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Rotational Mobility of an Erythrocyte Membrane Integral Protein Band 3 in Dimyristoylphosphatidylcholine Reconstituted Vesicles and Effect of Binding of Cytoskeletal Peripheral Proteins†

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ABSTRACT: Band 3 protein was isolated from human erythrocyte membranes, purified, and reconstituted into a well-defined phospholipid bilayer matrix (dimyristoylphosphatidylcholine). The preparation yielded uniform single-bilayered vesicles of the diameter 40–80 nm. The rotational motion of band 3 was studied by saturation transfer electron spin resonance (ESR) spectroscopy of covalently attached maleimide spin-labels. The rotational mobility changed in response to the host lipid phase transition. The rotational correlation time was in a range from 73 (37 °C) to 94 μs (26 °C) in the fluid phase and from 240 (15 °C) to 420 μs (5 °C) in the solid phase. The motion was analyzed based on the anisotropic rotation of band 3 in the reconstituted vesicles. To obtain information on the rotational diffusion constant around the axis parallel to the membrane normal, we made an attempt to measure the angle between the spin-label magnetic axis and the membrane normal. The result gave 3.9×10^4 s^{-1} at 37 °C as a rough estimate for the diffusion constant. This is compatible to anisotropic rotation of a cylinder of radius 3.3 nm in a two-dimensional matrix with inner viscosity 2 P and inner thickness 4 nm. The cytoskeletal peripheral proteins caused a definite increase in the rotational correlation time (from 73 to 180 μs at 37 °C, for example). The restriction of the rotational mobility was shown to be due to the ankyrin-linked interaction between band 3 and spectrin–actin–band 4.1 proteins in the reconstituted membranes.

Transmembrane control is a key mechanism in cellular response to outer signals. The molecular tool for the control must include interactions of the cell membrane integral protein with the peripheral proteins and the cytoplasmic fibrous structures. Human erythrocytes provide a simple model system for studying the interactions between integral and peripheral proteins since they lack cytoplasmic fibrous structures. Another advantage in using the erythrocytes is that the molecular components are exceptionally well characterized (Steck, 1974). The anchoring role of band 2.1 has recently been elucidated by Bennett & Stenbuck (1979, 1980) and Tyler et al. (1979, 1980). This protein anchors the integral protein band 3 and also binds to spectrin on the other hand, thus playing a key role in connecting the integral protein to the cytoskeletal network structure consisting of spectrin, actin, and band 4.1 (Lux, 1979).

Morphological studies of erythrocytes have been carried out that give evidence for linking of the integral proteins to the peripheral cytoskeletal structure (Nicolson & Painter, 1973; Pinto da Silva & Nicolson, 1974; Elgsaeter & Branton, 1974). We are studying this subject by measuring dynamic properties of the integral protein, rotational mobility of band 3 with saturation transfer ESR[1] spectroscopy of covalently attached spin-labels (Thomas et al., 1976), and lateral mobility with photobleaching recovery measurements of covalently attached fluorescent probes (Koppel et al., 1976). We are studying the protein–protein interactions by measuring the effect of binding of the cytoskeletal peripheral proteins on mobility. In this paper, we describe the results of a rotational study on a reconstituted membrane system, purified band 3 in dimyristoylphosphatidylcholine (DMPC). The results of a lateral study appear in an accompanying paper (Chang et al., 1981). In this series of papers, we have been able to make a detailed analysis of the rotational and lateral motions of an integral protein in a well-defined pure lipid matrix and also able to show a definite interaction between the integral protein and the cytoskeletal component proteins for the first time using such a simplified system.

Similar studies have been carried out with erythrocyte ghost membranes. Cherry and his collaborators investigated the rotational motion of band 3 by using flash-induced dichroism of covalently attached eosin probes. They concluded from the spectrin-depletion experiments that the spectrin–actin network has little or no effect on band 3 rotation in the ghost membranes, throwing doubt on the possibility of direct physical linking between them (Cherry et al., 1976; Nigg & Cherry, 1979). In a recent paper, however, they have reported restriction of the rotational mobility by the cytoskeletal components including band 2.1 does occur (Nigg & Cherry, 1980). Lateral diffusion of band 3 in erythrocyte membranes has been studied, and restriction of the mobility by the spectrin–actin

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DTAC, dodecyltrimethylammonium chloride; EDTA, ethylenediaminetetraacetic acid; MSL, a short-chain maleimide spin-label, 3-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy; (1,14)MSL, a long-chain maleimide spin-label, ester of 16-doxylstearic acid and *N*-(2-hydroxyethyl)maleimide; 5-doxylstearic acid, *N*-oxy-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; ESR, electron spin resonance.

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system has been indicated (Sheetz et al., 1980; Golan & Veatch, 1980). Our present studies on the simple reconstituted membranes should give a basis for understanding the protein–protein interactions in a more complex cellular system.

Materials and Methods

Preparation of Band 3–DMPC Reconstituted Vesicles. Ghosts were prepared by hypotonic hemolysis of human erythrocytes in 5 mM phosphate buffer at pH 8.0 and dialyzed against 0.5 mM EDTA and 0.1 mM dithiothreitol, pH 8.0, at 4 °C overnight. After incubation at 37 °C for 1 h, the pellet was resuspended in 5–10 volumes of 0.01 N NaOH, kept for 15 min at room temperature, and centrifuged at 4 °C for 1 h at 50000*g*. The pellet was washed twice with 5 mM phosphate at pH 8.0. The alkaline-treated ghosts were dissolved by adding an equal volume of 0.3 M DTAC in 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM imidazole–HCl at pH 7.4. After being gently stirred on ice for 30 min, it was centrifuged for 1 h at 50000*g*. The supernatant was charged onto a concanavalin A–Sepharose (Pharmacia) column, and the detergent was changed to 50 mM cholic acid. After elution of lipids and proteins, 0.15 M methyl α-D-mannoside (Nakarai Chemicals) was added to elute band 3.

DMPC (Sigma) was added to the purifed band 3 in 50 mM cholic acid, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM imidazole–HCl (pH 7.4) at a molar ratio of 200/band 3 and gently stirred. The solution was dialyzed at 4 °C against two changes of 150 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 5 mM imidazole–HCl, pH 7.4, and then against five to six changes of the same buffer without NaCl. The reconstitution method is similar to that of Barratt et al. (1977) and Ross & McConnell (1978) but different from the latter's in changing the detergent to cholic acid.

The detergents used were purified by recrystallization: DTAC (Tokyo Kasei) by adding acetone and cholic acid (Wako Chemicals) by adding water to the ethanol solutions, respectively. Protein was assayed by the method of Lowry et al. (1951) or by fluorometry of attached fluorescamine (Udenfriend et al., 1972). Phospholipid was determined according to Bartlett (1959). $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. (1971). Electron microscopic observations of the reconstituted vesicles were done after negative staining with 1% phosphotungstate at pH 7.4 with a JEOL Model 100 B.

Spin-Labeling of Reconstituted Vesicles. A maleimide spin-label reagent (MSL) was added to the reconstituted vesicles suspended in 1 mM EDTA and 5 mM imidazole–HCl, pH 7.4, at a molar ratio of 10/band 3 and reacted at 4 °C for 5–6 h. The mixture was then dialyzed twice against 130 mM KCl, 10 mM NaCl, and 5 mM imidazole–HCl, pH 7.4, to remove unreacted MSL and centrifuged for 1 h at 100000*g*. The pellet was placed in a quartz capillary tube for ESR measurements. The ESR spectrum was recorded with a JEOL Model FE-2X spectrometer. Spectral accumulations and integrations were carried out with an EC-100 computer connected to the spectrometer.

Reconstituted vesicles of band 3 labeled with a long-chain MSL [(1,14)MSL] were prepared as described elsewhere (T. Sakaki, A. Tsuji, P. F. Devaux, and S. Ohnishi, unpublished results). Briefly, the alkaline-treated ghosts were first labeled with (1,14)MSL, and the spin-labeled band 3 was extracted, purified, and reconstituted with DMPC as described in the preceding section.

Labeling of reconstituted vesicles with 5-doxylstearic acid was done by incubation at a molar ratio of 100 DMPC/spin-label for 1–2 h at 4 °C, followed by washing. MSL and

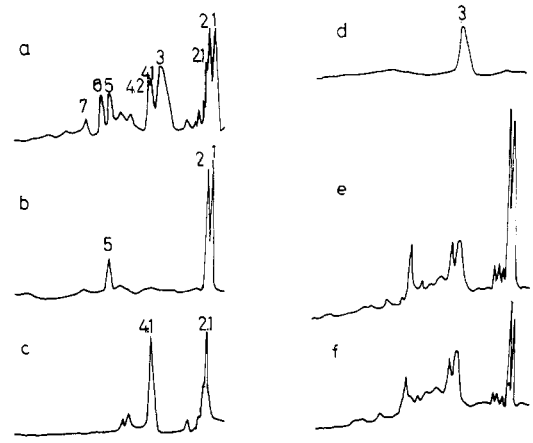

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of ghosts (a), spectrin and actin (b), bands 2.1 and 4.1 (c), band 3–DMPC reconstituted vesicles (d), and reconstituted vesicles incubated in isotonic phosphate buffer with the peripheral proteins at a weight ratio of band 3:(spectrin + actin):(bands 2.1 + 4.1) = 1:6:2 (e). (f) Sample e was incubated at 37 °C for 30 min in low ionic strength extraction buffer (0.3 mM sodium phosphate, 0.2 mM EDTA, and 0.2 mM DFP, pH 7.6).

5-doxylstearic acid were synthesized in this laboratory. (1,14)MSL is a gift of Devaux.

For determination of the number of covalently attached spin-labels, the reconstituted vesicles were solubilized with 5% $NaDodSO_4$ in isotonic imidazole buffer at pH 7.4. The ESR spectrum of the solution was measured and integrated twice. The integrated area was compared with that of a known concentration of tempocholine in glycerol–water. The difference in the sample-cavity Q factor was calibrated.

Binding of Peripheral Proteins to Reconstituted Vesicles. Spectrin and actin were extracted by adding an equal volume of a low ionic strength extraction buffer (0.3 mM sodium phosphate, 0.2 mM EDTA, and 0.2 mM DFP, pH 7.6) to the band 6 depleted ghosts (Yu & Steck, 1975) and incubating for 20 min at 37 °C. After centrifugation for 40 min at 40000*g* at 4 °C, KCl was added to the supernatant to a final concentration of 20 mM, and this solution was used as spectrin and actin (Figure 1b). The pellet was treated again with a large volume of the extraction buffer to remove spectrin and actin more completely. Band 2.1 (ankyrin) and band 4.1 were extracted from the spectrin-depleted ghosts by incubation in 1 M KCl, 5 mM phosphate, and 0.4 mM DFP, pH 7.6, according to Tyler et al. (1979) (see Figure 1c).

The peripheral proteins were added to the reconstituted vesicle suspension in isotonic phosphate buffer (130 mM KCl, 10 mM NaCl, and 5 mM sodium phosphate, pH 7.6) at a weight ratio of band 3:(spectrin + actin):(anhyrin + band 4.1) = 1:6:2 and incubated for 90 min at 4 °C. After centrifugation at 4 °C for 1 h at 100000*g*, the pellet was used for ESR measurements. The gel electrophoresis of the pellet showed binding of the peripheral proteins to the vesicles (Figure 1e). For some control experiments, ankyrin and band 4.1 were omitted from the binding mixture. Spectrin and actin also bound to the reconstituted vesicles although the amount of binding was less than that in the presence of ankyrin and band 4.1 (data not shown). In one experiment, ankyrin and band 4.1 were heat-treated at 60 °C for 10 min in 20 mM KCl, 1 mM EDTA, 0.4 mM DFP, and 5 mM phosphate, pH 7.6, according to Bennett & Stenbuck (1980), and these heat-denatured proteins were used for the binding together with intact spectrin and actin.

Estimation of Rotational Correlation Time. Satuation transfer ESR spectra of covalently attached spin-labels were

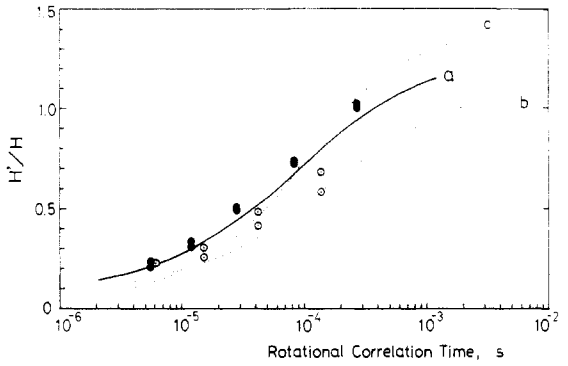

FIGURE 2: High-field peak height ratio H'/H of saturation transfer ESR spectrum vs. rotational correlation time for MSL–bovine serum albumin (●) and MSL–hemoglobin (⊙). Data are taken essentially from Kusumi et al. (1980). Curve a is some average of the data for the two kinds of proteins and used for calculation of rotational correlation time. The dotted curves b and c are taken from Thomas et al. (1976); b is for MSL–hemoglobin and c is from computer-simulated spectra, assuming axially symmetric hyperfine and g tensors.

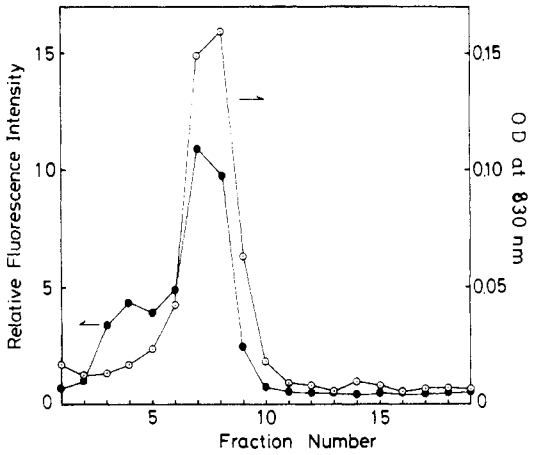

FIGURE 3: Fractionation of the dialyzate by a sucrose density gradient centrifugation. Band 3–DMPC–cholic acid solution was dialyzed, and the product (0.2 mL) was overlaid on 4 mL of a 2–40% sucrose gradient with 0.5 mL of a 50% sucrose cushion. After centrifugation at 4 °C for 20 h at 190000g, fractions were collected and assayed for phospholipid by OD at 830 nm and for protein by fluorescence intensity as described under Materials and Methods.

measured with a JEOL FE-2X spectrometer modified to detect second harmonic out-of-phase signals. The incident microwave power was adjusted to calibrate changes in the sample-cavity Q factor due to different water contents of samples and changes in the sample temperature as described previously (Kusumi et al., 1980). The high-field peak height ratio H'/H of the saturation transfer spectrum (see Figure 5B) was measured to monitor the rotational mobility. Since the high-field peaks are weaker than the central and low-field ones, the high-field region was accumulated several times on the computer. Rotational correlation time τ was estimated from the H'/H ratio by referring to a diagram made with MSL–bovine serum albumin and MSL–hemoglobin in glycerol–water (Figure 2). Data points for MSL–hemoglobin appear to systematically deviate from those for bovine serum albumin. This may be due to difference in the axial ratio of the proteins. However, we used some averages of the two data (curve a in Figure 2) as a reference since we assume isotropic rotation of these proteins. In Figure 2 we also present data by Thomas et al. (1976) for MSL–hemoglobin (curve b) and results of theoretical simulation (curve c) for comparison.

Results

Characterization of Band 3–DMPC Reconstituted System. The dialysis product of the cholic acid solution of band 3 and DMPC was centrifuged in a sucrose density gradient and analyzed for protein and phospholipid after fractionation (Figure 3). Both protein and phospholipid are present mainly in the fractions around 7 and 8. There are also minor fractions from 3 to 5 that contain some amounts of protein and less phospholipid.

When the dialysis product was centrifuged in isotonic imidazole buffer for 1 h at 100000g, 90% of the phospholipid and 60% of the protein were found in the pellet. The molar ratio of protein to phospholipid in the pellet was 1:250. This pellet was used in most of the following experiments. The electron micrograph of a negatively stained specimen of the pellet showed spherical vesicles with the diameter ranging from 40 to 80 nm (data not shown). This indicates formation of rather uniform single-bilayered reconstituted vesicles in the dialyzate. Multilamellar liposome structure of much larger dimensions was also observed but rarely. When we did not change detergent to cholic acid for reconstitution and used DTAC throughout, the product consisted only of multilamellar structures. Yu & Branton (1976) reconstituted band 3 in egg yolk phosphatidylcholine by using Triton X-100 for solubilization and cholate for reconstitution and obtained single-bilayered vesicles of the same size as in this study.

$NaDodSO_4$–polyacrylamide gel electrophoresis of the pellet showed an intense band at a position of M_r 90000 (Figure 1d). Only very weak bands were observed at higher molecular weight positions due to band 3 aggregates. The residual cholic acid in the reconstituted system was estimated as less than 1 mol % of phospholipid with tritiated derivative. Band 3 proteins in the reconstituted vesicles retained anion transport activity. MSL–band 3–DMPC vesicles were incubated for 1 h at 37 °C in 100 mM D-mannitol, 10 mM Na_2SO_4, and 5 mM phosphate, containing $^{35}SO_4$, and filtered through cellulose acetate with average pore diameter 0.2 μm (Sartorius). The radioactivity measurements showed that the vesicles contained $^{35}SO_4$ at a concentration close to that of the surrounding medium. The vesicles pretreated with 2,2′-diisothiocyanostilbene-4,4′-disulfonic acid contained less sulfate anion.

A conventional ESR spectrum of 5-doxylstearic acid intercalated into the reconstituted vesicles was measured to examine their temperature characteristics (Figure 4A). The results show a large change in the overall splitting around 22 °C, corresponding to the solid-to-fluid phase transition of the host phospholipid.

Rotational Motion of Band 3 in DMPC Bilayer Membranes. Figure 5 shows both conventional and saturation transfer ESR spectra of MSL–band 3 in DMPC reconstituted vesicles. The number of covalently attached spin-labels was 0.96 mol/mol of band 3. The conventional spectrum and the number of attached spin-labels were similar to those of Ross & McConnell (1978) obtained by spin-labeling band 3 in the detergent.

The conventional spectrum has a large overall splitting of 68 G, indicating strong immobilization of the attached spin-labels relative to the protein. The splitting value was little dependent on temperature, changing only from 68 to 66 G in a temperature range from 5 to 37 °C (Figure 4B). This justifies discussion of the protein rotational mobility based on the attached spin-label motion. There was a small fraction of a narrow component in the conventional spectrum (see arrow in Figure 5A). This component is due to weakly immobilized spin-labels, whose fraction did not change with temperature (from 5 to 37 °C).

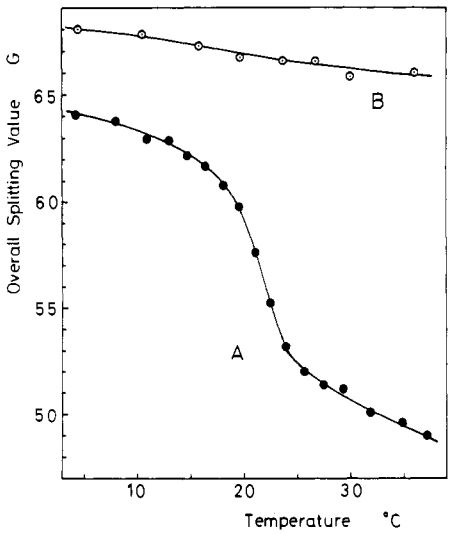

FIGURE 4: Temperature dependence of the conventional ESR spectrum of band 3–DMPC reconstituted vesicles labeled with 5-doxylstearic acid (A) or MSL (B). The overall splitting value of the spectrum is plotted in the ordinate. The vesicles contained band 3 and DMPC at a molar ratio of 1:250 and are packed in isotonic imidazole–HCl buffer at pH 7.4.

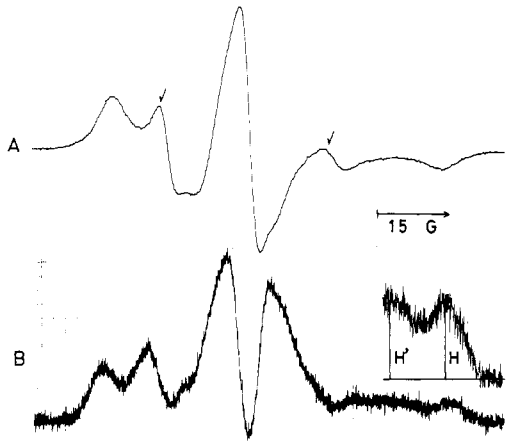

FIGURE 5: Conventional (A) and saturation transfer (B) ESR spectra of MSL–band 3 in DMPC reconstituted vesicles. The inset shows the high-field peak positions H and H' (H' being 11.5 G lower than H) used to estimate the rotational correlation time.

Since the narrow component in conventional spectra interferes with estimation of rotational correlation time from the low-field and central regions of saturation transfer spectra, we used the high-field peak height ratio to monitor the rotational motion. More than six independent series of experiments were carried out, and the results gave data in satisfactory agreement with each other. The reproducibility of the H'/H ratio was well within ±10% in most cases.

Figure 6 (curve A) shows the temperature dependence of the H'/H ratio. It decreased from 1.02 to 0.65 as the temperature increased from 5 to 37 °C. The rotational correlation time decreased from 420 to 73 μs in that temperature range (Table I). There was a broad discontinuous change around 19 °C. This change corresponds to the host lipid phase transition, although the midpoint temperature was somewhat lower than the phase transition temperature.

When the long-chain MSL, (1,14)MSL, was used to study band 3 in the reconstituted vesicles, some interesting differences were noted (T. Sakaki, A. Tsuji, P. F. Devaux, and S. Ohnishi, unpublished results). A narrow component appeared in the conventional spectrum only in the fluid phase, and its relative intensity increased with temperature. This change was attributed to dissociation of band 3 oligomers in the fluid phase; oligomeric proteins give a strongly immobilized spectrum and dissociated proteins give the 3 narrow component. The saturation transfer spectrum for the (1,14)MSL–band 3 in DMPC vesicles did not change significantly with temperature. The H'/H ratio remained to be 1.00–1.14; the rotational correlation time was 375–1050 μs in a temperature range from 4 to 40 °C (Table I). It is remarkable to note that no change in the rotational mobility corresponding to the host lipid phase transition was detected by (1,14)MSL, in contrast to the definite change observed by the short-chain MSL.

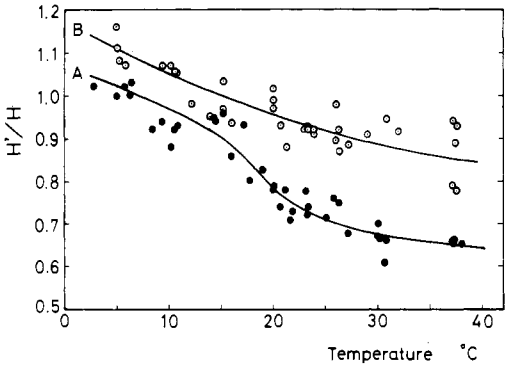

FIGURE 6: Temperature dependence of the rotational mobility of band 3 in DMPC reconstituted vesicles (A) and effect of binding of ankyrin, spectrin, actin, and band 4.1 on the mobility (B). Saturation transfer ESR spectra of MSL–band 3–DMPC vesicles (molar ratio of protein to lipid = 1:250) and those incubated with the peripheral proteins in isotonic phosphate buffer at a weight ratio of band 3:(spectrin + actin):(ankyrin + band 4.1) = 1:6:2 were measured at various temperatures. The ordinate plots the high-field peak height ratio H'/H of the spectrum.

Table I: Rotational Mobility of Band 3 in DMPC Reconstituted Vesicles and Effect of Binding of Cytoskeletal Component Proteins[a]

temp (°C)	band 3–DMPC[b]		band 3–DMPC bound with ankyrin, spectrin, actin, and band 4.1	
	H'/H	τ (μs)	H'/H	τ (μs)
5.0	1.02	420	1.11	810
10	0.97	315	1.06	550
15	0.92	240	0.99	360
20	0.78	125	0.96	300
26	0.71	94	0.92	240
30	0.68	83	0.89	210
37	0.65	73	0.86	180

[a] Rotational correlation time τ was estimated from the H'/H ratio of the saturation transfer spectrum for the covalently attached MSL by referring to Figure 2 (curve a) (see Materials and Methods for details). [b] The values for covalently attached (1,14)MSL are as follows: H'/H 1.13, τ 960 μs, 5 °C; 1.00, 375 μs, 20 °C; 1.11, 810 μs, 30 °C; 1.14, 1050 μs, 37 °C.

Effect of Binding of Peripheral Proteins on Rotational Motion of Band 3. MSL–band 3–DMPC reconstituted vesicles were bound with ankyrin, spectrin, actin, and band 4.1, and the saturation transfer ESR spectrum was measured at various temperatures. Figure 6 (curve B) shows temperature dependence of the H'/H ratio. It decreased from 1.11 at 5 °C to 0.86 at 37 °C; rotational correlation time decreased from 810 to 180 μs (see Table I). Comparison of curves A and B clearly shows that the binding caused an increase in the H'/H ratio, i.e., increase in the rotational correlation time (for example, from 73 to 180 μs at 37 °C). The effect was larger in the host lipid fluid phase and less marked in the solid phase. As a consequence, the discontinuous change in the band 3 rotational mobility in response to the lipid phase transition almost disappeared.

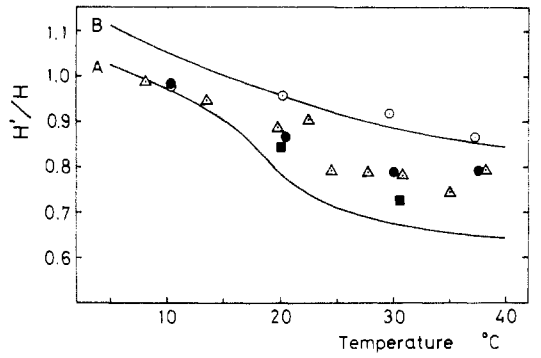

FIGURE 7: Effect of various treatments on band 3 rotational mobility in DMPC reconstituted vesicles. The high-field peak height ratio H'/H for MSL–band 3 is shown against temperature. The reconstituted vesicles bound with ankyrin, spectrin, actin, and band 4.1 (curve B) were incubated for 30 min at 37 °C in low ionic strength extraction buffer (●) or in isotonic phosphate buffer for a control (⊙). Heat-treated ankyrin and band 4.1 (10 min at 60 °C) and intact spectrin and actin were used for the binding (■). The reconstituted vesicles were incubated with spectrin and actin only in isotonic phosphate buffer (△). Curve A for band 3–DMPC vesicles and curve B are taken from Figure 6.

The reconstituted vesicles bound with ankyrin, spectrin, actin, and band 4.1 were incubated in low ionic strength extraction buffer to see if spectrin and actin are released as in the case of ghosts. The result showed release of about 50% of the initially bound spectrin and actin (Figure 1f). This release of spectrin and actin resulted in an increase in the band 3 rotational mobility as shown in Figure 7 (●); the H'/H ratio decreased from 0.89 to 0.79 and the rotational correlation time τ from 210 to 135 μs at 30 °C, for example. There remains to be some restriction in the mobility (Figure 7 and also compare with the parameter values for band 3–DMPC in Table I). A control experiment, incubation of the reconstituted vesicles bound with the peripheral proteins in isotonic phosphate buffer, did not cause any changes in the amount of bound spectrin and actin as well as in the restriction of band 3 rotational mobility [Figure 7 (○)].

When the reconstituted vesicles were incubated with heat-denatured ankyrin and band 4.1 and intact spectrin and actin, the band 3 rotational mobility was not much hindered [Figure 7 (■)]; $H'/H = 0.73$ and $\tau = 100$ μs at 30 °C, for example. The restriction was definitely smaller than that caused by the binding of intact ankyrin, spectrin, actin, and band 4.1 for which $H'/H = 0.89$ and $\tau = 210$ μs. When the reconstituted vesicles were bound with spectrin and actin in the absence of ankyrin and band 4.1, the rotational mobility was also restricted to some extent [Figure 7 (△)]; $H'/H = 0.78$ and $\tau = 130$ μs at 30 °C, for example. The restriction was not so large as that caused by binding of the full component. These results indicate that the observed restriction of band 3 rotational mobility by binding of ankyrin, spectrin, actin, and band 4.1 includes ankyrin-mediated interaction with the cytoskeletal proteins. The rotational mobility was also restricted by ankyrin-independent nonspecific binding of spectrin and actin, but the restriction was definitely smaller.

Binding of the full component of peripheral proteins did not affect the conventional ESR spectrum of MSL–band 3–DMPC vesicles. The overall splitting of the spectrum and the fraction of the narrow component were not affected.

When studied with the long-chain (1,14)MSL, the binding of ankyrin, spectrin, actin, and band 4.1 to the reconstituted vesicles did not affect both the conventional and saturation transfer ESR spectra. The narrow component in the conventional spectrum appeared in the host lipid fluid phase, and its relative intensity increased in the same way as that for the vesicles without the peripheral proteins. The H'/H ratio of the saturation transfer spectrum was almost the same as that for the vesicles in the absence of the peripheral proteins: 1.11 at 30 °C and 1.14 at 37 °C.

Discussion

The rotational mobility of band 3 in DMPC reconstituted membranes changes in response to the host lipid phase transition, although the response is not sharp. The rotational correlation time was 73–94 μs in the fluid matrix and 240–420 μs in the solid phase. Let us analyze the rotational motion in some detail, although the absolute value of the correlation time has some uncertainty (see Materials and Methods). The motion of band 3 in the lipid bilayer should be anisotropic as for rhodopsin in disc membranes (Kusumi et al., 1978). The rotation around the axis parallel to the bilayer normal should be much faster than that perpendicular to it; $D_\parallel > D_\perp$ where $D_\parallel$ and $D_\perp$ are the rotational diffusion constant for the parallel and perpendicular motions, respectively. The perpendicular rotation is very much hindered, and its characteristic time should be much larger than the observed order of magnitude of the correlation time (probably much longer than milliseconds). In order to estimate $D_\parallel$ from the observed correlation time τ, we must know orientation of the magnetic tensor axis of the covalently attached spin-label with respect to the protein rotational tensor axis. A theoretical study by Robinson & Dalton (1980) showed that, for nitroxide spin-labels at X-band frequencies, the high-field region of saturation transfer spectra, and hence the H'/H parameter, for systems undergoing anisotropic rotational motion will be virtually indistinguishable from spectra and parameter values obtained for isotropic rotation. The "apparent" rotational correlation time τ, obtained by analyzing spectra for anisotropic rotation in terms of an isotropic model system, will approach a conventional parameter τ_{iso} at longer correlation times (for example, $\tau \geq 50$ μs for $D_\parallel/D_\perp = 100$)

$$1/\tau \approx 1/\tau_{iso} = 6D_\perp + 3D_\parallel \sin^2 \theta \qquad (1)$$

where θ is the angle between the spin-label z axis and the bilayer normal.

A preliminary attempt was made to determine the angle θ by measuring anisotropy of ESR spectrum of films made from the spin-labeled reconstituted vesicles. The vesicles were first dialyzed against water and put on a flat quartz plate drop by drop and dried. After the plate was dipped in phosphate-buffered saline, the film was covered with a thin quartz plate and annealed at 40 °C under a 10-kg weight. ESR spectrum of the films made of vesicles labeled with 5-doxylstearate (but with nonlabeled band 3) changed depending on whether the external magnetic field was parallel or perpendicular to the film. The anisotropy was the same as that of a film made of pure DMPC containing 5-doxylstearate. The result therefore suggests that the reconstituted vesicles form multibilayered membranes parallel to the quartz plate. The ESR spectrum of the films made of MSL–band 3–DMPC vesicles also showed an anisotropy, but the reproducibility was poor (only 3 preparations out of 15). Orientation of the covalently attached MSL was not complete, and a signal due to unoriented MSL was always observed. However, the oriented MSL showed a simple three-line spectrum with a large hyperfine splitting of 32–33 G, when the magnetic field was parallel to the film normal. With the magnetic field perpendicular to the normal, the spectrum consisted of several lines in the central part with hyperfine splitting of the order of 13 G. This anistropy of spectrum is consistent with the angle θ of $\sim 20°$. A rough

estimate for $D_\parallel$ can now be made from eq 1 by using the approximate θ value and by putting 73 μs for τ at 37 °C. We neglect the $6D_\perp$ term compared with the second term. The result gives $D_\parallel = 3.9 \times 10^4$ s^{-1}.

Saffman & Delbrück (1975) derived an equation for lateral and rotational diffusion of a cylinder in a two-dimensional matrix. The rotational diffusion constant obtained above is compatible to anisotropic rotation of a cylinder with radius 3.3 nm in membranes with inner viscosity 2 P and inner thickness 4 nm. The lateral diffusion constant of the cylinder in the membrane should be 2.1×10^{-8} cm^2 s^{-1} according to their equation. This value is close to the diffusion constant of 1.6×10^{-8} cm^2 s^{-1} obtained for band 3 in DMPC reconstituted membranes at 30 °C (Chang et al., 1981). Therefore, our measurements of lateral and rotational diffusions of band 3 in simple DMPC bilayer membranes indicate that the proteins move in the two-dimensional matrix as a cylinder with radius 3.3 nm.

There are a few other motions that can affect the saturation transfer ESR spectrum of the reconstituted vesicles. The vesicle rotation as a whole is one such example. If we calculate the rotational correlation time for spherical vesicles with radius 30 nm in water (viscosity 0.01 P), the result gives 26 μs at 37 °C, which is smaller than the observed correlation time in the whole temperature range. Probably, the vesicle rotation may be hindered in our highly packed samples obtained by a high-speed centrifugation. The decrease in the rotational correlation time on the solid-to-fluid phase transition is not compatible to the vesicle rotation since the vesicle diameter should increase on the transition. Second, rapid lateral diffusion of band 3 in highly curved spherical vesicles can appear as rotation. The rotational diffusion constant D_R due to this effect can be estimated by a relationship $D_R = D_T/a^2$ where D_T is the lateral diffusion constant and a is the vesicle radius. $D_R = 3.6 \times 10^3$ s^{-1} for $a = 30$ nm and 8×10^3 s^{-1} for $a = 20$ nm are obtained by putting $D_T = 1.6 \times 10^{-8}$ cm^2 s^{-1} for 30 °C. These values are 1 order of magnitude smaller or one-fifth of the observed rotational diffusion constant. These considerations support our analysis of the spectra based on anisotropic rotational motion of band 3 in the reconstituted vesicles.

When the rotational motion of band 3 was investigated with long-chain spin-label (1,14)MSL, the rotational correlation time did not change much with temperature and remained large (several hundred microseconds), even when the bilayer matrix was fluid. The apparent discrepancy between MSL and (1,14)MSL can be reasonably explained by dissociation of band 3 in the fluid phase. The local motion of (1,14)MSL becomes largely freed on dissociation, as can be seen by the appearance of the narrow component in the conventional spectrum. Therefore, the spin-labels attached to dissociated band 3 proteins would not contribute to the H'/H ratio of saturation transfer spectra. Only those spin-labels attached to associated proteins contribute to the ratio. On the other hand, the local motion of MSL remained restricted in dissociated band 3 in the time scale of conventional spectroscopy, as seen by only slight temperature dependence of the overall splitting (Figure 4B). Spin-labels attached to both dissociated and associated proteins contribute to the H'/H ratios in this case. The observed decrease in rotational correlation time in the fluid phase is therefore largely due to faster rotational motion of the dissociated band 3 proteins.

Are the dissociated band 3 proteins dimers or monomers? Several lines of evidence have shown the presence of dimer in erythrocyte membranes (Yu & Steck, 1975; Kiehm & Ji, 1977) and in reconstituted vesicles with egg yolk phosphatidylcholine (Yu & Branton, 1976). The radius of 3.3 nm deduced from this analysis (for 37 °C) is compatible to the presence of a dimer in the fluid DMPC bilayer medium. Nigg & Cherry (1979) analyzed the dichroism decay curve based on the coexistence of rapidly and slowly rotating band 3 in erythrocyte membranes and assigned the former component (with $1/D_\parallel = 150$ μs) to the dimer and the latter to aggregate proteins. Our present results are therefore in qualitative agreement with their analysis. The magnitude of the rotational correlation time appears to be smaller in the present system ($1/D_\parallel = 26$ μs to be compared with 150 μs). This difference is at least partly due to lower inner viscosity of DMPC bilayer compared to that of erythrocyte ghost membranes; the overall splitting value for 5-doxylstearate in band 3–DMPC was 50 G at 30 °C (Figure 4A) while it was 59 G at 30 °C in erythrocyte ghost membranes [see also Chang et al. (1981)].

This study has shown a definite effect of the cytoskeletal component proteins on the rotational mobility of band 3 in DMPC bilayer matrix. The rotational correlation time increased from 73 to 180 μs at 37 °C. In principle, this increase can be caused by a restriction of rotational motion and also by a decrease in the angle θ (see eq 1). It is difficult to conclude from these results alone which factor predominates. Measurements of ESR anisotropy of the films made of the reconstituted vesicles bound with peripheral proteins should be able to distinguish between them, but the preparation of such oriented films is very difficult. However, our recent measurements of lateral diffusion of band 3 in DMPC membranes clearly showed a restriction caused by the peripheral proteins. The diffusion constant decreased from 1.6×10^{-8} to 9×10^{-9} cm^2 s^{-1} at 30 °C (Chang et al., 1981). Therefore, the increased correlation time can be at least partly due to restriction of the rotational motion of band 3. This restriction is caused by the ankyrin-linked interaction of band 3 with the peripheral proteins, as evidenced by experimental results in Figure 7, and therefore may well reflect the interaction in native erythrocyte membranes.

In the reconstituted vesicles, band 3 proteins probably dispose randomly, about a half of them exposing the ankyrin-binding site on the outer surface. Saturation transfer spectra of the reconstituted vesicles incubated with the peripheral proteins may therefore be a sum of two types of MSL–band bound and unbound with the proteins. Actual restriction of the rotational mobility by the peripheral proteins may be larger than the observed increase in the rotational correlation time, if we take into account this factor.

Binding of the peripheral proteins did not affect dissociation of band 3 in fluid DMPC bilayer matrix since the conventional spectrum for (1,14)MSL was not affected. Therefore, the effect of peripheral proteins is mainly to restrict rotational mobility of dissociated band 3 proteins in the fluid matrix. Rotational motion of associated band 3 proteins was also restricted, but the effect was observed as small. This conclusion is in agreement with a recent paper by Nigg & Cherry (1980) that proteolytic cleavage of band 3 as well as extraction of bands 2.1 and 4.1 caused an increase in the rapidly rotating fraction of band 3 in erythrocyte ghost membranes.

Information obtained from the present series of our studies on rotational and lateral motions of band 3 in a simplified reconstituted membrane and effect of the peripheral proteins on the motions should give a basis for understanding interactions between integral protein and cytoskeleton and elucidating transmembrane control mechanisms in more complex cellular systems.

Acknowledgments

We thank Dr. Akihiro Kusumi for providing us with spectral data for Figure 2 and for his advice, Professor Philippe Devaux for his discussions, and S. B. Sato for his help during this study. We also thank Dr. H. Saji for the supply of red blood cells.

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Carbon-13 Nuclear Magnetic Resonance Studies of Cyanocobalamin and Several of Its Analogues†

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ABSTRACT: The carbon-13 nuclear magnetic resonance spectrum of cyanocobalamin in aqueous solution has been interpreted. The assignments are based on the earlier biosynthetic studies with carbon-13-enriched precursors and on the present systematic analysis of the spectra of cyanocobalamin, cyanocobalamin lactone, cyanocobalamin lactam, cyanoepicobalamin, and several cyanocobalaminmonocarboxylic acids. The interpretation of the spectrum of cyanocobalamin greatly simplifies the structure determination of new corrinoids and should prove very helpful in future studies of these compounds. The structures of two cyanocobalamindicarboxylic acids and a cyanocobalaminmonocarboxylic acid lactone have been determined by comparing their carbon-13 magnetic resonance spectra with that of cyanocobalamin.

Vitamin B_{12} (cyanocobalamin) (Figure 1) is one of the most complex nonpolymeric molecules found in nature. The final elucidation of its structure in the 1950s by Hodgkin and coworkers (Hodgkin, 1965) required X-ray crystallographic studies because chemical degradative procedures were not sufficient for the determination of a structure as complex and as chemically inert as the corrin ring.

At present, nuclear magnetic resonance spectroscopy (NMR), and in particular ^{13}C NMR, is undoubtedly the most powerful technique for the elucidation of the structure of complex molecules. Doddrel & Allerhand (1971) determined the ^{13}C NMR spectrum of cyanocobalamin and made several assignments. Subsequent assignments were based on the la-

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0006-2960/82/0421-2372$01.25/0