emic lysozyme has been solved and refined at 1.5 Å (Artymiuk & Blake, 1981). Crystals of the HT lysozyme have been grown by essentially the same method (Osserman et al., 1969): a drop consisting of 5 μL of 7 M NH_4NO_3 and 5 μL of 20 mg/mL HT lysozyme was allowed to vapor-diffuse against a reservoir of 1 mL of 7 M NH_4NO_3, both buffered at pH 4.7. The characteristic clusters of crystals of up to 0.2 × 0.2 × 0.8 mm^3 in size appeared after several weeks (Figure 6). Fifteen-degree precession photographs (Figure 7) revealed the cell dimensions to be a = 57.2 Å, b = 61.2 Å, and c = 32.9 Å ($\alpha = \beta = \gamma = 90°$), in very good agreement with those of human leukemic lysozyme crystals (a = 57.1 Å, b = 61.0 Å, and c = 32.9 Å). The diffraction intensities appear to be identical.

NMR Studies (C. M. Dobson and C. Redfield). A 300-MHz proton NMR spectrum of HT lysozyme has been obtained: it appears essentially identical with the spectra of human leukemic lysozyme and human milk lysozyme (Figures 8 and 9). Both techniques therefore indicate that HT lysozyme is indistinguishable from the leukemic or the milk enzyme.

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Rotational Diffusion and Self-Association of Band 3 in Reconstituted Lipid Vesicles†

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ABSTRACT: Band 3, the anion transporter of the human erythrocyte membrane, has been purified and reconstituted into phospholipid vesicles of varying composition. Rotational diffusion of band 3 in these vesicles was measured by observing flash-induced transient dichroism of an eosin triplet probe covalently bound to the protein. In egg phosphatidylcholine vesicles of high lipid/protein ratio at temperatures well above the gel to liquid-crystalline phase transition, the absorption anisotropy decays to a constant value of 12 ± 1% (expressed as a percentage of the initial anisotropy). However, higher values of the residual anisotropy and a slower decay are observed upon decreasing the temperature, even though the lipids remain in a liquid-crystalline phase. A similar effect is observed upon decreasing the lipid/protein ratio at constant temperature. It is concluded that self-association of band 3 occurs which is dependent on temperature and protein concentration in the bilayer. It is very probable that similar effects occur in the erythrocyte membrane, where a strong temperature dependence of band 3 rotational mobility is also observed. Reconstitution of band 3 into dioleoylphosphatidylcholine vesicles yields results similar to those obtained with egg phosphatidylcholine. When dimyristoylphosphatidylcholine is used for reconstitution, band 3 is immobilized below the lipid phase transition and remains partially associated above the transition. A precise analysis of the anisotropy decay curves is hampered by the presence of multiple rotating species. Under conditions which favor maximum dissociation of band 3, the data are consistent with the major fraction of band 3 having a rotational relaxation time ($\phi_\parallel$) of ≈40 μs (where $\phi_\parallel$ is defined as the reciprocal of the diffusion coefficient for rotation about the membrane normal). This would be a reasonable value for either the dimer or the tetramer of band 3.

Rotational and lateral diffusion of integral membrane proteins has been extensively investigated in recent years. These studies have yielded much information concerning membrane dynamics and protein–protein interactions [for reviews, see Cherry (1979), Edidin (1981), Hoffmann & Restall (1983), Peters (1983), and Axelrod (1983)]. Some of the most detailed investigations have been performed with band 3, the anion-exchange protein in the erythrocyte membrane. Rotational diffusion of band 3 has been measured by using triplet probes (Cherry et al., 1976; Nigg & Cherry, 1979a; Austin et al., 1979; Johnson & Garland, 1981), while lateral diffusion measurements have involved fluorescence photobleaching recovery (FPR)[1] and fusion techniques (Peters et al., 1974; Golan & Veatch, 1980; Koppel et al., 1981; Schindler et al., 1980; Fowler & Branton, 1977). Lateral and rotational diffusion of band 3 has also been measured in reconstituted vesicles by FPR and saturation-transfer electron-spin resonance, respectively (Chang et al., 1981; Sakaki et al., 1982).

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[1] Abbreviations: eosinyl-MA, eosinyl-5-maleimide; egg PC, egg phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DTE, dithioerythritol; L/P, lipid/protein ratio(s) (w/w); PBS, phosphate-buffered saline; 5PB, 5 mM phosphate buffer; FPR, fluorescence photobleaching recovery; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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Measurements of the rotation of band 3 in the erythrocyte membrane depend on the fact that anionic triplet probes such as eosinyl-5-maleimide (eosinyl-MA) selectively label band 3 when reacted with intact erythrocytes. Rotation may then be measured by either transient dichroism (Nigg & Cherry, 1979a), phosphorescence (Austin et al., 1979), or fluorescence depletion (Johnson & Garland, 1981) methods. In all cases, the investigations exploit the long lifetime of the triplet state of the probe to measure the relatively slow rotation of band 3 in the membrane.

These measurements have proved particularly useful for elucidating protein–protein interactions in the erythrocyte membrane. In particular, dimeric association of band 3 (Nigg & Cherry, 1979b), complex formation between band 3 and glycophorin (Nigg et al., 1980), interactions between band 3 and cytoskeletal proteins (Nigg & Cherry, 1980), and binding of glyceraldehyde-3-phosphate dehydrogenase (band 6) to band 3 (Matayoshi et al., 1983) have been studied.

It is clear from these investigations that rotational motion of band 3 is in part restricted by interactions with peripheral membrane proteins. The restriction can be removed by mild proteolysis with trypsin, which selectively cleaves off the 40 000-kDa segment of band 3 which protrudes from the cytoplasmic surface of the membrane (Steck et al., 1976). Even then, the situation is far from simple. Analysis of transient dichroism experiments indicates that band 3 may exist in different sized aggregates. The equilibrium between these aggregates can be influenced by varying the cholesterol content of the membrane (Mühlebach & Cherry, 1982) and by varying the temperature (Nigg & Cherry, 1979a). The effect of temperature is particularly marked. At 45 °C, most of band 3 rotates in a time on the order of 100 μs, while below 20 °C band 3 is essentially immobile over the time range 0–2 ms. Most probably, this immobilization is due to self-aggregation of band 3 and may reflect temperature-dependent properties of the lipids, although it should be emphasized that the bulk of the lipids remains in a liquid-crystalline phase down to at least 4 °C (Gottlieb & Eanes, 1974; Maraviglia et al., 1982).

In the present study, we have employed transient dichroism to investigate the rotational mobility of purified band 3 reconstituted into lipid vesicles of varying composition. The aim of these experiments is to gain insight into the behavior of band 3 in the native erythrocyte membrane, especially with respect to the conditions under which self-aggregation occurs.

Materials and Methods

Materials. Eosinyl-5-MA was purchased from Molecular Probes, Triton X-100 was from Packard, ^{3}H-Triton X-100 was from NEN, egg PC was from Lipid-Products, DOPC and DMPC were from Fluka, and Amberlite XAD-2 and the latex beads (LB3 and LB1) were from Sigma.

Labeling of Erythrocytes and the Ghost Preparation. Labeling of band 3 and preparation of ghosts were carried out as described by Nigg & Cherry (1979a). In brief, recently outdated blood (normally O^+) was washed 2–3 times in 5 mM phosphate buffer, pH 7.5, containing 150 mM NaCl (PBS) and once in isotonic phosphate buffer, pH 7.5. The washed cells were incubated for 30 min at room temperature with eosinyl-MA (1 mg/5 mL of packed erythrocytes) to selectively label band 3. Unreacted label was removed by two washes of the cells with 10–15 volumes of PBS (all volumes are based on the volume of packed red blood cells). The cells were then hemolyzed in 20–30 volumes of 5 mM phosphate buffer, pH 7.5 (5PB), and washed 2–4 times with the same buffer.

High-Salt Wash and Triton X-100 Extraction. Ghosts were suspended in 20 volumes of PBS, pH 8, and centrifuged at 18000*g* for 20 min. The supernatant was removed, and the ghosts were suspended 2 more times in 6 volumes each of the same buffer and centrifuged at 24000*g* for 7 min. The pellet was resuspended in 2 volumes of 36 mM Na_2HPO_4 and 0.5% Triton X-100 (v/v), pH 7.5, and stirred for 20 min at room temperature. The suspension was centrifuged at 27000*g* for 20 min to remove cytoskeletal proteins, and the supernatant was immediately applied to an ion-exchange column.

The purification of band 3 is a slightly modified version of the method described by Lukacovic et al. (1981).

Ion-Exchange Chromatography. One hundred milliliters of Triton extract was applied to a Whatman DE-52 anion-exchange column (2.3 × 5 cm; flow rate ~70 mL/h, room temperature). The column was washed with 30 mL of 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.5, to elute contaminating hemoglobin. The remaining protein, comprising band 3, band 4.2, and glycophorin, was eluted with 150 mM Na_2HPO_4, 150 mM NaCl, and 0.5% Triton, pH 7.5, and this fraction was immediately applied to the affinity column.

Affinity Chromatography. [[[4-(Hydroxymercuri)-benzoyl]oxy]ethyl]agarose 4B was prepared exactly as described by Lukacovic et al. (1981). The partially purified fraction obtained from the ion-exchange column was applied to this affinity column (2.1 × 2.3 cm; flow rate ~70 mL/h). The column was first eluted with 30 mL of 150 mM Na_2HPO_4, 150 mM NaCl, and 0.5% Triton, pH 7.5, and then with 30 mL of 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.5. Pure band 3 was subsequently eluted with 0.5–0.8 mM cysteine added just before use to 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.5. The detection of band 3 was simplified by its pink color arising from the eosin label. Pink fractions (10–15 mL) containing 0.5–1 mg of protein/mL were collected and immediately used for reconstitution.

Reconstitution of Band 3 into Lipid Vesicles. The appropriate amount of phosphatidylcholine in up to 3 mL of chloroform/methanol was rotated under vacuum in a Florentine flask and dried further for several hours under high vacuum to obtain a thin layer of lipids coating the flask. Six milligrams of pure band 3 was diluted with 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.5, to 0.25 mg/mL (24 mL) and with 36 mM Na_2HPO_4, 1 mM DTE, and 0.1 mM EDTA, pH 7.5, to 0.1 mg of protein/mL (60 mL). This solution (now containing 0.2% Triton) was added to the flask, and the contents were swirled until the lipids were suspended. The clear suspension was filled into dialysis tubes (exclusion limit 12–14 kDa) and dialyzed for 6 days at 4 °C against 2–3 L of 36 mM Na_2HPO_4, 1 mM DTE, 0.1 mM EDTA,and 0.02% NaN_3 containing Amberlite XAD-2 to absorb Triton. The Amberlite was changed daily, reducing the amount from 6 to 1 g. The removal of Triton was monitored by adding a tracer amount of radioactive labeled Triton to the dialysate. After 6 days, the vesicles were collected by centrifugation for 1 h at 35 000 rpm.

Determination of Vesicle Size Distribution. The size of egg PC and DOPC vesicles was examined by gel filtration on Sephacryl S-1000 as described by Nozaki et al. (1982). The column (1.6 × 45 cm; flow rate 5.2 mL/h) was calibrated with latex beads of defined mean diameter (109 ± 2.7 and 305 ± 8.4 nm) in 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.5.

The pellet obtained at the end of the reconstitution procedure was resuspended to a total volume of 2 mL in 36 mM Na_2HPO_4, 1 mM DTE, and 0.1 mM EDTA, pH 7.5, and applied to the column. The column was run overnight at 4 °C with 36 mM Na_2HPO_4, 1 mM DTE, 0.1 mM EDTA, and 0.02% NaN_3, pH 7.5, and fractions of 2.6 mL (30 min) were collected. The absorption of each fraction at 532 nm (eosin)

was measured, and hence the amount of band 3 present in different sized vesicles was determined.

Vesicles with a diameter larger than 100 nm were concentrated by centrifugation and used for measurement of band 3 rotational diffusion.

DMPC vesicles were not analyzed on the Sephacryl S-1000 column for these vesicles absorbed to the column and could only be removed by washing the column with 0.5% Triton buffer. They were, however, pelleted by low-speed centrifugation, and in the case of high L/P, large aggregates were detectable in the phase-contrast microscope.

General Methods. Protein was determined according to Lowry et al. (1951) with addition of 0.5% SDS to the alkaline buffer. Phospholipids (after dialysis against a phosphate-free buffer) were determined according to Chen et al. (1956), and proteins were analyzed on SDS–PAGE as described by Laemmli et al. (1970). For eosinyl-MA, we determined an extinction coefficient of 1.0×10^5 cm^2 mol^{-1} at 532 nm in the presence of BSA in isotonic phosphate buffer at pH 7.5. This value was used for spectrophotometric determination of eosin in the protein and membrane samples.

Transient Dichroism Measurements. The transient dichroism apparatus used to measure rotational motion was similar to that described in detail elsewhere (Cherry et al., 1978). However, excitation was by a Nd-YAG laser (JK Lasers, Ltd.) using the frequency-doubled emission at 532 nm. The pulse width was about 15 ns and the repetition rate 16 Hz. Transient absorbance changes at time t after the flash arising from ground-state depletion were simultaneously recorded at 515 nm for light polarized parallel [$A_\parallel(t)$] and perpendicular [$A_\perp(t)$] with respect to the polarization of the exciting flash. Up to 512 signals were averaged in a Datalab DL 102A signal averager. Further averaging was achieved by combining the data from three or four different samples of the same preparation. Data were analyzed and plotted by calculating the absorption anisotropy, $r(t)$, defined by

$$r(t) = \frac{A_\parallel(t) - A_\perp(t)}{A_\parallel(t) + 2A_\perp(t)} \quad (1)$$

$r(t)$ is independent of the signal lifetime and depends only on rotational motion, provided the absorption transient exhibits a single exponential decay. Data analysis and curve fitting (using a nonlinear least-squares procedure) were performed on a Hewlett Packard 9825A desk-top computer.

For a membrane protein rotating only about the membrane normal, $r(t)$ is given by

$$r(t) = \frac{r_0}{A_1 + A_2 + A_3}[A_1 \exp(-t/\phi_\parallel) + A_2 \exp(-4t/\phi_\parallel) + A_3] \quad (2)$$

where $\phi_\parallel = 1/D_R$ (D_R is the diffusion coefficient for rotation about the membrane normal), r_0 is the experimental anisotropy at $t = 0$ and A_1, A_2, and A_3 are constants which depend only on the orientation of the transition dipole moment of the absorption band used for the measurement (Cherry, 1978; Kawato & Kinosita, 1981). This equation only applies when a single rotating species is present. If the population of rotating proteins is heterogeneous, the anisotropy decay contains two additional exponential terms for each additional component. Since resolution of the decays into more than two components is rarely feasible, it is usual to analyze the data by the following simplified equation:

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) + r_3 \quad (3)$$

In this case, ϕ_1 and ϕ_2 are not exact relaxation times but may give an estimate for the faster and slower components that are present.

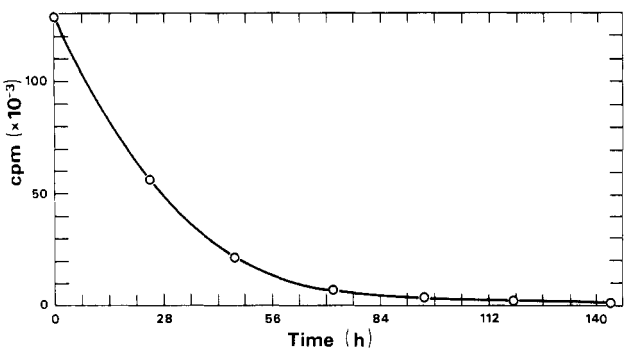

FIGURE 1: Removal of Triton X-100 by slow dialysis. A tracer amount of ^{3}H-Triton was added to the solution, giving 2200 cpm/100 μL. Aliquots of 100 μL were taken every day to determine remaining radioactivity.

Measurements were normally performed with vesicles suspended in 36 mM Na_2HPO_4, 1 mM DTE, and 0.1 mM EDTA, pH 7.5, buffer containing varying amounts of glycerol. All samples were flushed with argon prior to transient dichroism measurements to remove oxygen. The eosin concentration was typically 1–2 μM.

Results and Analysis

Isolation and Reconstitution. The isolation of band 3, according to Lukacovic et al. (1981), yielded an essentially pure band 3 suitable for reconstitution experiments. We usually obtained between 5 and 15 mg of protein from 50 mL of packed red blood cells. The eosin/band 3 ratio in the purified preparation was 0.97 ± 0.10. This confirmed the ratio of 1 to 1 which was previously determined with eosinyl-MA-labeled erythrocyte membranes (Nigg & Cherry, 1979a). Minor contamination by band 4.2 present after elution from the affinity column was no longer detectable in the reconstituted vesicles. Band 3 is reported to exist as an equilibrium of monomers, dimers, and tetramers in Triton X-100 (Pappert & Schubert, 1983). This equilibrium is presumably initially reversible, but irreversible aggregation occurs with time. To minimize this effect, isolation and purification of band 3 were performed as rapidly as possible. Even so, it was not possible to totally prevent irreversible aggregation. Some dimers were detectable on SDS–PAGE after the purification procedure, and tetramers and probably higher aggregation states were present at the end of the reconstitution. Although it was difficult to quantify how much of band 3 had undergone irreversible aggregation, it was clear that only a small fraction was involved.

Reconstitution of band 3 into phosphatidylcholine vesicles was achieved by slow dialysis of Triton X-100 and adsorption of the detergent to Amberlite XAD-2. The decrease of the detergent concentration was monitored with a trace of tritium-labeled Triton X-100 and is shown in Figure 1. We usually found less than 0.005% (v/v) Triton X-100 after 140 h of dialysis, well below the critical micellar concentration of 0.015%. One hundred percent of protein and 70–90% of lipids were recovered by centrifugation. A freeze–fracture electron micrograph of the reconstituted vesicles is shown in Figure 2.

The anion transport function of band 3 was tested in vesicles reconstituted in an identical manner but with unlabeled band 3. These vesicles exhibited a rapid sulfate exchange, which was absent in vesicles reconstituted with eosinyl-MA-labeled band 3 (Mühlebach, 1984). Eosinyl-MA was previously observed to be a potent inhibitor of anion transport in red blood cells (Nigg & Cherry, 1979a).

FIGURE 2: Freeze–fracture electron micrograph of reconstituted egg PC vesicles with L/P = 7.2. The sample was frozen from 20 °C in a jet of liquid propane. Freeze–fracture was carried out in a Balzer's 300 apparatus, and specimens were replicated with Pt/C and backed with SiO for transmission electron microscopy. Magnification 500000×.

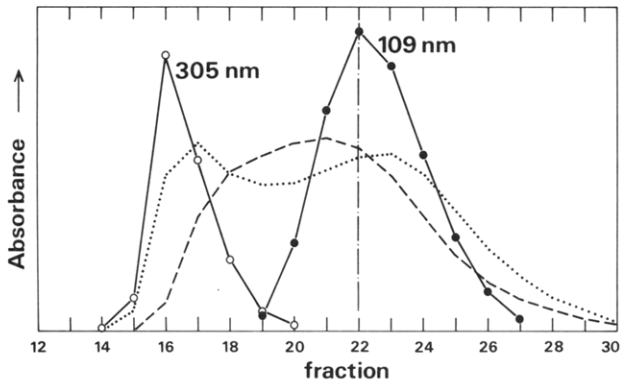

FIGURE 3: Vesicle size distribution of egg PC vesicles used for rotational diffusion measurements: elution pattern on a Sephacryl S-1000 column. Latex beads: (○) LB3, diameter 305 nm; (●) LB1, diameter 109 nm. Egg PC vesicles: (- - -) L/P = 7.5; (···) L/P = 1.1. The vertical line at fraction 22 represents the cutoff for vesicles larger than 100 nm used for protein rotation measurements.

Vesicle Size. In contrast to most previously reported reconstitution procedures for band 3 (Yu & Branton, 1976; Lukacovic et al., 1981; Darmon et al., 1983; Wolosin, 1980; Sakaki et al., 1982), the slow dialysis of the detergent results in the formation of large vesicles with diameters in the range between 50 and 300 nm, as judged by electron microscopy. The vesicle size distribution was characterized in more detail by using gel filtration chromatography. Calibration of the Sephacryl column with latex beads and the vesicle size distribution of two typical samples are shown in Figure 3. No significant difference in vesicle size was observed between reconstitutions with different L/P. Vesicle diameters were typically in the range between 50 and 250 nm for egg PC and DOPC. DMPC vesicles were not analyzed on the Sephacryl column for they absorbed to the column material. The egg PC vesicles were principally unilamellar according to freeze–fracture electron micrographs, and the protein was equally distributed in all vesicles. To minimize the contribution of vesicle tumbling to the anisotropy decay, we only used vesicles with a diameter larger than 100 nm for measurements of rotational diffusion.

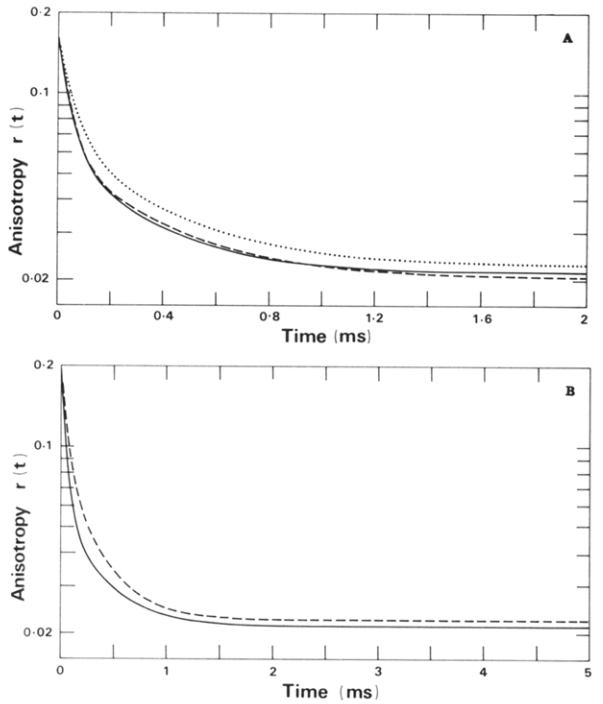

FIGURE 4: Influence of glycerol on the anisotropy decay of eosinyl-MA-labeled band 3 in egg PC vesicles with L/P = 7.5 at 20 °C. (A) Vesicle diameter >100 nm, time range 0–2 ms: (- - -) 65% glycerol (w/w), 15 cP; (—) 70% glycerol (w/w), 23 cP; (···) 75% glycerol (w/w), 36 cP. (B) Vesicle diameter >150 nm, time range 0–5 ms: (—) 70% glycerol (w/w), 23 cP; (- - -) 80% glycerol (w/w), 62 cP.

Some reconstitutions were performed by adding Amberlite XAD-2 beads directly to the detergent mixture. Although the process results in more rapid removal of Triton X-100, the vesicles formed in this way were usually smaller than 100 nm in diameter and hence less well suited to the protein rotation measurements. A further disadvantage was the loss of more than 50% of the protein by adsorption to the beads.

Measurements of Rotational Diffusion. The measurements of rotational diffusion were performed within 2 days after collection of the vesicles because it was found that prolonged storage promoted irreversible aggregation of band 3, thus slowing down its rotational motion.

For better clarity, the data points have been omitted from most of the figures containing anisotropy decay curves. The curves shown in the figures were obtained by fitting the data by use of eq 3 as described under Materials and Methods. Data points are included on one curve in Figure 5 to illustrate the typical signal to noise ratio obtained in these experiments. For comparison of anisotropy decays under different conditions, small variations in the initial anisotropy (r_0) were compensated by normalizing curves to the same value of r_0 in the figures. The values of the parameters r_1, r_2, and r_3 are expressed as a percentage of r_0 throughout.

Influence of Glycerol. Glycerol is reported to have no effect on the rotational diffusion of band 3 in ghost membranes at a concentration of 70% (Nigg & Cherry, 1979a). To minimize vesicle tumbling, which could contribute to the anisotropy decay, all measurements were therefore carried out in glycerol-containing media of high viscosity. The influence of glycerol on the rotational diffusion of band 3 in egg PC vesicles with an L/P of 7.5 is demonstrated in Figure 4.

Figure 4A shows that the $r(t)$ curves for band 3 in egg PC vesicles are essentially the same for vesicles in 65% glycerol (15 cP) and 70% glycerol (23 cP). However, there is a noticeable slowing down of the anisotropy decay at 75% glycerol. A calculation based on the vesicle size distribution and the

Stokes–Einstein equation showed that the decay in the anisotropy in 70% glycerol due to vesicle tumbling is less than 3% over 2 ms. Thus, the change in $r(t)$ observed above 70% glycerol must reflect changes in the rotation of band 3 in the membrane. This is confirmed by the results shown in Figure 4B, which compares the anisotropy decays in 70% and 80% glycerol over 5 ms. Although the initial decay is slower in 80% glycerol, the two curves fall to almost the same value of r_3. Since the viscosities of 70% and 80% glycerol differ by a factor of about 3, this result can only occur if vesicle tumbling has essentially been eliminated. The optimum glycerol concentration for these experiments therefore appears to be 70%, which is sufficiently high to eliminate the effects of vesicle tumbling without apparently affecting the rate of protein rotation.

Determination of the Residual Anisotropy. The minimum value of r_3, known as r_∞, is of particular interest in rotational diffusion measurements with membrane proteins. The value of r_∞ depends on the orientation of the chromophore with respect to the membrane normal. Measured values of r_3 greater than r_∞ indicate the presence of rotationally immobile proteins on the time scale of the measurement. In order to obtain a good value for r_∞, it is essential to ensure that no significant decay of the anisotropy due to vesicle tumbling occurs over the time range of the experiment. As discussed in the previous section, this condition is fulfilled at glycerol concentrations of 70% and above. As an additional precaution, some measurements were performed with vesicles of diameter >150 nm and the data collected over 5 ms to determine whether a constant residual anisotropy was achieved. Figure 4B shows that the anisotropy does indeed remain constant after ~2 ms. The small difference in r_∞ between 70% and 80% glycerol is within experimental error and shows that the value of r_∞ is essentially independent of the glycerol concentration over this range. The value obtained for r_∞ is 12 ± 1%.

We regard this as the maximum possible value of r_∞; the actual value could conceivably be less if a contribution from immobile aggregates of band 3 is present. This, however, is unlikely because a constant residual anisotropy over the range 2–5 ms is observed in Figure 4B. Any aggregates which showed no mobility over this time range would have to be quite large (~10 times the diameter of the smallest oligomer present). It is clear from freeze–fracture electron micrographs (Figure 2) that such large aggregates are not present in vesicles of high L/P at 20 °C.

In those cases where r_3 reached the minimum value of 12%, attempts were made to fit the data by the exact equation (eq 2) for anisotropy decay for a single rotating species (Cherry & Godfrey, 1981). In no case could a good fit be obtained, as judged by the sum of the squares of the residuals and their autocorrelation function. It is thus probable that multiple rotating species (e.g., different sized oligomers) of band 3 are present in the reconstituted vesicles, even at very high L/P.

Effect of Temperature on Rotational Diffusion of Band 3 in Egg PC Vesicles. Figure 5 shows the decay of the absorption anisotropy $r(t)$ measured in 70% glycerol at three temperatures, 20, 10, and 2.6 °C, for band 3 incorporated in egg PC vesicles at an L/P of 7.5. The first section of Table I summarizes the time constants ϕ_1 and ϕ_2 and the coefficients r_1, r_2, and r_3 obtained from fitting the data by use of eq 3. It is clear that decreasing the temperature results in a decrease in the rotational motion of band 3. In particular, the increasing value of r_3 with decreasing temperature indicates the formation of an immobile fraction of band 3. Since the gel to liquid-crystalline phase transition of egg PC is below 0 °C, this immobilization can only be due to the formation of aggregates. The low-temperature aggregation was found to be completely reversible. Above 20 °C, r_3 remained at 12% up to about 28 °C (not shown). Measurements were not made at higher temperatures because of the probability of an increasing effect of vesicle tumbling as the aqueous phase viscosity decreases.

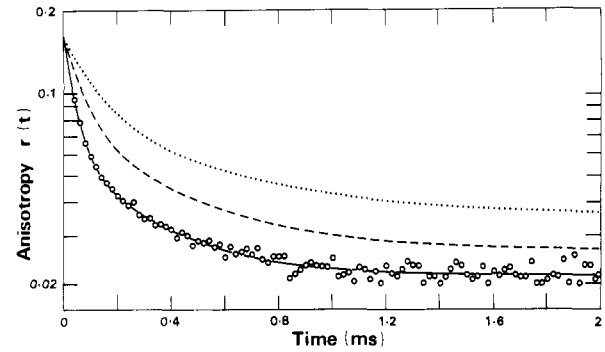

FIGURE 5: Temperature dependence of the anisotropy decay of eosinyl-MA-labeled band 3 in egg PC vesicles with L/P = 7.5 in 70% glycerol (w/w): (O) 20 °C; (---) 10 °C; (···) 2.6 °C.

Table I: Analysis of Anisotropy Decay Curves for Eosinyl-MA-Labeled Band 3 in Egg PC Vesicles[a]

	L/P = 7.5 temp (°C)		
parameter	20	10	2.6
ϕ_1 (μs)	40 ± 8	73	115
ϕ_2 (μs)	282 ± 23	386	502
r_1 (%)	60 ± 6	52	44
r_2 (%)	28 ± 6	32	34
r_3 (%)	12 ± 1	17	22
	L/P = 2.2 temp (°C)		
parameter	20	10	1.3
ϕ_1 (μs)	73 ± 3	104	168
ϕ_2 (μs)	475 ± 20	617	721
r_1 (%)	57 ± 2	47	38
r_2 (%)	26 ± 2	32	29
r_3 (%)	18 ± 1	21	33
	L/P = 1.1 temp (°C)		
parameter	20	10	0.9
ϕ_1 (μs)	121 ± 7	126	124
ϕ_2 (μs)	763 ± 76	775	792
r_1 (%)	25 ± 2	16	9
r_2 (%)	33 ± 2	33	27
r_3 (%)	42 ± 3	51	64

[a] Vesicles in 70% glycerol (w/w) at different temperatures and L/P. The parameters were obtained from the best fit to eq 3 by using a nonlinear least-squares regression analysis. Values at 20 °C are the mean and standard deviation of four measurements. Values at lower temperatures are the mean of two measurements.

When multiple rotating species are present, a precise analysis of the anisotropy decay curve is very complex (Cherry & Godfrey, 1981). Nevertheless, it is clear that there is an increase in ϕ_1 and ϕ_2 and an increase in r_2 relative to r_1 with decreasing temperature, again consistent with aggregation effects. It should be emphasized that the data cannot solely be explained by the temperature dependence of membrane viscosity. Although this factor may in part account for the effect on ϕ_1 and ϕ_2, only changes in aggregation equilibria can explain the temperature dependence of r_1, r_2, and especially r_3.

Effect of L/P on Rotational Diffusion of Band 3 in Egg PC Vesicles. Figure 6 shows anisotropy decays measured in

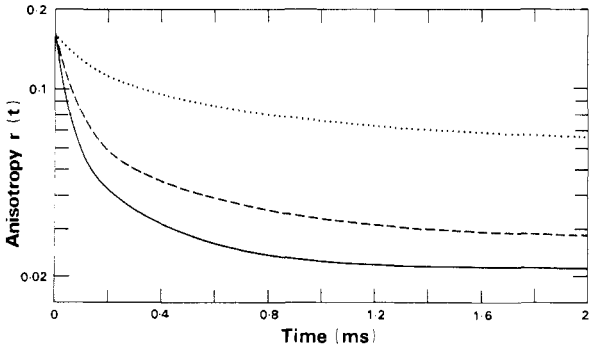

FIGURE 6: Dependence on L/P of the anisotropy decay of eosinyl-MA-labeled band 3 in egg PC vesicles at 20 °C in 70% glycerol (w/w): (—) L/P = 7.5; (- - -) L/P = 2.2; (···) L/P = 1.08.

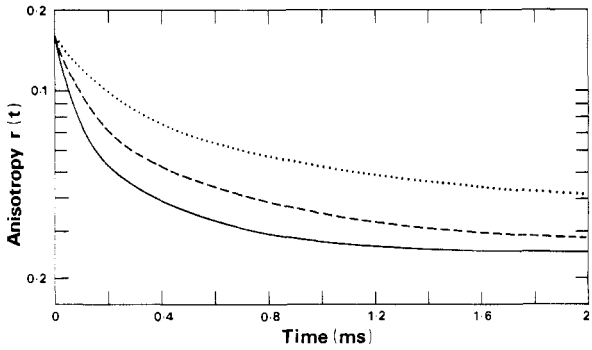

FIGURE 7: Temperature dependence of the anisotropy decay of eosinyl-MA-labeled band 3 in DOPC vesicles with L/P = 8.8 in 70% glycerol (w/w): (—) 20 °C; (- - -) 10 °C; (···) 2 °C.

70% glycerol at 20 °C for egg PC vesicles with L/P = 7.5, 2.2, and 1.1. Measurements were also made at different temperatures and the complete set of parameters obtained from fitting the data by use of eq 3 is given in Table I. It is clear from Table I that the effect of temperature on vesicles with L/P = 2.2 and 1.1 is similar to that described in the previous section for L/P = 7.5.

In addition, Figure 6 shows that there is a striking dependence of the anisotropy decay on L/P. The loss of rotational mobility with decreasing L/P must again result from band 3 aggregation as the concentration of protein in the membrane increases. The percentage of band 3 which is immobile over 2 ms, I, can be calculated from

$$I = \frac{100(r_3 - r_\infty)}{100 - r_\infty} \quad (4)$$

where r_3 and r_∞ are both expressed as the percent of r_0. This calculation shows that at 20 °C, 6.5% of band 3 is immobile at L/P = 2.2 while at L/P = 1.1 the immobile fraction rises to 35% (taking r_∞ = 12%).

Reconstitution with Other Lipids. Because of the heterogeneity of acyl chains in egg PC, it is of interest to compare the behavior of band 3 when incorporated into vesicles formed from a single lipid species. Experiments were therefore performed with DOPC, and anisotropy decays obtained with band 3 incorporated in vesicles of L/P = 8.3 are shown in Figure 7 for vesicles in 70% glycerol as a function of temperature. The results are qualitatively similar to those shown in Figure 5 for egg PC and are similarly interpreted as reflecting self-aggregation of band 3 as the temperature is lowered. It is found, however, that the time constants ϕ_1 = 57 μs and ϕ_2 = 346 μs, at 20 °C, are somewhat higher than those obtained for the egg PC vesicles (compare Table I) and that the residual anisotropy r_3 = 15.6% does not reach the minimum value. This

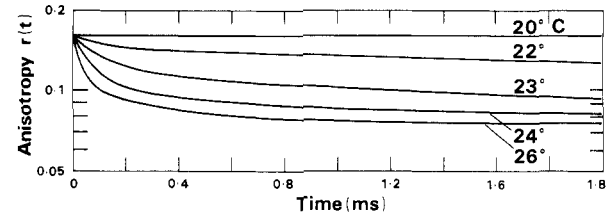

FIGURE 8: Temperature dependence of the anisotropy decay of eosinyl-MA-labeled band 3 in DMPC vesicles with L/P = 9.3 in 50% glycerol (w/w).

indicates a slightly increased tendency for self-aggregation of band 3 in a pure lipid environment compared with the egg PC mixture.

This effect is even more marked when band 3 is reconstituted into vesicles of DMPC. Figure 8 shows data obtained at different temperatures from band 3 reconstituted with DMPC with L/P = 9.3.

As mentioned previously, it was not possible to analyze the size distribution of DMPC vesicles by column chromatography, but the presence of predominantly large vesicles or clusters of vesicles was indicated by the high turbidity of the preparation and optical microscopy. In fact, the $r(t)$ curve obtained below the lipid phase transition in 50% glycerol shows no decay, demonstrating an absence of vesicle tumbling effects. All measurements with these vesicles were therefore performed in 50% glycerol.

Above the lipid phase transition, which occurs at 23 °C in DMPC, the anisotropy clearly decays much less than in the case of egg PC and DOPC at 20 °C. A more appropriate comparison, however, may be with egg PC and DOPC at 1–2 °C since it is likely that the number of degrees above the phase transition is more significant than the absolute temperature. In fact, band 3 appears to behave differently in DMPC because increasing the temperature up to 30 °C does not result in a further significant decrease in r_3. It thus appears that there is a large fraction of aggregated band 3 in DMPC vesicles which is not dissociated by increasing temperature. The alternative explanation, that r_∞ changes due to an altered orientation of band 3 in the membrane, appears less likely.

The value for r_3 for the curve at 26 °C in Figure 8 is 47%, from which an immobile fraction of 40% is calculated from eq 3, assuming r_∞ remains at 12%. The time constants ϕ_1 = 37 μs and ϕ_2 = 335 μs at this temperature are in the same range at those in egg PC at 20 °C.

Figure 8 shows that band 3 is completely immobilized in the gel phase at 20 °C. When the temperature is raised, band 3 becomes mobile with the major change occurring between 22 and 24 °C, clearly reflecting the effect of the lipid phase transition. Measurements at low L/P (not shown), however, indicated that the phase transition was broadened with protein mobility persisting down to about 18 °C. This is an effect similar to that previously observed with bacteriorhodopsin in DMPC vesicles (Heyn et al., 1981).

Discussion

Interpretation of Anisotropy Decay Curves. The present measurements were performed with highly purified band 3 reconstituted into lipid vesicles. Most measurements were performed with the lipids in a liquid-crystalline phase. In this simple system, the rotational relaxation time of band 3 depends only on the size of the rotating particle and the viscosity of the membrane.

Two observations indicate that the population of rotating particles is heterogeneous. First, the parameter r_3 was found not to be constant but to vary with L/P and temperature

(Table I). Values of r_3 higher than its minimum value of 12% indicate the existence of a population of band 3 which is immobile on the time scale of the experiment. Second, for a homogeneous population, the anisotropy decay contains two exponential terms which differ by a factor of 4 in their exponents (eq 2). Table I shows that for the best fit of the data by eq 3, the two exponents differ by a factor considerably greater than 4. In fact, forcing the ratio ϕ_2/ϕ_1 to be equal to 4 in the curve fitting results in poor fits as judged by the sum of the squares of the residuals and their autocorrelation function.

It should be emphasized that the data cannot be explained by heterogeneity of the eosin binding sites on band 3. We therefore conclude that band 3 must be present as different sized aggregates in the membrane. The proportion of aggregates which are sufficiently large to be immobile on the time scale of the experiment varies with L/P and temperature. Here, we use the word aggregate to include both oligomers and nonspecific aggregates. It is important to distinguish between reversible and irreversible aggregation. We were not able to prevent a small amount of irreversible aggregation of band 3 occurring during the purification and reconstitution procedure. This may account for the observation that the rotating population of band 3 is not homogeneous even at high L/P and well above the lipid phase transition. In addition to this factor, it is clear that substantial aggregation of band 3 occurs in the membranes in response to decreasing temperature or decreasing L/P. In the case of the temperature response, this aggregation is shown to be completely reversible.

When the rotating population is heterogeneous, each compound contributes two exponential terms to the anisotropy decay. In practice, however, the fit to the data is not further improved by adding terms to eq 3. The parameters r_1, r_2, ϕ_1, and ϕ_2 obtained from the curve fittings are therefore difficult to precisely interpret. They are, however, useful for comparative purposes since increasing aggregation is revealed by a decreasing fraction of the faster component.

The remaining parameter, r_3, is susceptible to more detailed analysis. Provided all rotating species become rotationally randomized around the membrane normal during the time of the measurement, then r_3 will fall to its minimum value of r_∞. As discussed under Results and Analysis, the value of $r_\infty = 12 \pm 1\%$, which we obtain at high L/P in egg PC vesicles at 20 °C, is probably close to the correct value. Further interpretation of r_∞ depends on the nature of the eosin binding site(s) on band 3. In fact, the total inhibition of the anion transport function of band 3 at an eosin/band 3 ratio of 1/1 indicates a specific binding site (Nigg & Cherry 1979a). Analysis of proteolytic fragments of eosin-labeled band 3 reveals that the label is confined to the same 15-kDa chymotryptic fragment as is labeled by other anion transport inhibitors (M. J. A. Tanner and R. J. Cherry, unpublished results). If there is a specific site, then it may be possible to relate r_∞ to the orientation of the transition dipole moment of the eosin absorption band by

$$r_\infty/r_0 = (1/4)(3\cos^2\theta - 1)^2 \qquad (5)$$

where θ is the angle between the transition dipole moment and the membrane normal (Cherry, 1978; Kawato & Kinosita, 1981). Evidence from time-resolved fluorescence polarization measurements indicates that restricted "wobbling" of the probe in its binding site on band 3 occurs in the time range of a few nanoseconds. (R. J. Cherry, unpublished results). In this case, r_0 obtained in the microsecond time range by transient dichroism measurements is reduced although the ratio r_∞/r_0 is unaltered. The angle θ corresponds to the mean orientation of the transition dipole moment, i.e., the orientation of the axis for the wobbling motion with respect to the membrane normal.

Substituting the measured value of $r_\infty/r_0 = 12\%$ into eq 5 gives two solutions of $\theta = 41°$ or $\theta = 71°$. Fluorescence depletion measurements by Johnson & Garland (1981) with eosin-labeled band 3 in single ghosts and linear dichroism studies with oriented erythrocyte membranes (Mühlebach, 1984) both suggest that the angle of 41° is more likely to be the correct value. If this is true, the coefficient of the term $\exp(-t/\phi_\parallel)$ is much greater than that of the term $\exp(-4t/\phi_\parallel)$ in the expression for $r(t)$. In this case, we could tentatively assign the value of $\phi_1 = 40$ μs obtained in egg PC at 20 °C in vesicles of high L/P (i.e., the conditions favoring maximum disaggregation) to being close to the true relaxation time $\phi_\parallel$ for the major component present. Monomers of bacteriorhodopsin have a rotational relaxation time of about 15 μs in a fluid lipid bilayer (Cherry & Godfrey, 1981). Although the band 3 monomer is considerably larger, much of the protein mass is external to the lipid bilayer, and the diameters of the membrane-spanning segments of the two proteins may well be similar. The value of 40 μs might therefore indicate that dimers or tetramers of band 3 are the major species present.

It should be noted that time-resolved fluorescence polarization measurements failed to detect any segmental motion involving the eosin binding site (R. J. Cherry, unpublished results). A further argument against the presence of such a motion is the observation that band 3 is completely immobilized by cross-linking glycophorin, with which it probably forms a complex (Nigg et al., 1981).

Comparison of the Erythrocyte Membrane with the Model System. One aim of the present work was to gain insight into the behavior of band 3 in the erythrocyte membrane. A particular property which requires further elucidation is the loss of rotational mobility of band 3 in the erythrocyte membrane as the temperature is decreased below 37 °C. The proposal that this is due to self-aggregation of band 3 (Nigg & Cherry, 1979a) was strengthened by the finding that the temperature effect remains after removal of the 40-kDa cytoplasmic segment of band 3 by mild proteolysis (Nigg & Cherry, 1980). This segment is the site of interaction with peripheral proteins (Bennett & Stenbuck, 1980; Steck, 1974), and its removal therefore considerably reduces the possibility of restrictions arising from interactions with other proteins. Nevertheless, the correct interpretation of the temperature effect remains uncertain because many structural features of the complex erythrocyte membrane remain to be elucidated.

The present study shows that the temperature dependence of band 3 rotational mobility in simple lipid bilayers is qualitatively similar to that observed in the erythrocyte membrane. The results with egg PC and DOPC show that the effect occurs well above the lipid phase transition. In the model system, the only protein–protein interaction which can account for the effect is self-association of band 3 molecules. In view of this finding, it is highly probable that temperature-dependent self-association of band 3 occurs in the erythrocyte membrane too. This behavior is independent of glycophorin, which is not present in the model system.

A possible difference between the erythrocyte membrane and reconstituted vesicles is that band 3 is likely to be much more symmetrically oriented in the reconstituted system. This in turn could lead to different interactions between parallel and antiparallel proteins although this is a difficult point to test experimentally.

The present results also show that the aggregation of band 3 depends on the L/P in the membrane. Similar behavior has

previously been observed with cytochrome oxidase (Kawato, J., et al., 1982), cytochrome P-450 (Kawato, S., et al., 1982), and bacteriorhodopsin (Cherry & Godfrey, 1981). In the human erythrocyte membrane, the L/P is about 1/1, but after allowance for peripheral proteins, the ratio of lipid to integral membrane proteins is closer to 2/1. The results at this ratio with egg PC are therefore probably the most comparable with the erythrocyte membrane although a detailed comparison awaits further studies using erythrocyte lipids for reconstitution.

Dorst & Schubert (1979) have argued on the basis of the behavior of band 3 in detergent micelles that band 3 exists in the erythrocyte membrane as a monomer–dimer–tetramer equilibrium. The present study supports the conclusion that different sized aggregates of band 3 can occur in lipid bilayers. Whether or not all the aggregates present can be described as oligomers is not clear. Certainly, aggregates larger than the tetramer must be present under conditions where r_3 is found to be greater than r_∞.

The value for r_∞ of 12 ± 1% obtained in the present studies is in excellent agreement with that observed with eosin-labeled band 3 in the erythrocyte membrane (Mühlebach & Cherry, 1982). However, this low value is only obtained after preincubation of intact erythrocytes for 25 h at 37 °C prior to ghost preparation and mild proteolysis with trypsin to release restrictions from peripheral proteins. If the preincubation is omitted, the value of r_∞ after trypsin treatment is 23 ± 2%. The restriction to motion which is removed by the preincubation is not understood at the present time.

Sakaki et al. (1982) previously investigated the rotational mobility of purified band 3 in DMPC vesicles by saturation transfer ESR. Their value for $\phi_\parallel = 26$ μs obtained above the lipid phase transition is comparable with the time constant of the fast component of the anisotropy decay (33 μs) reported here for the same phospholipid (although using a somewhat different procedure for band 3 purification and reconstitution). However, the two studies give different results below the lipid phase transition, where we find the protein is immobilized ($\phi_\parallel$ > 10 ms), while the spin-label method detects rotational mobility in the time range below 1 ms. A possible explanation is that vesicle tumbling is not completely eliminated in the ESR experiments, which rely on tight packing of vesicles by centrifugation to overcome the problem. A further point revealed by the present experiments is that even above the lipid phase transition, band 3 remains much more aggregated in DMPC compared with unsaturated lipids. A different aggregation behavior of band 3 in different lipid systems could conceivably account for the lipid dependence of anion transport reported by Köhne et al. (1983).

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Registry No. DOPC, 10015-85-7; DMPC, 13699-48-4; glycerol, 56-81-5.

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Structure and Motion of Phospholipids in the Chromatophore Membrane from *Rhodospirillum rubrum* G-9†

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ABSTRACT: The structure and motion of the phospholipids in chromatophore membranes from *Rhodospirillum rubrum* G-9 have been investigated by using differential scanning calorimetry (DSC), ^{31}P NMR, and electron spin resonance (ESR) spin-labeling. ^{31}P NMR shows that the phospholipids in chromatophore membranes are present essentially as a bilayer between the growth temperature (25–30 °C) and −20 °C; furthermore, the total lipids extracted from chromatophore membranes form a smectic lamellar phase over the same temperature range when dispersed in aqueous solvents. DSC indicates that the lipids in chromatophore membranes undergo a broad, reversible, endothermic phase transition of low enthalpy between about −10 and 10 °C. There are several irreversible, endothermic transitions between about 40 and 90 °C which have been tentatively assigned to membrane proteins. In protein-free lipid bilayers made from the lipid extract, the phase transition is shifted to lower temperatures by about 20 °C. At the growth temperature, the chromatophore membrane is therefore functioning well above the lipid phase transition, possibly in the liquid-crystalline state. ^{31}P NMR indicates that the motionally averaged conformation of the phosphodiester group in chromatophore membranes is very similar to that in liquid-crystalline bilayers made from the lipid extract or from pure phosphatidylethanolamine or phosphatidylglycerol. Lipid–protein interactions in chromatophore membranes, therefore, have no detectable effect on this average conformation. ESR spin-labeling shows that at the growth temperature phospholipid bilayers made from the lipid extract are tightly packed and highly ordered. In chromatophore membranes, the phospholipids are even more highly ordered, and the value of the order parameter, $S_{33} = 0.75$, is among the highest reported so far and comparable to that measured in the purple membrane of *Halobacterium halobium*. The effect of membrane protein is to increase the packing order by ~20% and to raise the lipid phase transition temperature by about 20 °C.

In recent years, some components of the photosynthetic membrane of purple, non-sulfur bacteria such as *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides* have been isolated and some of their properties studied in isolated systems (Clayton, 1978; Cogdell, 1982). However, little is known about the physical structure and thermodynamic state of the photosynthetic membrane. In this paper, some fundamental structural and motional properties of the phospholipids in the intact membrane and in their extracted form have been investigated by using differential scanning calorimetry, ^{31}P NMR, and electron spin resonance (ESR) spin-labeling.

Materials and Methods

Cultures of the carotenoidless mutant *Rhodospirillum rubrum* G-9 were grown at low light intensity, and chromatophores were prepared as described previously (Snozzi & Bachofen, 1979). Freeze–fracture electron microscopy revealed that the membrane preparation consists of vesicles bounded by a single membrane, the chromatophore membrane. The size ranged between 50 nm and 0.5 μm, with most vesicles being between 50 and 200 nm (data not shown). Unless otherwise stated, chromatophore membranes were dispersed in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and 5 mM ethylenediaminetetraacetic acid (EDTA) and handled in the dark. Protein concentrations were determined by using the modified Lowry assay of Peterson (1977) with bovine serum albumin as the standard. The native state of the chromatophores was checked by the near-IR absorption spectrum. When stored at 4 °C, they were stable over several days.

Preparation of the Extracted Lipids. Chromatophores (0.4 g of total protein dispersed in 18 mL of buffer) were extracted twice under nitrogen with 65 mL of CH_2Cl_2/CH_3OH (1:2 v/v) until the protein pellet was free of bacteriochlorophyll (Ames, 1968). The blue organic phase obtained after centrifugation was dried under a stream of N_2 to a small volume (approximately 4 mL), applied to a Sephadex LH 20 column (30 × 2.5 cm) equilibrated with CH_2Cl_2/CH_3OH (3:1 v/v), and eluted with the same solvent. This separated the lipids from

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