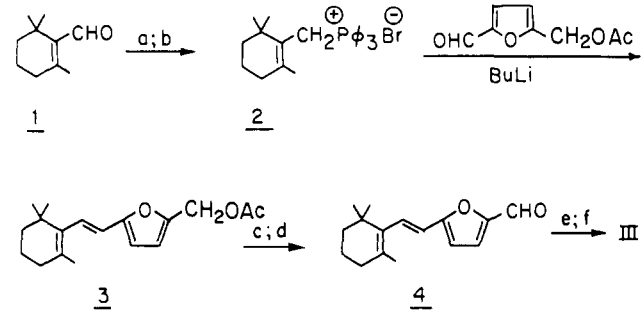
The biologically active, light-adapted, modification of bacteriorhodopsin (the protein pigment in the purple membrane of *Halobacterium halobium*) contains an *all-trans*-retinyl chromophore, bound to the bacteriorhodopsin protein via a protonated Schiff base linkage with a lysine residue. The light-driven proton pump in the pigment bR_{570}[1] is induced by a photoprocess centered in the polyene chromophore [see Stoeckenius et al. (1979) and Ottolenghi (1980) for reviews]. Light absorption is followed by a cascade of structural transformations involving both the polyene and the protein. Obviously, a detailed description of all these events is a prerequisite for formulating a molecular model for the function of bacteriorhodopsin.

Of primary importance is the primary event, associated with the red-shifted K_{610} intermediate, which stores a substantial amount of the photon energy (Honig et al., 1979 Warshel et al., 1982). Accumulated, though indirect, evidence suggests that this process is based on isomerization about the C_{13}=C_{14} double bond in the polyene and on the resulting charge separation between the protonated Schiff base and its counterion (Honig et al., 1979).

Looking for direct evidence supporting this model, we have previously carried out experiments with an artificial pigment made by recombining bacterioopsin with a synthetic retinal analogue in which the C_7=C_8 double bond rotation is blocked by a six-membered ring (Umadevi et al., 1983). In this work, this approach is extended to retinal derivatives in which all other C=C double bonds (i.e., C_9=C_{10}, C_{11}=C_{12}, and C_{13}=C_{14}) are alternatively blocked by appropriate five-membered epoxy rings. A basically similar work was first carried out by Akita et al. (1980), who tested the C_{11}=C_{12} cis → trans photoisomerization model for visual pigments, using an artificial bovine rhodopsin based on a seven-membered ring retinal analogue.

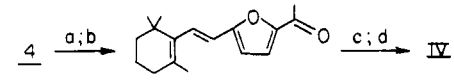
Parallel to the selective blocking of C=C bond rotations, we have carried out a complementary set of experiments with artificial pigments based on short-chain aromatic retinal

Scheme I[a]



[a] (a) $NaBH_4$/EtOH, 30 min, 0 °C. (b) Ph_3P·HBr/THF, 25 °C, 12 h. (c) KOH/EtOH, 0 °C, 2 h. (d) MnO_2/CH_2Cl_2, 25 °C, 12 h. (e) $(EtO)_2POCH_2CN$/NaH, THF, 25 °C, 1 h. (f) DIBAH/hexane, −78 °C, 1 h/silica, H_2O.

Scheme II[a]



[a] (a) MeLi/EtOH, 0 °C, 1 h. (b) MnO_2/CH_2Cl_2, 25 °C, 12 h. (c) $(EtO)_2POCH_2CN$/NaH, THF, 25 °C, 1 h. (d) DIBAH/hexane, −78 °C, 1 h/silica, H_2O.

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[1] Abbreviations: bR_{570}, bacteriorhodopsin, the subscript denoting the wavelength of maximum absorption; THF, tetrahydrofuran; DIBAH, diisobutylaluminum hydride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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