Evidence from Deuterium Nuclear Magnetic Resonance for the Temperature-Dependent Reversible Self-Association of Erythrocyte Band 3 in Dimyristoylphosphatidylcholine Bilayers†

Christopher E. Dempsey, Nicholas J. P. Ryba, and Anthony Watts*

Biochemistry Department, University of Oxford, Oxford OX1 3QU, United Kingdom

Received September 24, 1985; Revised Manuscript Received December 3, 1985

ABSTRACT: Band 3, isolated from human erythrocytes, has been reconstituted into bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) deuterated in the terminal methyl groups of the choline head group. By use of Triton X-100 for selective extraction and purification of band 3 and then cholate for subsequent solubilization with the lipid, a number of reconstituted complexes were produced by exhaustive detergent dialysis with protein:lipid weight ratios of between 0.32:1 and 1.25:1. Electron micrographs of negatively stained complexes showed that this method produced large vesicles of greater than 300-nm diameter. Deuterium nuclear magnetic resonance (NMR) spectra from the choline methyl deuterons in bilayer lipid above the liquid-crystal–gel phase transition temperature were shown to change systematically with increasing concentrations of band 3 in the bilayers. The measured quadrupole splittings, taken as the separation of the turning points in the recorded spectra, decreased from a value of 1.28 kHz for pure lipid to 0.98 kHz for bilayers with a protein:lipid ratio of 1.25:1 at 26 °C. At 35 °C, a more pronounced decrease in the quadrupole splittings was measured. The data from the complexes with protein:lipid ratios up to 0.7:1 (w/w) obey the mathematical treatment for a rapid two-site exchange between lipids at the protein–lipid interface and the bulk lipid phase. The temperature dependence of the measured quadrupole splitting with respect to the protein:lipid ratio indicates that the amount of lipid at the protein–lipid interface increases with increasing temperature. This is interpreted in terms of a reversible temperature-dependent self-association of band 3 within the bilayer with the exclusion of lipid from the associating intramembranous surfaces of the protein. An upper limit of 70 lipids per protein monomer has been estimated at 35 °C, a number which suggests that the lipids having perturbed ^{2}H NMR properties are in more than a single shell surrounding the total hydrophobic perimeter of the protein. The apparent self-association properties of band 3 in DMPC bilayers were independent of the main gel to liquid-crystal phase transition of the lipid. However, band 3 caused a marked broadening of the lipid phase transition which was suppressed to lower temperatures at high protein:lipid ratios. It is suggested that the temperature-dependent self-association behavior of band 3 in membranes is an intrinsic property of the protein which is qualitatively independent of the properties of the supporting lipid.

The interactions of membrane proteins with phospholipids in reconstituted systems have been studied by deuterium nuclear magnetic resonance (^{2}H NMR)[1] spectroscopy using selectively deuterated phospholipids for a number of protein–lipid systems [reviewed in Smith & Bloom (1985)]. The results with chain-deuterated lipids have rather uniformly been interpreted as indicating that the lipid order is relatively unperturbed by integral membrane proteins (Devaux, 1983; Smith & Oldfield, 1984; Smith & Bloom, 1985). Hydrogen-bonding or electrostatic interactions at the membrane surface have been less extensively studied, but recent reports on the interactions of myelin basic protein with negatively charged lipids (Sixl et al., 1984) and of bovine retinal rhodopsin with the phosphatidylcholine head group (Ryba et al., 1986) have shown that significant interactions between membrane proteins and phospholipid head groups occur at the membrane surface and can be studied by deuterium NMR.

In this paper, we report a further example of the use of deuterium NMR to study the interaction of an integral membrane protein, the anion-exchange protein from human erythrocytes (band 3), with the polar head group of phosphatidylcholine in reconstituted bilayers. Natural or reconstituted membranes containing purified band 3 have not previously been studied by deuterium NMR. The rotational mobility of band 3 (labeled with an eosin probe) in the native erythrocyte membrane (Nigg & Cherry, 1979) and in reconstituted bilayer membranes of egg phosphatidylcholine, DOPC, and DMPC (Mühlebach & Cherry, 1985) has been analyzed by using flash-induced transient dichroism measurements. These studies have shown that both in situ and in reconstituted systems, band 3 undergoes reversible self-association which is dependent both on temperature and on the cholesterol content of the membrane. The self-association of band 3 purified in nonionic detergents has been studied by ultracentrifugation (Pappert & Schubert, 1983), and a reversible equilibrium self-association between monomers, dimers, and tetramers of band 3 was found. Nonionic detergents are expected to have a minimal effect on the aggregation

† This research was supported by SERC Grants GR/D/02218 and GR/B/69111 (A.W.) and a research studentship (N.J.P.R.).

[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; *p*-CMB, *p*-(chloromercuri)benzoic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

0006-2960/86/0425-2180$01.50/0

properties of membrane proteins (although this idea remains to be substantiated), and it was proposed that a similar self-association equilibrium of band 3 is present in the native erythrocyte membrane. On the other hand, saturation transfer ESR spectroscopy of spin-labeled band 3 reconstituted in DMPC bilayers gave no evidence for a reversibly self-associating species of band 3 which was estimated to be present as dimers (Sakakai et al., 1982). Little information on the nature of the interactions between self-associating species of band 3 at the molecular level has yet been found, and the important question of whether the interactions occur between the integral domains of band 3 within the membrane (Kopito & Lodish, 1985) or are mediated largely by interactions between regions of the polypeptide chain that lie outside the membrane has not been answered. In this study, we show that deuterium NMR of head-group-deuterated DMPC is sensitive to the incorporation of band 3 within DMPC vesicles and that the apparent self-association of band 3 that results in the exclusion of bilayer lipid from the intramembranous protein surface can be studied by this method.

Materials and Methods

Materials. Triton X-100 was purchased from Aldrich or Fluka and stored in the dark at room temperature. Cholic acid (Aldrich) was recrystallized from 70% ethanol where necessary to remove a yellow impurity and was converted to the sodium salt during preparation of buffers. All detergent solutions were prepared immediately before use. Recently outdated packed erythrocytes were obtained from a local blood bank. Biobeads SM2 (Bio-Rad) were prepared as described by Holloway (1973) and Amberlite XAD beads (BDH) by washing in acetone and then in boiling water. The preparation of *N*-methyl-deuterated (DMPC-d_9) or -tritiated (DMPC-t_9) dimyristoylphosphatidylcholine has been described (Ryba et al., 1986). Radioactive sodium [^{14}C]cholate was from Amersham International.

Purification of Band 3 from Human Erythrocytes. Slightly pink ghosts were prepared by hypotonic lysis of packed, washed erythrocytes in phosphate buffer according to Dodge et al. (1963). The lysis solutions contained PMSF (20 μg mL^{-1}) and 0.5 mM EDTA. Ghosts were used fresh (within 2 days at 4 °C) or stored frozen at −20 °C and used within 3 weeks.

Band 3 was isolated by selective extraction into Triton X-100 by the method of Wolosin (1980) with modifications to improve the purity of the preparation to remove endogenous lipid and exchange detergent to sodium cholate. All manipulations were performed on ice and in buffers containing PMSF (20 μg mL^{-1}). Ghosts were washed twice in 6 volumes of 20 mM borate and 150 mM NaCl (pH 8.0) to release residual hemoglobin and band 6 (glyceraldehydephosphate dehydrogenase). The pellet (15 min, 27000*g*) was resuspended in a small volume of 1:5 diluted isotonic borate buffer and extracted for 30 min with 3 volumes (based on the original ghost volume) of 0.05% Triton X-100 and 0.2 mM dithiothreitol (DTT) in 1:5 diluted borate with N_2 gassing. The supernatant was removed after pelleting (20 min, 60000*g*) and the pellet reextracted for 15 min with 1 volume of extraction buffer (without DTT) containing 0.5 mM *p*-CMB. This step resulted in the release of further glycophorin, band 4.2, and a variable amount of cytoskeletal proteins (bands 1, 2, 4.1, and 5). The pellet was washed with 1:5 diluted isotonic borate and extracted for 1 h on ice with extraction buffer containing 0.5% Triton X-100. The supernatant obtained after pelleting (30 min, 80000*g*) was immediately concentrated 5–6-fold by ultrafiltration at 4 °C over an Amicon PM 10 membrane (to give 0.5–1 mg mL^{-1} protein) and chromatographed on a Sephadex G-75 column (4 × 60 cm) equilibrated in dialysis buffer (20 mM borate, 50 mM NaCl, 0.2 mM DTT, 0.1 mM EDTA, and 20 μg mL^{-1} PMSF, pH 8.0) containing 30 mM sodium cholate. This step resulted in the removal of endogenous phospholipid (see Results) and a small and variable amount of low molecular weight protein and also effected the exchange of Triton X-100 for sodium cholate. The protein eluant was concentrated to about 0.5–1 mg mL^{-1} by ultrafiltration and used immediately for reconstitution.

Reconstitution of Band 3 with DMPC-d_9. DMPC-d_9, suspended in dialysis buffer by warming above the lipid phase transition temperature (24 °C), was solubilized at a final concentration of 10 mg mL^{-1} by addition of 0.5 volume of 150 mM sodium cholate in dialysis buffer. An aliquot of solubilized lipid was added to the band 3 solution to give the desired initial protein:lipid weight ratio, and the resulting mixture was dialyzed at 4 °C. The formation of large vesicles was promoted by decreasing the rate of dilution of cholate by dialyzing against 5 mM external cholate until vesicle formation was obvious from the increased turbidity of the suspension, usually after 3–4 days. The dialysis solution was then replaced by cholate-free dialysis buffer and the release of detergent enhanced by addition of Biobeads SM2 and Amberlite XAD beads to the external solution. The critical micelle concentration (cmc) of sodium cholate is approximately 15 mM under the conditions of dialysis, but the protein and lipid remained solubilized (as determined by the inability to sediment protein or lipid at 150000*g*) down to 8 mM cholate, presumably due to partitioning of detergent into protein–lipid–detergent mixed micelles.

After dialysis, protein–lipid recombinants were pelleted, resuspended in a small volume of dialysis buffer, and sedimented on a continuous sucrose density gradient (10–40% sucrose in dialysis buffer). The recombinants sedimented as uniform bands except at high protein:lipid ratios (>1.25:1.0 w/w) in which case a granular band of high density (aggregated protein) was obtained in addition to vesicles. The protein:lipid ratio was variable over a range of 0 to about 1.25:1 (w/w), and the starting ratio before dialysis was maintained in the final vesicle preparation over this range. Protein:lipid ratios above about 1.25:1 (w/w) could not be obtained and invariably resulted in the formation of aggregated protein.

NMR Spectroscopy. NMR experiments were performed immediately after recovery of the reconstitutions from the density gradients. The samples were washed twice in dialysis buffer and then suspended in the same buffer (0.4 mL) prepared in deuterium-depleted water. Before the NMR experiments, the samples were incubated at 37 °C for 1 h. Omission of this preincubation step resulted in hysteretic effects in the temperature dependence of the deuterium NMR spectra of band 3 containing reconstitutions.

Deuterium and ^{31}P NMR spectra were obtained on a Bruker WH-300 NMR spectrometer using single 90° pulses of 29-μs duration as described earlier (Ryba et al., 1986). Spectra were also obtained on a Bruker CXP-200 spectrometer using a quadrupole echo sequence (Davis, 1983) and a 90° pulse length of 4–5 μs. Spectral intensity measurements using an insert were done as described earlier (Ryba et al., 1986). A comparison of the temperature dependence of the deuterium spectral intensity of the reconstituted complexes was made by using the automatic intensity facility of the WH-300 spectrometer and DMPC-d_9 as a control.

Protein and Phosphate Analysis. Protein concentrations in membrane suspensions or detergent solutions were measured by using a modified Lowry procedure (Markwell et al., 1981).

Neither band 3 nor glycophorin gives color values in the Markwell modified assay equivalent to the color value of the standard protein, bovine serum albumin. By use of a value for the absorption coefficient of band 3 in nonionic detergent of $A = A^{1\%}_{280\ nm} - 1.95A^{1\%}_{320\ nm} = 7.7$ (Pappert & Schubert, 1983) for the absorption coefficient of band 3 in sodium cholate solutions, a Lowry value (relative to BSA) of 0.6 was determined and used for the measurement of protein concentrations (i.e., band 3 protein concentrations were corrected by multiplying by 1.67 the value determined in the Lowry assay with BSA as a standard). The relative Lowry value of glycophorin was determined from weighed samples of purified lyophilized glycophorin to be 0.18. Glycophorin was purified by the method of Taraschi et al. (1982). Phospholipid determinations from inorganic phosphate analysis after acid hydrolysis were done by using the assay of Rouser et al. (1975) or by scintillation counting of detergent-solubilized reconstitutions doped with small amounts (≤0.1%) of DMPC-t_9.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed on 7.5% gels according to the method of Laemmli (1970), and protein bands were identified by Coomassie or silver staining (Morrissey, 1981). The amount of glycophorin in band 3 samples was estimated by comparing the intensity of the glycophorin band with graded amounts of pure glycophorin run on the same gel after staining with silver.

RESULTS

Purification and Reconstitution of Band 3. The purification procedure used was a modified version of the method of Wolosin (1980). Borate buffers (instead of phosphate) were used throughout to allow determination of lipid concentrations using the phosphate assay and gave identical results in the purification of band 3 as were achieved in phosphate-buffered solutions. The reextraction of glycophorin-depleted membranes with 0.5 mM *p*-CMB resulted in the release of some residual glycophorin remaining after the 0.05% Triton X-100 extraction and also some (but not all) band 4.2. The major impurity in the final band 3 preparation was glycophorin which was particularly prominent on silver-stained gels. Glycophorin stains much more strongly with silver than other erythrocyte membrane proteins, and this property enabled a good estimation of the glycophorin content in band 3 preparations to be made from a comparison of the intensity of the glycophorin band with graded amounts of purified glycophorin run on the same gel. The amount of glycophorin was estimated to be 3–5 μg/100 μg of total protein, corresponding to one glycophorin molecule for seven or eight band 3 molecules in the reconstitution solution. The protein used for reconstitution was estimated to be 85–90% pure with respect to band 3. Minor amounts of cytoskeleton proteins (bands 1, 2, and 5) sometimes contaminating the band 3 preparation were never detectable in the final protein–DMPC complexes. The minor amounts of glycophorin and band 4.2 were reconstituted with band 3 in similar proportions as were present in the reconstitution solution.

An important modification in the reconstitution method was the inclusion of the rapid gel filtration of Triton X-100 solubilized band 3 on a Sephadex G-75 column preequilibrated in sodium cholate. This resulted in the efficient exchange of Triton X-100 for cholate (Figure 1). Residual Triton X-100 was estimated from the absorbance at 280 nm (after subtracting the absorbance due to band 3 determined from the Lowry assay) to be less than 1% by weight of Triton X-100 with respect to protein. Residual endogenous phospholipids were undetectable by phosphate analysis, indicating a residual level of less than 1 mol/mol of band 3, although this does not preclude the presence of residual unphosphorylated erythrocyte membrane lipids.

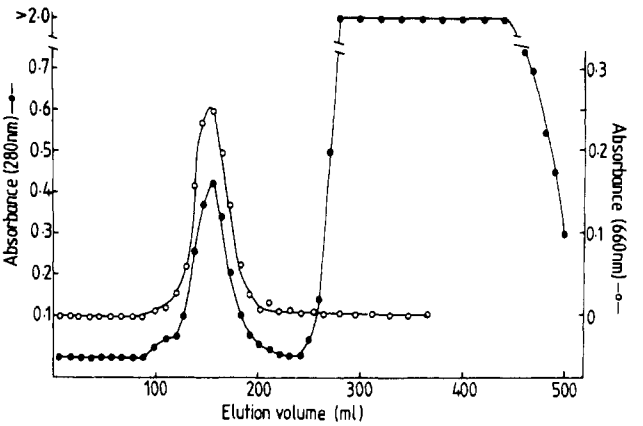

FIGURE 1: Elution profile for the exchange of Triton X-100 for sodium cholate in Triton X-100 solubilized band 3 solution on Sephadex G-75 (45 × 2.5 cm). The elution buffer was 20 mM borate, 50 mM NaCl, 20 mM sodium cholate, 0.2 mM DTT, and 20 μg mL^{-1} PMSF, pH 8.0, with a flow rate 75 mL h^{-1}. Absorbance at 660 nm (○) is the protein determination using the modified Lowry assay, and absorbance at 280 nm (●) is the absorbance in the elution profile due to band 3 and Triton X-100.

Detergent dialysis of cholate-solubilized band 3 and DMPC-d_9 resulted in the formation of recombinants in which the protein:lipid ratio could be varied over a wide range, between that of pure lipid and an upper limit of about 1.25:1 (w/w). Over this range, the initial protein:lipid ratio before dialysis was maintained. At starting protein:lipid weight ratios greater than about 1.25:1, complexes with a limiting final ratio were produced, and protein in excess of that required to generate this ratio was recovered as a dense aggregate on density gradients. Protein and lipid were recovered in 50–60% yield after the reconstitution and density gradient steps.

Sodium cholate, rather than Triton X-100, was used for both the storage of band 3 and the reconstitution with DMPC-d_9. Band 3 was found to be more stable in cholate solutions (as determined by PAGE and the inability to sediment protein stored for up to 3 weeks at 4 °C at 150000*g* for 1 h). It was particularly noticeable that band 3 retains its monomeric molecular weight on SDS gels after purification in sodium cholate whereas band 3 purified and stored in the nonionic detergents Triton X-100 or $C_{12}E_9$ rapidly forms high molecular weight aggregates (greater than dimers) that are nondissociable in SDS even in the presence of 1 mM DTT. Sodium cholate could be removed from reconstitutions by dialysis to less than 1 mol % of reconstituted lipid as determined by scintillation counting of samples doped with radioactive cholate. Reconstitution of band 3 from cholate solutions also gave larger vesicles than we were able to achieve by reconstitution from Triton X-100. The formation of large recombinants was enhanced by decreasing the rate at which sodium cholate was removed by dialysis, and vesicle size was largely dependent on this factor (rather than on the protein:lipid ratio). Although protein-containing vesicles were of a similar size to pure lipid vesicles, they were more heterogeneous with small proportions of vesicles having diameters of less than 100 nm or more than 10 μm (Figure 2 shows an electron micrograph of a representative sample but without any of the extremely large vesicles sometimes obtained). Protein-containing vesicles of different sizes sedimented uniformly on sucrose density gradients indicating that size heterogeneity was not related to heterogeneity in the protein:lipid ratios of the complexes.

NMR Spectroscopy. The residual quadrupole splitting ($\Delta\nu_Q$) for DMPC-d_9 in bilayers is small [about 1.2 kHz at 30

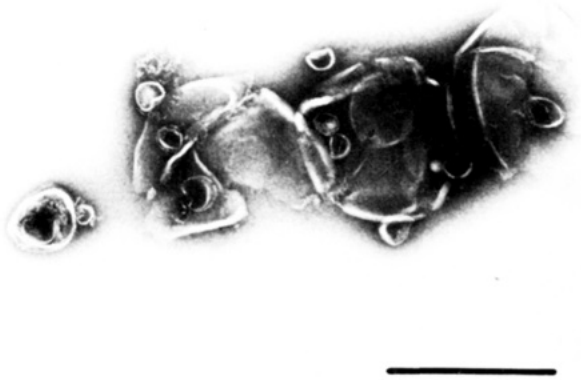

FIGURE 2: Electron micrograph of negatively stained band 3–DMPC vesicles formed by cholate dialysis as described under Materials and Methods. The protein:lipid ratio is 0.48:1 (w/w). Vesicles were diluted in distilled water, spread on Teflon grids, and stained with uranyl acetate, pH 5.0. Grids were examined with a Philips EM 400 electron microscope at 80 kV. The scale bar is 500 nm.

°C compared to the maximum of 127 kHz (Davis, 1983)], and the NMR spectrum is thus sensitive to motional effects having correlation times of the order of 1 ms or faster. To reduce the small residual anisotropy through vesicle rotation or lipid lateral diffusion, it can be calculated that the vesicles must be less than about 300 nm in diameter (Burnell et al., 1980; Ryba et al., 1986). Since the majority of the lipid in the band 3-DMPC complexes is in particles with diameters greater than 300 nm, we assume that the spectral changes observed in the complexes compared with pure lipid reflect the orientational and dynamic properties intrinsic to the lipid head group and its interactions with protein. The range of vesicle sizes is likely to contribute to spectral broadening, however, and we have not attempted to assign the contribution to line broadening from motional effects intrinsic to the head group or arising from the effects of protein incorporation. Small vesicles probably give rise to some of the intensity in the center of the NMR spectra, which is particularly prominent at high temperatures. However, the shoulders of the spectra that we define as the apparent quadrupole splitting, $\Delta\nu_Q$, arise from the largest vesicles, and these are minimally affected by motional averaging. This parameter is thus a valid criterion by which to probe the effects of incorporation of band 3 into DMPC-d_9 bilayers. The apparent quadrupole splitting was not affected by any base-line distortions arising from the low power of the WH 300-MHz spectrometer, as shown by a comparison of spectra taken on a high-power CXP-200 spectrometer using a quadrupole-echo sequence and short radio-frequency pulses (Figure 3). Determination of the intensity of deuterium contributing to the recorded spectra from band 3–DMPC-d_9 complexes by comparison with a coaxial insert containing a pure DMPC-d_9 dispersion showed that in all cases measured, all the labeled phospholipid was observable over the spectral range of the pure lipid (within an estimated error of 10%). There was no variation in the temperature dependence of the net deuterium intensity, indicating (within an error of about 10%) that temperature-dependent changes observed did not result in the formation of a population of trapped lipid, either highly ordered or motionally restricted, giving highly broadened spectra.

At all protein:lipid ratios measured, the incorporation of band 3 caused a decrease in the apparent quadrupole splitting of the DMPC-d_9 deuterons (Figures 5 and 6) when compared with protein-free DMPC-d_9 vesicles (Figure 4). The effect of band 3 on the recorded ^{2}H NMR spectra depended on the

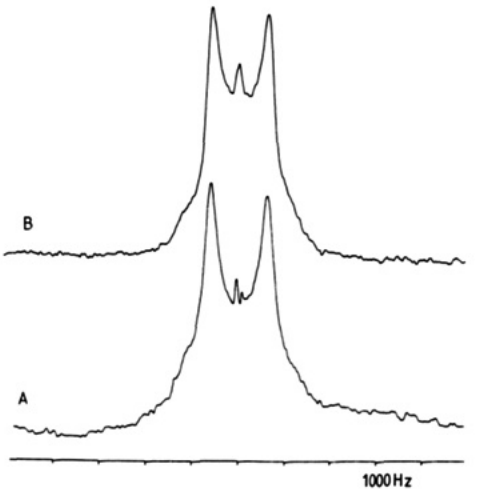

FIGURE 3: Comparison of ^{2}H NMR spectra from reconstituted band 3 in DMPC-d_9 (0.71:1 protein:lipid, w/w) recorded at 23 °C by using the Bruker WH 300-MHz spectrometer (29-μs 90° pulse) operating at 46.1 MHz (A) and the high-power CXP-200 spectrometer (30.7 MHz; 4.5-μs 90° pulse) using a quadrupole echo sequence with 40-μs echo delays (B). The base-line distortions resulting from the long 90° pulse of the low-power spectrometer do not affect the measured quadrupole splitting which is 1160 + 15 Hz in each case.

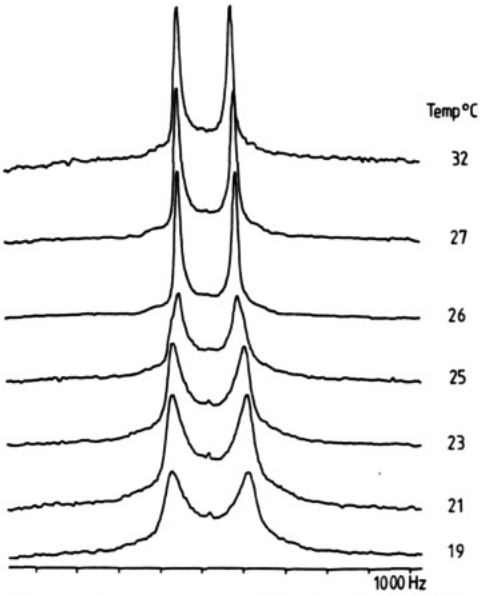

FIGURE 4: Temperature dependence of the deuterium NMR spectrum of DMPC-d_9 in deuterium-depleted dialysis buffer at 46.1 MHz showing the sensitivity of the terminal end of the choline head group to the main gel to liquid-crystalline phase transition at 24 °C.

protein:lipid ratio. At low protein:lipid ratios (less than about 0.4:1 protein:lipid, w/w), narrow, well-defined spectral edges could be resolved at all temperatures measured, with a small degree of broadening in the central spectral region (Figure 5). At temperatures below the DMPC-d_9 phase transition temperature of 24 °C, little difference in the spectral shape between protein-free and protein-containing complexes was observed, although the values of $\Delta\nu_Q$ were somewhat smaller for the band 3 complexes. For complexes containing more protein (more than about 0.48:1 protein:lipid, w/w) (Figure 6), a noticeable increase in line broadening in the central regions of the spectra was observed which was more apparent at higher temperatures. Below 29 °C, the spectra appear rather similar to those in Figure 5 for less concentrated protein-containing complexes.

The apparent quadrupole splittings for all six vesicle dispersions examined are shown in Figure 7 as a function of

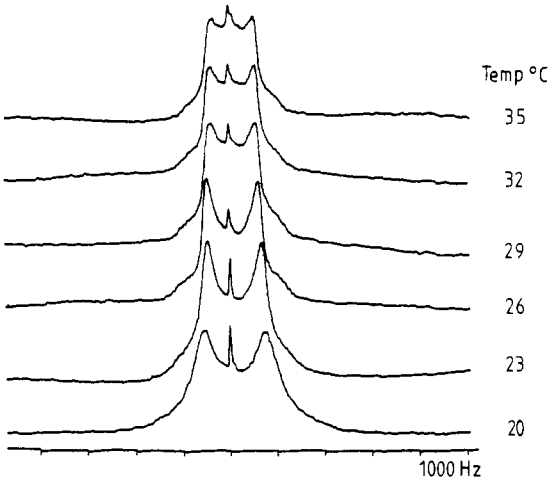

FIGURE 5: Temperature dependence of the deuterium NMR spectrum of reconstituted band 3–DMPC-d_9 (0.4:1 w/w) in deuterium-depleted dialysis buffer at 46.1 MHz.

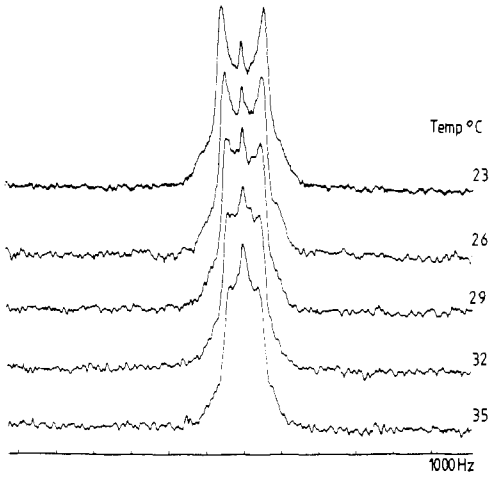

FIGURE 6: Temperature dependence of the deuterium NMR spectrum of reconstituted band 3–DMPC-d_9 (0.71:1 w/w) in deuterium-depleted dialysis buffer at 30.7 MHz (CXP-200 spectrometer) using a quadrupole echo pulse sequence with 40-μs pulse delays.

temperature. Here it can be seen that band 3 broadens the phase transition of the bilayer as sensed by the choline methyls of DMPC-d_9. At very high protein:lipid ratios (greater than about 0.7:1 protein:lipid, w/w), the measured quadrupole splittings decreased monotonically with decreasing temperature, indicating either a highly broadened phase transition or the suppression of the transition to temperatures below those measured (17 °C). In common with other integral membrane proteins (Kang et al., 1979; Tamm & Seelig, 1983; Ryba et al., 1986), a successive decrease of $\Delta\nu_Q$ with increasing content of band 3 within the bilayer was observed.

The values of $\Delta\nu_Q$ at different temperatures are shown in Figure 8 for each of the lipid:protein ratios. The variation of $\Delta\nu_Q$ with protein:lipid ratio is linear for all temperatures, indicating that the lipid at the protein surface is in fast exchange throughout all environments of the complexes at all protein:lipid ratios as shown previously for other reconstituted proteins (Kang et al., 1979; Sixl et al., 1984; Ryba et al., 1986). A marked difference between the present and previous studies is that the slopes of the plots in Figure 8 vary with temperature and diverge with increasing protein concentration (increasing protein:lipid ratio). At the highest protein:lipid ratio (1.25:1 w/w) and high temperatures, the measured quadrupole splitting levels off to a constant value of about 760 Hz (Figures 7 and 8) although the intensity in the center of the spectra

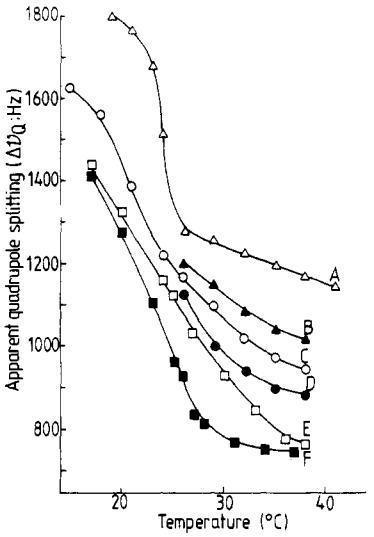

FIGURE 7: Temperature dependence of the apparent quadrupole splitting in the ^{2}H NMR spectra of band 3–DMPC-d_9 complexes. All samples were preincubated at 37 °C for 1 h before the spectra were recorded (see Materials and Methods), and the temperature variations were reversible over the temperature ranges illustrated. The protein:lipid weight ratios are (A) pure DMPC-d_9, (B) 0.32:1 (w/w), (C) 0.4:1 (w/w), (D) 0.48:1 (w/w), (E) 0.71:1 (w/w), and (F) 1.25:1 (w/w).

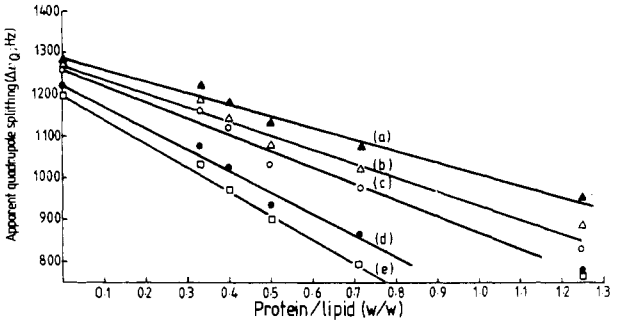

FIGURE 8: Variation in measured quadrupole splitting as a function of the band 3:DMPC-d_9 weight ratio ($1/n_1$) constructed from data similar to those of Figure 7. The temperatures are 26 (▲), 27 (△), 29 (○), 32 (●), and 35 °C (□).

still increased with increasing temperature. This effect may be due to the high degree of spectral broadening for this complex.

For all the complexes, the measured variations of $\Delta\nu_Q$ with temperature were completely reversible provided that the annealing step (1 h at 37 °C) was performed before the NMR measurements. Complexes which were measured 7 days after the initial measurements gave spectra that were not the same as those recorded on the day of recovery from dialysis with indications of lipid hydrolysis having occurred.

The incorporation of band 3 in DMPC-d_9 bilayers did not affect the spin–lattice relaxation time (T_1) of the N-methyl deuteron which was 50 ± 3 ms at 32 °C over the range of protein:lipid ratios measured. Phosphorus NMR spectra were obtained for all complexes (not shown) and were typical of those arising from bilayer lipid. Within the limitations of the signal to noise ratio in the ^{31}P NMR spectra, there was no change in the phosphorus chemical shift anisotropy for any protein:lipid ratio compared with protein-free vesicles.

DISCUSSION

The results presented here provide further demonstration that the head groups of phospholipids in membrane bilayers are sensitive to interaction with proteins. As in the case of

rhodopsin (Ryba et al., 1986), the incorporation of band 3 into bilayers of DMPC-d_9 results in a broadening of the spectral lines and a decrease in the apparent quadrupole splitting. Significantly, the effect of band 3 on the ^{2}H NMR spectrum of DMPC-d_9 is markedly temperature dependent, the spectral changes at a given protein:lipid ratio being greater at higher temperatures than at lower temperatures.

There are several possible contributions to the effect of band 3 on the ^{2}H NMR spectrum of DMPC-d_9 in reconstituted bilayer vesicles. It is possible that partial averaging of the residual spectral anisotropy (with a resulting decrease in quadrupole splitting) may arise from the rotation of vesicles or the lateral diffusion of lipid molecules around the surface of small vesicles, although we have argued against this on electron microscopic evidence (see Results). The extreme shoulders of the spectra, whose separation we define as the apparent quadrupole splitting, contain no contribution from the population of small vesicles (about 20%) which may give rise to the broad central component in the spectra which is particularly prominent at higher temperatures (Figures 5 and 6).

It is also unlikely that residual detergent in the protein–lipid complexes can be responsible for the changes observed in the ^{2}H NMR spectra. The exchange of Triton X-100 for sodium cholate by gel filtration results in levels of Triton X-100 of less than 1 wt % with respect to band 3 even before dialysis, and the addition of this amount of the detergent to DMPC-d_9 bilayers had no effect on the measured quadrupole splitting. Residual sodium cholate concentrations (less than 1 mol % of reconstituted lipid) were well below levels required to affect the spectrum of DMPC-d_9 (Ryba et al., 1986).

The major effects of band 3 on the ^{2}H NMR spectra can then be attributed to interactions between the protein and bilayer lipid. As with the interaction of myelin basic protein with negatively charged lipids (Sixl et al., 1984) and of rhodopsin with DMPC-d_9 (Ryba et al., 1986), it is possible to analyze the spectral changes in terms of a two-site fast exchange of lipids between the bulk lipid phase (n_f/n_t being the proportion of lipid in the complex not associating with protein) and lipids at the protein surface (n_c/n_t being the proportion of lipids interacting with protein) such that $n_f + n_c = n_t$, where n_t is the total lipid:protein ratio for the complex. Such an analysis predicts a linear relationship between the experimentally observed quadrupole splitting, Q_0, and the inverse of the chemically determined value of n_t from the proportionality relationship $Q_0 = (n_c/n_t)Q_c + (n_f/n_t)Q_f$, where Q_c and Q_f are the values of $\Delta\nu_Q$ for lipids interacting with the protein and for lipids in the remaining bulk phase, respectively. As shown in Figure 8, Q_0 is linear with respect to $1/n_t$ at least for protein:lipid ratios up to 0.7:1 (w/w). At very high protein:lipid ratios at 33 and 35 °C, the observed values of $\Delta\nu_Q$ deviate from linearity and tend toward a limiting value.

Deviations from linearity at high protein:lipid ratios in the application of the two-site exchange model to protein–lipid interactions have previously been observed (Kang et al., 1979; Sixl et al., 1984; Ryba et al., 1986). This effect can be understood in the nature of the bulk lipid phase in which the conformational and dynamic properties of the lipid molecules that give rise to the characteristic quadrupole splittings have contributions from interactions (which may be cooperative) between lipids. At very high protein:lipid ratios, the size of the cooperative units of bulk lipid may be diminished to an extent where these interactions are significantly altered and their cooperative nature decreased. The protein-associated lipid will then be in rapid exchange with a bulk lipid phase whose properties may be quite different from those of pure lipid.

The assumption that the lipid head groups sample a homogeneous protein surface is also likely to be an oversimplification of the protein–lipid interaction. While rapid exchange on the ^{2}H NMR time scale required for averaging of the residual quadrupolar anisotropy (faster than about 10^4 Hz) onto and off the protein surface seems to occur (there is no evidence for "protein-immobilized" lipid in the present or our previous work), it is unlikely that a given lipid molecule can experience all possible interactions with the protein surface on this time scale. This heterogeneity of the protein sites during sampling by a lipid polar group will probably contribute some degree of inhomogeneous spectral broadening (Bienvenue et al., 1982). The broadening of the spectra that is particularly prominent at high protein:lipid ratios and high temperatures (Figure 5) may arise from such inhomogeneous broadening and contributes to the apparent leveling of measured quadrupole splittings to a limiting value at protein:lipid ratios of 1.25:1 (Figures 7 and 8). Within these limitations, the linearity of the plots in Figure 8 indicates that the fast exchange model is a reasonable approximation for the interaction of band 3 with DMPC in reconstituted bilayers at protein:lipid weight ratios up to 0.7:1.

In the case of rhodopsin–DMPC-d_9 interactions (Ryba et al., 1986), the observed quadrupole splittings converge to a common value at all temperatures when plotted against the protein:lipid ratio. The comparable results for band 3 (Figure 8) are rather different from those obtained with rhodopsin. Since in the application of the proposed fast exchange model the slopes of the plots in Figure 8 have a value of $n_c(Q_c - Q_f)$, the changes in slope with respect to temperature imply that any or all of the three parameters, n_c, Q_c, and Q_f, are temperature dependent. The value of Q_f actually *decreases*, although by less than 10% (by 90 Hz), from 26 to 35 °C as seen from Figure 8 at $1/n_t = 0$. However, since the slopes of Figure 8 *increase* with increasing temperature, the largest contribution to the change in slopes will be from changes in n_c or Q_c. Previously we have suggested that Q_c may be close to zero for lipids interacting with rhodopsin and myelin basic protein (Ryba et al., 1986; Sixl et al., 1984). If such an assumption is valid for DMPC-d_9 deuterons when interacting with band 3, then the significant increase in the value of $n_c(Q_c - Q_f)$ in Figure 8 (from 240 Hz at 26 °C to 548 Hz at 35 °C) suggests that the number of lipids interacting with each protein monomer *increases* on average with increasing temperature.

It has previously been shown that band 3 undergoes a reversible self-association in reconstituted bilayers of egg PC, DOPC, and DMPC (Mühlebach & Cherry, 1985). On aggregation of an integral membrane protein, the protein surface available for lipid association may remain constant (if aggregating protein retains its "solvating" lipid) or might decrease (if lipid is excluded from the interface between self-associating protein molecules). The results in Figure 8 which show a temperature-dependent change in the number of lipid molecules interacting on average with band 3 indicate that the temperature-dependent self-association of band 3 occurs with exclusion of lipid molecules from the protein–lipid interface. Such an effect would be indistinguishable from associations involving the extramembranous regions of band 3 (Kopito & Lodish, 1985) in which lipid "solvating" the intramembranous regions was retained, when using methods such as ultracentrifugation (Pappert & Schubert, 1983), rotational correlation time measurements by flash-induced transient dichroism decay (Mühlebach & Cherry, 1985), and saturation-transfer ESR

(Sakaki et al., 1982) which give information about the bulk size of the associating species. The ^2H NMR method indicates that self-associating band 3 molecules in DMPC release a proportion of their solvating lipid, presumably that proportion interacting with the previously exposed protein–protein interface. The independence of the measured spectral intensity to changes in temperature indicates (within an error of about 10%) that no motionally restricted or highly ordered lipid is present between aggregated proteins.

Verma and Wallach (1976) showed that tryptophan residues of erythrocyte integral membrane proteins become more accessible to nitroxide spin-labeled fatty acids intercalated within the membrane upon increasing the temperature, with a transition to unrestricted nitroxide quenching of fluorescence above about 37 °C where the protein is minimally aggregated. In addition, proton NMR measurements have suggested an increased mobility in the methyl side chains of integral membrane proteins in the erythrocyte above 35 °C (Sheetz & Chan, 1972). These observations are consistent with the interpretation of a temperature-dependent self-association of band 3 involving the intramembranous apolar faces of the protein.

It is possible to make an estimate of n_c, the number of lipids released from the bulk lipid phase per molecule of band 3, at a given temperature from the slope of the straight lines in Figure 8, $n_c(Q_c - Q_f)$, and assuming (Sixl et al., 1984; Ryba et al., 1986) that Q_c is close or equal to zero. Using the value of Q_f at each temperature from the pure lipid spectra, the value of n_c changes by a factor of 2.5, from 28:1 to 70:1 (mol/mol, lipid:protein) at 26 and 35 °C, respectively, assuming a molecular weight of 100000 for band 3. In the strict application of the two-site exchange model, n_c may be taken as the number of lipids required to surround the maximally dissociated species of band 3 in DMPC-d_9 (at 35 °C). From a width of a lipid of 9.6 Å (Engelman, 1971), it can be calculated that 70 lipid molecules (35 in each leaflet of the bilayer) would surround a cylindrical protein with a diameter of about $(35 \times 9.6)/\pi$ = 107 Å. Even though band 3 is predicted (from the sequence of the murine protein) to contain 12 transmembrane helical sections (Kopito & Lodish, 1985), this value is rather larger than expected for the band 3 monomer by comparison with the limited information available for rhodopsin (seven helices; 35-Å diameter; Osborne et al., 1978) or bacteriorhodopsin (seven helices, 35 × 45 Å in cross section; Henderson & Unwin, 1975). It should be realized, however, that the value of n_c obtained by extrapolation to $\Delta\nu_Q = 0$ is unlikely to be a valid representation of a hypothetical *single* annulus of lipid molecules surrounding the hydrophobic perimeter of the protein (Ryba et al., 1986). Lipid molecules in shells not immediately adjacent to the protein surface will experience interactions that differ from those in bulk lipid, and the exchange of lipid molecules through these shells may generate contributions to inhomogeneous broadening that result in the reduction of the measured quadrupole splitting. In terms of the two-site exchange model, our best conclusion arising from the value of n_c is that the lipid in more than a single shell of lipids surrounding the protein contributes to the protein-induced exchange-averaged spectral perturbations. It should be pointed out that although we have related the "annulus" of lipids around the protein to the monomeric species of band 3, it is probably incorrect to assume that the maximally dissociated species occurring at 35 °C is a monomer. There is good evidence, for example, that band 3 is dimeric in membranes (Jennings, 1984), and in our preparations, a small proportion of protein is reconstituted in the form of aggregates that are undissociable in SDS. Assuming that lipid is excluded from the associating surfaces of any aggregates remaining under the conditions of maximal dissociation (high temperature), then the maximally dissociated species would be expected to have less annular lipid per mole of monomer than monomeric band 3. Our conclusion that more than a single shell of lipid is "perturbed" by the protein surface is therefore reinforced by consideration of residual self-association of band 3 under conditions of maximal dissociation.

The reversible self-association of band 3 observed by deuterium NMR is independent of the main gel–liquid-crystalline phase transition of DMPC-d_9 which occurs at 24 °C (see Figure 7). This transition, although broadened and suppressed at high protein:lipid ratios, takes place without apparently affecting the association of band 3 which is manifest at temperatures up to 35 °C, well above the phase transition of DMPC. Mühlebach and Cherry (1985) observed that in DMPC, band 3 at a lipid:protein ratio of 9.3:1 (w:w) was completely immobilized at 20 °C (below the lipid phase transition temperature) but became mobile at temperatures of about 22–24 °C, clearly reflecting the influence of the lipid phase transition. This effect need not be due to self-association, however, and may result from the increased microviscosity of the bilayer in the gel phase. These authors point out that at higher protein:lipid ratios the phase transition is broadened, as is observed here, with protein mobility persisting down to 18 °C. In addition to the independence of the self-association properties of band 3 on the phase transition of DMPC-d_9 observed here, a more general independence of band 3 self-association on phospholipid type is suggested from measurements of band 3 self-association in the erythrocyte membrane (Nigg & Cherry, 1979), in reconstituted single lipid bilayers (Mühlebach & Cherry, 1985; this work), and even in nonionic detergents (Pappert & Schubert, 1983). It is expected that any self-association equilibrium is a property of both the solute and supporting solvent, and in the case of proteins in lipids might be a function of relative association energies of protein–protein, protein–lipid, and lipid–lipid interactions. In the case of band 3, protein–lipid and lipid–lipid interactions appear to be relatively unimportant in determining the association properties of the protein, although the detailed nature of the association is clearly modulated by the lipid (Mühlebach & Cherry, 1985). The evidence that the temperature-dependent self-association of band 3 is an intrinsic property of the protein suggests that a temperature-dependent conformational transition between an association-promoting conformer and an association-inhibiting conformer may drive the self-association of the protein. Whether such a putative conformational equilibrium is related to conformational transitions observed by ESR spin-labeling (Ginsberg et al., 1981) and fluorescence energy transfer (Macara et al., 1983), and implied in the large activation volume measured for anion exchange (Canfield & Macey, 1984) that appears to be related to the function of the protein, remains to be determined. Significantly, however, inhibition of band 3 activity by the eosin maleimide probe leaves the self-associated properties of band 3 at least qualitatively unaffected (Nigg & Cherry, 1979; Mühlebach & Cherry, 1985).

ACKNOWLEDGMENTS

We thank the Oxford Enzyme Group for use of the WH-300 NMR spectrometer and Dr. C. M. Dobson for use of the CXP-200 NMR spectrometer. We acknowledge the excellent technical assistance of Peter Fisher.

Registry No. DMPC, 18194-24-6; cholic acid, 81-25-4.

REFERENCES
Bienvenue, A., Bloom, M., Davis, J. H., & Devaux, P. F. (1982) *J. Biol. Chem. 257*, 3032–3038.
Bloom, M., & Smith, I. C. P. (1985) in *Progress in Protein–Lipid Interactions* (Watts, A., & DePont, J. J. H. H. M., Eds.) Vol. I, pp 61–88, Elsevier, Amsterdam.
Burnell, E. E., Cullis, P. R., & DeKruijff, B. (1980) *Biochim. Biophys. Acta 603*, 63–69.
Canfield, V. A., & Macey, R. I. (1984) *Biochim. Biophys. Acta 778*, 379–384.
Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.
Devaux, P. F. (1983) in *Biological Magnetic Resonance* (Berliner, L. J., & Reuber, J., Eds.) Vol. V, Plenum Press, New York.
Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119–130.
Dorst, H.-J., & Schubert, D. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1605–1618.
East, J. M., Melville, D., & Lee, A. G. (1985) *Biochemistry 24*, 2615–2623.
Engelman, D. M. (1971) *J. Mol. Biol. 58*, 153–165.
Ginsburg, H., O'Connor, S. E., & Grisham, C. M. (1981) *Eur. J. Biochem. 114*, 533–538.
Henderson, R., & Unwin, P. N. T. (1975) *Nature (London) 257*, 28–32.
Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.
Jennings, M. L. (1984) *J. Membr. Biol. 80*, 105–117.
Kang, S. Y., Gutowsky, H. S., Hsung, J. C., Jacobs, R., King, T. E., Rice, D., & Oldfield, E. (1979) *Biochemistry 18*, 3257–3267.
Kopito, R. R., & Lodish, H. (1985) *Nature (London) 316*, 234–238.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Macara, I. G., Kuo, S., & Cantley, L. C. (1983) *J. Biol. Chem. 258*, 1785–1792.
Markwell, M. A. K., Haas, S. M., Tolbert, N. E., & Bieber, L. I. (1981) *Methods Enzymol. 72*, 296–303.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Mühlebach, T., & Cherry, R. J. (1985) *Biochemistry 24*, 975–983.
Nigg, E. A., & Cherry, R. J. (1979) *Biochemistry 18*, 3457–3465.
Osborne, H. B., Sardet, C., Michel-Villaz, M., & Charbre, M. (1978) *J. Mol. Biol. 123*, 177–206.
Pappert, G., & Schubert, D. (1983) *Biochim. Biophys. Acta 730*, 32–40.
Rouser, G., Fleischer, S., & Yamamoto, A. (1975) *Lipids 5*, 494–496.
Ryba, N. J. P., Dempsey, C. E., & Watts, A. (1986) *Biochemistry* (submitted for publication).
Sakaki, T., Tsuji, A., Chang, C.-H., & Ohnishi, S. (1982) *Biochemistry 21*, 2366–2372.
Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 548–555.
Sixl, F., Brophy, P. J., & Watts, A. (1984) *Biochemistry 23*, 2032–2039.
Smith, R. L., & Oldfield, E. (1984) *Science (Washington, D.C.) 225*, 280–288.
Tamm, L. K., & Seelig, J. (1983) *Biochemistry 22*, 1474–1483.
Taraschi, T. F., DeKruijff, B., Verkleij, A. J., & Echteld, C. J. A. (1982) *Biochim. Biophys. Acta 685*, 153–161.
Verma, S. P., & Wallach, D. F. H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3558–3561.
Wolosin, J. M. (1980) *Biochem. J. 189*, 35–44.

^{19}F Nuclear Magnetic Resonance Studies of Selectively Fluorinated Derivatives of G- and F-Actin†

Manfred Brauer* and Brian D. Sykes
Department of Biochemistry and Medical Research Council of Canada Group on Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received September 27, 1985; Revised Manuscript Received December 2, 1985

ABSTRACT: G-Actin is a globular protein (M_r 42 300) known to have three cysteine residues that are at least partially exposed and chemically reactive (Cys-10, -284, and -374). When G-actin was reacted with 3-bromo-1,1,1-trifluoropropanone, three resolvable ^{19}F resonances were observed in the ^{19}F NMR spectrum. This fluorinated G-actin derivative remained fully polymerizable, and its ^{31}P NMR spectrum was not significantly different from that of unmodified G-actin, indicating that the chemical modification did not denature the actin and the modified residues do not interfere with the extent of polymerization or the binding of adenosine 5′-triphosphate. One of the three ^{19}F resonances was assigned to fluorinated Cys-374 on the basis of its selective reaction with *N*-ethylmaleimide. This resonance was dramatically broadened after polymerization of fluorinated G-actin, while the other two resonances were not markedly broadened or shifted. Thus, Cys-10 and -284 are not involved in or appreciably affected by the polymerization of G-actin, while the mobility of the ^{19}F label at Cys-374 is markedly reduced.

Fluorine-19 nuclear magnetic resonance (NMR) has proven to be an exceptionally useful probe for studying the conformation and mobility of proteins, membranes, and other biological macromolecular systems (Sykes & Weiner, 1980; Gerig, 1978). It offers the advantages of high sensitivity (close to that of ^1H NMR), 100% natural abundance, a wide chemical shift range (~2000 ppm), and a low background of naturally occurring resonances. ^{19}F must, in general, be introduced into the system by bioincorporation of fluorinated

† This work was supported by the Medical Research Council of Canada Group on Protein Structure and Function and the Alberta Heritage Foundation for Medical Research.
* Address correspondence to this author at the Department of Applied Sciences in Medicine, University of Alberta.

0006-2960/86/0425-2187$01.50/0