Studies of Fc_γ Receptors of Human B Lymphocytes: Phospholipase A_2 Activity of Fc_γ Receptors†

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ABSTRACT: The presence of phospholipase A_2 activity within human B cell Fc_γ receptors was investigated. Lysate produced by detergent treatment of chronic lymphocytic leukemia cells that had 1% of the cells surface radioiodinated was subjected to affinity chromatography by using either *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphorylcholine–Sepharose (PC–Sepharose) or heat-aggregated human IgG–Sepharose 4B conjugate (IgG–Sepharose). The materials eluted from both adsorbants by ethylenediaminetetraacetate- or urea-containing buffer were further purified by gel filtration and isoelectric focusing in the presence of 6 M urea. Both isolated PC- and IgG-binding materials were homogeneous, when judged by gel filtration and isoelectric focusing, and had identical isoelectric points (p*I* = 6.5), peptide maps, and amino acid compositions. Furthermore, both preparations catalyzed equally the hydrolysis of phosphatidylcholine to release fatty acid from the 2 position. Optimal enzymatic activity depended on the presence of Ca^{2+}, was maximal at pH 9.5, and was augmented by Fc_γ fragments. Both preparations specifically bound to the Fc portion of IgG and inhibited human antibody-coated erythrocyte rosette formation by peripheral mononuclear cells. Our data thus demonstrate the identity of PC- and IgG-binding materials and suggest that a functional activity of the human B cell Fc_γ receptor is the generation of phospholipase A_2 activity within the plasma membrane.

The Fc_γ receptor ($Fc_\gamma R$)[1] is an integral membrane lipoprotein which specifically binds the Fc portion of IgG proteins at the surface of various cells, including B lymphocytes and macrophages (Basten et al., 1972a,b; Dickler & Kunkel, 1972; Anderson & Grey, 1974; Suzuki et al., 1980; Suzuki et al., 1981). The precise functions of $Fc_\gamma R$ at the cell surface in the immune response are not yet clearly defined, with one notable exception. This refers to $Fc_\gamma R$ present on K cells that were shown to be directly involved in antibody-dependent cell-mediated cytotoxicity (ADCC) (Perlman et al., 1972; Anderson & Grey, 1974; Revillard et al., 1975). However, the molecular mechanism of this phenomenon is not understood. Various potential roles of $Fc_\gamma R$ other than the involvement in ADCC have been proposed. These have assumed that $Fc_\gamma R$ may (1) act as a receptor for surface IgM on the B cell surface (Ramasamy, 1976), (2) help localize antigen into spleen (Brown et al., 1970; Miller et al., 1971), and (3) regulate the immune response (Dickler, 1976). It has also been suggested that the $Fc_\gamma R$ is closely related to or even identical with Ia antigens (Dickler & Sachs, 1974).

As reported earlier (Suzuki et al., 1980), we have developed a five-step procedure to isolate $Fc_\gamma R$ proteins from human CLL cell lysates in homogeneous and biologically active form. Biochemical characterization of such materials revealed that $Fc_\gamma R$ on human B cells is a single-chain lipoprotein of M_r near 30 000 which is associated specifically with phospholipids in a molar ratio of protein to phospholipid of 1:1. The fatty acids associated with $Fc_\gamma R$ proteins are mostly unsaturated (Suzuki et al., 1981). These findings and the results reporting the increased synthesis of prostaglandin E_2 by Fc fragment activated human macrophages (Passwell et al., 1979) prompted us to investigate the possibility of the presence of phospholipase A_2 activity within $Fc_\gamma R$ proteins.

The results presented in this paper will demonstrate that an integral membrane protein isolated as $Fc_\gamma R$ exhibits phospholipase A_2 activity and that an integral membrane protein isolated as phospholipase A_2 can specifically bind to the Fc portion of IgG protein. Two protein preparations isolated from CLL cells of a single patient by two different affinity chromatography systems were found to be identical with each other in their molecular size, isoelectric points, tryptic peptide maps, and amino acid compositions, thus demonstrating two functional properties of an isolated, homogeneous membrane molecule.

Experimental Procedures

Cells. CLL cells [(0.5–1.0) × 10^{12} cells] that have been shown by EA rosette assay to bear $Fc_\gamma R$ on their surface were obtained by leukopheresis from two different patients (JP and WA). Lymphocytes were further fractionated by the Ficoll–Hypaque gradient centrifugation method (Bøyam, 1968). Normal human peripheral blood mononuclear cells were obtained from heparinized blood by the same technique.

Surface Radioiodination. Approximately 1 × 10^9 CLL cells suspended in 10 mL of TBS were radioiodinated with 2 mCi of ^{125}I (Amersham) by the lactoperoxidase-catalyzed method (Morrison & Bayse, 1970) by using Enzymobeads (Bio-Rad) (Thorell & Johansson, 1971). After radioiodination, cells were washed five times with TBS (45-mL aliquot) at 4 °C. Viability, as determined by trypan blue exclusion, was greater than 90%.

Detergent Treatment. Cells (1 × 10^9 radioiodinated and 1 × 10^{11} untreated) were suspended in TBS containing 25 mM $CaCl_2$ and 1 mM each of IAM, EDTA, and PMSF at 0 °C. Triton X-100 (Amersham) was then added to a final con-

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[1] Abbreviations used: CLL, chronic lymphocytic leukemia; EA, antibody-coated erythrocytes; EDTA, ethylenediaminetetraacetate; $Fc_\gamma R$, Fc_γ receptor; IAM, iodoacetamide; IEF, isoelectric focusing; IgG–Sepharose, heat-aggregated human IgG–Sepharose 4B conjugate; PBS, phosphate-buffered saline; PC, *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphorylcholine; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TBS, Tris-HCl buffered saline; Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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centration of 1%. After the solution was stirred for 30 min at 0 °C, nuclei materials, unlysed cells, and debris were removed by centrifugation at 5 °C at 12000 rpm for 60 min. The clear supernatant solution, denoted as cell lysate, was immediately subjected to affinity chromatography.

Affinity Chromatography. In order to extract phospholipase A_2 from cell lysate, affinity chromatography media (PC–Sepharose 4B) were prepared essentially according to the method described by Rock & Snyder (1975). 1-Octadec-9-enyl-2-hexadecylglycero-3-phosphorylcholine (Calbiochem) was oxidized by the method described by Shimojo et al. (1974) to convert to *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphorylcholine (PC). The product migrated, as expected, slightly faster than the starting material when chromatographed on silica gel H layers with a solvent system consisting of chloroform/methanol/water (65:25:4 v/v/v). The PC was then coupled to AH–Sepharose 4B (Pharmacia) through the carboxyl moeity to provide PC–Sepharose 4B as follows. AH–Sepharose 4B (7.5 g) swollen in excess 0.5 M NaCl was washed with deionized water and resuspended in tetrahydrofuran. PC dissolved in 50% dioxane and *N,N'*-dicyclohexylcarbodiimide (Aldrich) were added to the gel. The coupling reaction was allowed to proceed at pH 5 for 24 h at 25 °C with gentle stirring. The gel was then washed with tetrahydrofuran followed by methanol, 1 M NaCl, and deionized water. The gel was packed in a column (2.5 × 10 cm) and equilibrated with the first buffer consisting of Tris-HCl, pH 7.5 (50 mM), $CaCl_2$ (25 mM), NaCl (0.2 M), EDTA (1 mM), and Triton X-100 (0.5%) (buffer 1).

Normal human IgG proteins, Fab and Fc fragments, heat-aggregated IgG, and heat-aggregated IgG- and Fc–Sepharose 4B were prepared as described earlier (Suzuki et al., 1980). Protein A–Sepharose CL-4B conjugate was purchased from Pharmacia (Uppsala, Sweden).

Other Physicochemical Methods. The method of isoelectric focusing in the presence of 6 M urea has been previously described (Uki et al., 1974; Suzuki et al., 1980; Suzuki et al., 1981). Radioiodination of the isolated $Fc_\gamma R$ or phospholipase A_2 was carried out in TBS containing 0.5% Triton X-100 by the lactoperoxidase-catalyzed method (Thorell & Johansson, 1971) by using Enzymobeads (Bio-Rad). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was carried out by the method of Weber & Osborne (1969). Amino acid analyses were perrformed with the use of an automated amino acid analyzer (JEOL 6AH) on samples that were hydrolyzed at 110 °C in constant-boiling HCl for various lengths of time (20–72 h) in evacuated Pyrex tubes. Tryptic peptide mapping followed the method described earlier (Suzuki et al., 1980; Suzuki et al., 1981). Approximate estimation of protein concentration in the presence of Triton X-100 containing buffer was made with the method based on Coomassie blue colorimetry (Bradford, 1976) by using a Bio-Rad protein assay kit. The more precise determination of protein concentration was carried out by nitrogen analysis (Kjeldahl). The nitrogen contents of the protein samples were assumed to be 16%.

Assay of Phospholipase A_2 Activity. The first method employed a pH stat titrator (Radiometer Model TTT 11 equipped with SBU1A, pH M 28, and SBR2C titrigraph). The substrate (12.5 μmol of egg yolk phosphatidylcholine, Sigma) was dissolved in 10 mL of the assay solution (10 mM $CaCl_2$, 20 mM KCl, and 0.5% Triton X-100) and equilibrated to 37 °C in a thermoregulated vessel under a gentle stream of nitrogen. After the addition of an aliquot of the enzyme preparation, the rate of hydrolysis was followed by titration with 5 mM NaOH. The rate of hydrolysis was linear for more than 10 min after the latency period.

Phospholipase A_2 activity was also assayed by thin-layer or silicic acid chromatography of hydrolysis products by using radioactive phosphatidylcholine as substrate. Preparation of 2-[^{3}H]oleylphosphatidylcholine followed the method described by Robertson & Land (1962). [9,10-^{3}H]Oleic acid (1 mCi) (Amersham) was suspended in 0.1 M sodium phosphate (pH 7.5) and briefly sonicated. Then ATP (200 μmol), coenzyme A (2 μmol), and a freshly prepared rat liver microsome suspension (50 mg of protein) were added. The mixture was stirred gently for 3 h at 37 °C, with additional ATP, CoA, and rat liver microsomes supplied at 0.5 and 2 h. Lipids were then extracted with chloroform/methanol (2:1 v/v). The lipid extract containing both unbound fatty acid and labeled phospholipid was fractionated over a column of silicic acid, previously equilibrated with chloroform/methanol (9:1 v/v). Labeled phosphatidylcholine was obtained by elution with chloroform/methanol (6:4 v/v) after free fatty acid had been removed by elution with chloroform/methanol (9:1 v/v).

In order to assay phospholipase A_2 activity, highly purified $Fc_\gamma R$ materials (100–300 μg) were incubated for 30 min to 6 h at 37 °C with phosphatidylcholine (250 nmol/assay), including a trace amount of the radioactive substrate, in the presence and absence of heat-aggregated IgG or Fc fragment (10–300 μg/assay) in a total volume of 100 mL of 0.2 ionic strength, pH 8.5, Tris-HCl buffer or 0.15 M KCl (pH 9.5), each containing 0.5% Triton X-100 and 5 mM $CaCl_2$. At the end of the incubation period, the lipids were extracted from the reaction mixture with chloroform/methanol (2:1 v/v), concentrated with a stream of nitrogen, and spotted on silica gel G coated glass plates together with various standards. Ascending chromatograms were developed in one dimension with a solvent system of chloform/methanol/acetic acid/water (25:15:4:1 v/v). Components separated were identified as fatty acid, phosphatidylcholine, and lysophosphatidylcholine by comparison with the standards. They were scraped off from the plates and counted with a scintillation counter (Packard). Lipid extracts from the reaction mixtures were also fractionated by passage over a column (1 × 5 cm) of silicic acid (4 g) equilibrated with chloroform. Fatty acids, phosphatidylcholine, and lysophosphatidylcholine were eluted from the column by 100 mL each of solvents containing 10% and 40% methanol in chloform and 100% methanol, respectively (Sweeley, 1969).

Assay of Phospholipase C and D. Phospholipase C activity was assayed by measuring the release of phosphorus from lecithin in Tris–maleate buffer, pH 7.2 (Ottolenghi, 1969). The assay for the phospholipase D activity followed the method described by Kates & Sastry (1969).

EA Rosette Assay. Human EA was prepared by sensitizing group O, Rh (+) human erythrocytes with a subagglutinating dose of human anti-D antibody (IgG class). Human peripheral mononuclear cells suspended in phosphate-buffered saline (PBS) (1 × 10^6 cells/mL) were added to an equal volume of 0.5% EA suspension in PBS. These were incubated for 30 min at 37 °C and then for 16 h at 4 °C. The mixtures were then gently dispersed by repeated pipetting and subjected to microscopic examination for rosette formation. Assay was routinely performed in triplicate in which the standard error was found to be less than 10%.

The Fc-binding activity of $Fc_\gamma R$ and phospholipase A_2 preparations was determined by the degree of inhibition of EA rosette formation. Potential inhibitors were preincubated at 4 °C for 1 h with EA preparations in the presence and absence

of varying amounts of heat-aggregated IgG, Fc_γ fragments, or Fab fragments. The mixtures were washed three times with PBS at 25 °C and then added to human peripheral blood mononuclear cell suspension to examine whether EA could be rosetted.

Results

Isolation of Phospholipase A_2 from CLL Lysate. In our series of exploratory experiments, highly purified $Fc_\gamma R$ obtained from patient WA (Suzuki et al., 1980) were assayed for phospholipase A_2 activity at pH 8.5 with the use of the substrate containing a trace amount of 2-[^{3}H]oleoylphosphatidylcholine by thin-layer chromatography of lipids extracted from the reaction mixtures. While about 70% of the radioactivity was recovered in total, about 12–18% of the recovered radioactivity was found in the free fatty acid fraction when assayed in the presence of heat-aggregated human IgG. In the absence of aggregated IgG, however, only about 2% of the recovered radioactivity was in the fatty acid fraction. Thus, the results of the initial experiments seemed to indicate the possible presence of phospholipase A_2 activity within $Fc_\gamma R$ materials.

If $Fc_\gamma R$ protein possesses both Fc-binding and phospholipase A_2 activity, such material should bind to PC–Sepharose as well as to IgG–Sepharose. The physicochemical properties and biological activities of the materials isolated by the two different affinity chromatography methods could be readily compared.

To examine this possibility, the surface proteins of 1×10^9 B lymphocytes obtained from a CLL patient (JP) were first radioiodinated. The labeled cells were extensively washed with TBS and added to prewashed, unlabeled cells (1×10^{11}). The cell lysate, prepared as described under Experimental Procedures, contained 67% of the radioactivity as Cl_3CCOOH-precipitable materials and was first passed through a column (1 × 20 cm) of protein A–Sepharose CL-4B to remove intrinsic IgG proteins. The fraction that did not bind to protein A–Sepharose was divided into two equal volumes of 480 mL, each containing about 7.8 g of protein (3.6×10^7 cpm as Cl_3CCOOH–precipitable materials). They were immediately passed over the two separate columns containing PC–Sepharose (2.5 × 10 cm) and IgG–Sepharose (2.5 × 30 cm), respectively, both columns being previously equilibrated against buffer 1 at 4 °C. The columns were then extensively washed with buffer 1 until the radioactivity of the effluent became less than 50 cpm/mL. The bound materials were first eluted with TBS containing 50 mM EDTA and 0.5% Triton X-100 until the radioactivity of the effluents became less than 50 cpm/mL. The eluates thus obtained were denoted as an EDTA eluate. The materials that remained bound to affinity media were then eluted with 6 M deionized urea made in TBS containing 0.5% Triton X-100. Such eluates were denoted as a urea eluate. Figure 1A,B illustrates the results obtained by this elution scheme. In all cases, the radioactivity peaks were totally superimposable on the protein peaks, suggesting the cell-surface origin of these proteins. On the basis of the Cl_3CCOOH-precipitable radioactivity, approximately 3% of the radioactivity present in the lysate was found to be adsorbed on IgG–Sepharose while 2.6% was bound to PC–Sepharose. About 18.7% of the materials bound to PC–Sepharose 4B were found in the EDTA eluate while the EDTA eluate from IgG–Sepharose comprised only 2.5% of the total bound materials.

Gel Filtration of Affinity Chromatography Products. In order to further purify the products isolated by affinity chromatography, they were first passed separately through a

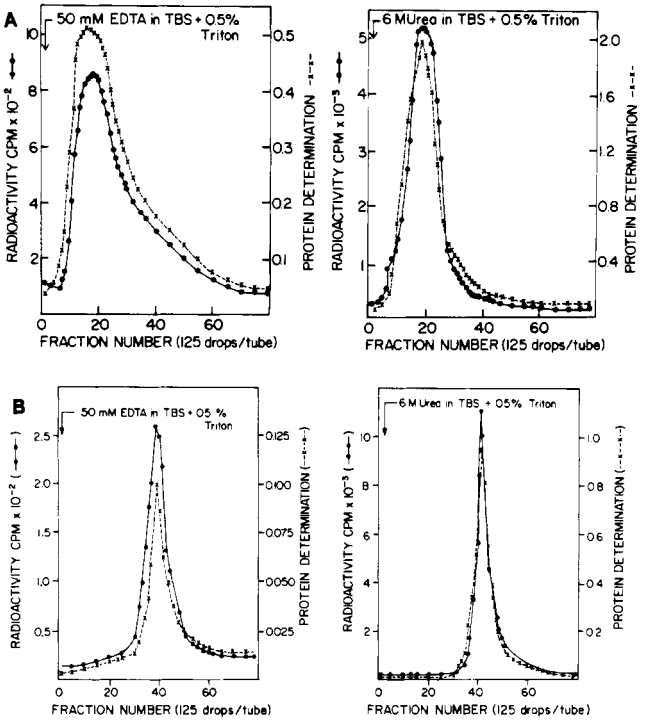

FIGURE 1: Elution profiles of PC-binding materials from PC–Sepharose 4B (A) and IgG-binding materials from IgG–Sepharose (B).

column (2.5 × 45 cm) of Sephadex G-100 previously equilibrated against 0.15 M potassium chloride containing 20 mM $CaCl_2$ and 0.5% Triton X-100. A typical gel filtration pattern obtained with the EDTA eluate from PC–Sepharose is shown by Figure 2A. Results with the EDTA eluate from IgG–Sepharose were essentially identical with this. On the basis of the Cl_3CCOOH-precipitable radioactivity, approximately 86% and 70% of the EDTA eluates from PC– and IgG–Sepharose, respectively, were found in the first fraction. Since the radioiodination was carried out by the lactoperoxidase-catalyzed method, the radiolabeled fractions must contain solubilized phospholipase A_2 and $Fc_\gamma R$, respectively, that were present at the cell surface. The second peaks that were not associated with the radioactivity must have been either devoid of radioiodinatable tryosines or derivable from cytoplasmic compartments and were not studied further.

Sephadex G-100 gel filtration patterns of the urea eluates from PC– and IgG–Sepharose were also essentially identical. As seen in Figure 2B, which depicts a typical result obtained with the urea eluate from PC-Sepharose, the urea eluates were again separated into two peaks as in the case of the EDTA eluates. The majority of radiolabeled materials (78–98%) were eluted in the first fraction.

Isoelectric Focusing of the PC- and IgG-Binding Materials. The charge properties of approximately 10–15 mg of the radiolabeled fractions obtained by gel filtration of the EDTA eluates from PC– and IgG–Sepharose were investigated by the isoelectric focusing method. Since these proteins had relatively low radioactivities due to initial surface radioiodination, about 1 mg of each protein was first separately reiodinated with 1 mCi of ^{125}I as described under Experimental Procedures. More highly radiolabeled materials would permit the detection of minor components that may be present in these protein preparations. The reiodinated proteins were extensively dialyzed against TBS containing 0.5% Triton X-100 and were separated from residual protein-unbound ^{125}I by passage over a column of Sephadex G-100. More than 98% of the radioactivity of the reiodinated proteins was found to be Cl_3CCO-

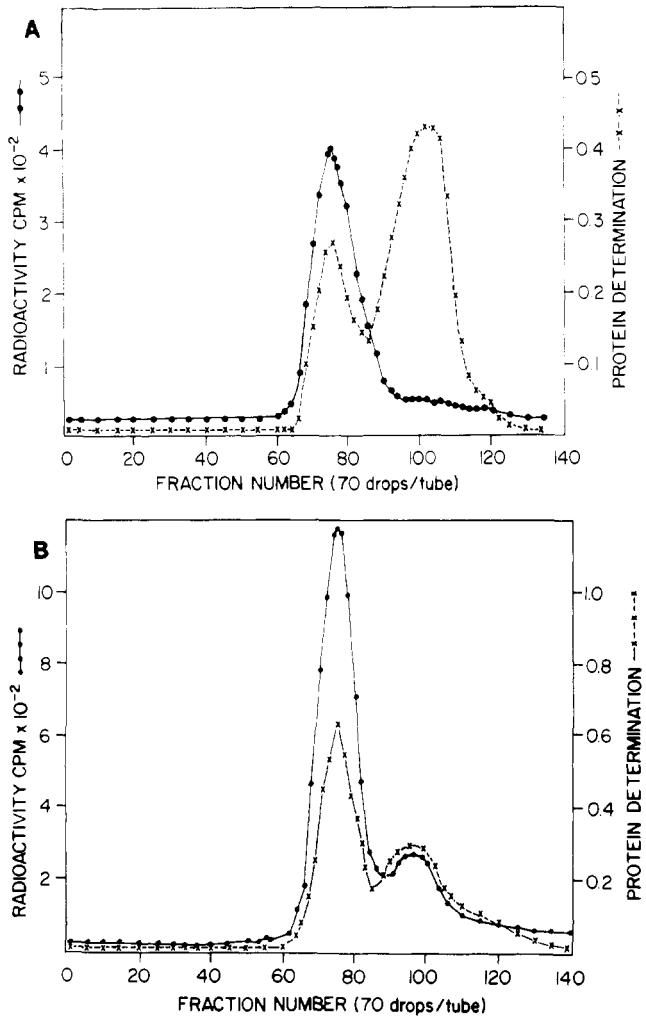

FIGURE 2: Sephadex G-100 gel filtration patterns of the EDTA (A) and urea eluates (B) from PC–Sepharose. A column (2.5 × 50 cm) of Sephadex G-100 was previously equilibrated against 0.15 M KCl, 0.025 M $CaCl_2$, and 0.5% Triton X-100. The gel filtration patterns of the EDTA- and urea-eluates from IgG–Sepharose were essentially identical with the corresponding patterns shown.

OH precipitable. These materials were pooled with the rest of the surface-radiolabeled materials and subjected to isoelectric focusing. The result obtained with PC-binding material is shown in Figure 3, which was essentially identical with that for IgG-binding material. Thus, both PC- and IgG-binding materials eluted with EDTA were found to be sharply focused at a pH near 6.5, indicating charge homogeneity and the identity of these proteins.

In order to investigate the nature of the materials that were bound tightly to the affinity media and could be eluted with 6 M urea, 20–25 mg of these eluates from the IgG- and the PC–Sepharose affinity columns was also electrofocused. Their electrofocusing patterns were similar not only to each other but also to those of the EDTA-eluates shown by Figure 3.

The above procedure yielded the final electrofocused materials of 14 and 1.5 mg from the EDTA eluate from PC- and IgG–Sepharose, respectively. The yields of the electrofocused urea-eluates from PC- and IgG–Sepharose were 30 and 40 mg, respectively.

Amino Acid Compositions of the Electrofocused Materials. Table I illustrates the amino acid compositions of the EDTA and urea eluates that were gel filtered and electrofocused as described above. It is clear that the amino acid compositions of the PC- and IgG-binding materials are essentially identical within experimental error. They are also very similar to those

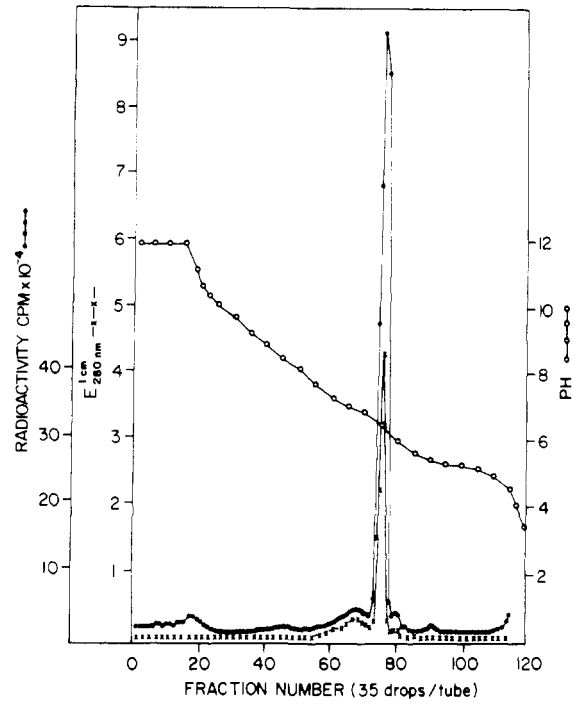

FIGURE 3: Isoelectric focusing pattern of the EDTA eluate from PC–Sepharose. The electrofocusing patterns of the urea eluate from PC–Sepharose and the EDTA and urea eluates from IgG–Sepharose were essentially identical with the pattern shown.

Table I: Amino Acid Compositions of PC-Binding (EDTA and Urea Eluates) and IgG-Binding (EDTA and Urea Eluates) Materials Obtained from JP Cell Lysates[a]

	JP PC-binding		JP IgG-binding		
amino acids	EDTA eluate	urea eluate	EDTA eluate	urea eluate	WA FcγR[b]
Lys	18.0	18.1	18.2	18.1	16.4
His	7.0	6.9	6.4	6.4	5.9
Arg	15.0	14.3	15.1	15.0	14.2
Asp	25.0	25.2	24.7	24.0	23.3
Thr	14.0	13.5	13.9	14.0	11.8
Ser	21.0	21.2	20.8	20.0	19.1
Glu	34.0	35.1	34.1	33.0	33.4
Pro	12.0	11.7	12.1	12.0	12.9
Gly	24.0	24.0	24.0	24.0	24.0
Ala	20.0	19.5	20.0	20.0	17.8
$^1/_2$-Cys	3.0	2.7	3.1	3.0	2.9
Val	17.0	16.8	17.1	17.0	14.2
Met	4.0	4.9	4.2	4.0	4.4
Ile	11.0	10.9	11.2	11.0	10.9
Leu	26.0	26.7	26.8	27.0	26.1
Tyr	7.0	6.5	7.6	7.9	6.9
Phe	10.0	9.2	9.5	10.0	10.3
$GlcNH_2$	0	0	0	0	0
$GalNH_2$	0	0	0	0	0

[a] The values listed are the average of six experiments computed on the basis of an assumed M_r of 30 000 and expressed as the number of residues per mole of protein. The values for Thr, Ser, and Tyr are those extrapolated to 0 h from the results obtained with 20-, 44-, and 68-h hydrolyses. [b] The values for WA FcγR were reported elsewhere (T. Suzuki, T. Taki, K. Hachimin and R. Sadasivan, unpublished experiments) and are lised for comparison.

of the highly purified, biologically active Fc$_\gamma$R proteins obtained from the other patient (WA) that are listed for comparison. However, minor differences exist in the contents of Lys, Thr, Ala, and Val. This may be due to the presence of either minor impurities or possible idiotype differences. In keeping with the previously reported data on the high purified human B cell Fc$_\gamma$R proteins (Suzuki et al., 1981), both PC- and IgG-binding materials had no detectable hexosamine,

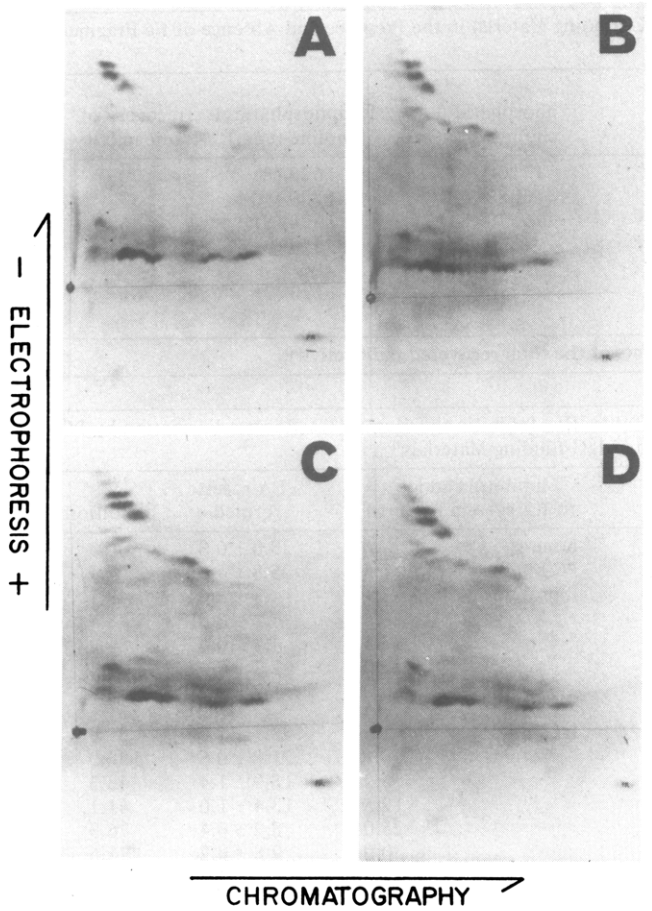

FIGURE 4: Tryptic peptide maps of the PC-binding materials, (A) EDTA eluate and (B) urea eluate, and the IgG-binding materials, (C) EDTA eluate and (D) urea eluate.

which is usually associated with glycoproteins. A point of interest is the relatively low cysteine content (1.7% of total residues, in contrast to those of both bovine pancreatic phospholipase A_2 (11.4%) (Fleer et al., 1978) and *Bitis gabonica* venom enzymes (10.2%) (Botes & Viljoen, 1974).

Tryptic Peptide Maps. The tryptic peptide maps of the EDTA and urea eluates from PC–Sepharose that were further purified by gel filtration and electrofocusing are presented as Figure 4A,B and appear to be essentially identical with those of the corresponding IgG-binding materials (Figure 4C,D). In each case, about 35 different peptides were identified by ninhydrin staining. This number of peptides agrees with what is expected from the number of lysyl and arginyl residues (see Table I).

Biological Properties of the PC- and IgG-Binding Materials. (1) Phospholipase A_2 Activity. If phospholipase A_2 activity is an intrinsic property of $Fc_\gamma R$ proteins, both PC- and IgG-binding materials should exhibit phospholipase A_2 activity. To determine this, both PC- and IgG-binding materials that were purified by electrofocusing were extensively dialyzed against 0.15 M KCl containing 25 mM $CaCl_2$ and 0.5% Triton X-100. They were then assayed for their phospholipase A_2 activity by the pH stat method with egg yolk phosphatidylcholine as substrate.

As seen in Figure 5A, the electrofocused EDTA eluate from PC–Sepharose was capable of hydrolyzing phosphatidylcholine between pH 7.5 and 10, with the optimal pH being near 9.5. Figure 5B shows that the maximum enzymatic activity at pH 9.5 was obtained in the presence of 5 mM Ca^{2+}. The enzymatic activity observed was dependent on Ca^{2+}, and the addition of EDTA to a level of 5 mM completely abolished the

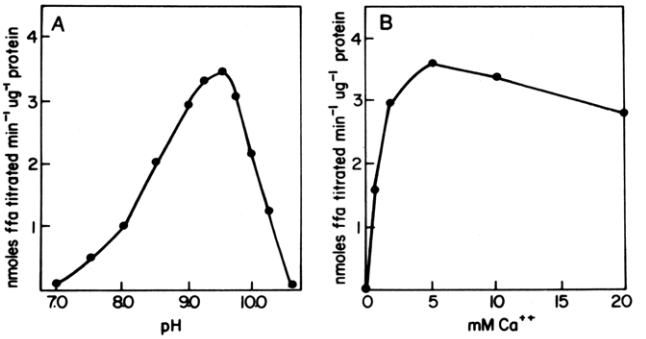

FIGURE 5: (A) Effect of pH on the hydrolysis of egg yolk lecithin (10 mg) by EDTA eluate from PC–Sepharose (10 μg) as determined by the pH stat method. (B) Effect of Ca^{2+} on the hydrolysis of egg yolk lecithin (10 mg) by EDTA eluate from PC–Sepharose (10 μg) at pH 9.5 as determined by the pH state method.

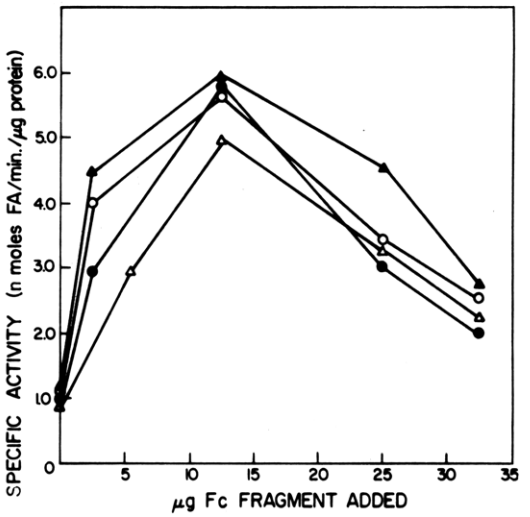

FIGURE 6: Effects of Fc fragments on phospholipase A_2 activity of PC- and IgG-binding materials. Enzymatic activity of various samples was assayed with the pH stat method at pH 9.5 at 37 °C in the presence of 5 mM $CaCl_2$. Various amounts of human Fc fragments were incubated at 37 °C at pH 9.5 with 10-μg aliquots of PC- or IgG-binding materials prior to addition to the substrate (egg yolk lecithin) solution. (●) EDTA eluate from PC–Sepharose; (○) urea eluate from PC–Sepharose; (▲) EDTA eluate from IgG–Sepharose; (△) urea eluate from IgG–Sepharose.

activity. Mg^{2+} could not substitute for Ca^{2+}. The enzymatic activity of the urea eluate from PC–Sepharose, and of the EDTA and urea eluates from IgG–Sepharose, was then assayed at pH 9.5 in the presence of k mM Ca^{2+}. The specific activity of about 1.0 μmol min^{-1} mg^{-1} protein was obtained for each preparation (see Figure 6). This value was close to that found for phospholipase A_2 from *Crotalus admanteus* venom (3.0 μmol min^{-1} mg^{-1} protein) (Rock & Snyder, 1975) but is considerably higher than that for phospholipase A_2 of Krebs ascites cell plasma membrane (1.5 pmol min^{-1} μg^{-1} protein) (Record et al., 1977). The PC–Sepharose affinity media should have bound additional substances in the cell lysates that have an affinity for phosphorylcholine moieties. However, none of the material showed any detectable phospholipase C or D activity when 100 μg of each material was used.

The pH stat method utilized for the assay does not discriminate phospholipase A_1 from A_2 activity. In order to demonstrate that the enzymatic activity observed above indeed represents the latter type activity, the release of [^{3}H]oleic acid specifically from 2-[^{3}H]oleoylphosphatidylcholine by PC-binding materials (EDTA eluate) was examined. About 100 μg of PC-binding materials was first incubated with or without

Table II: Specific Hydrolysis of 2-[^{3}H]Oleoylphosphatidylcholine by JP PC-Binding Material in the Presence and Absence of Fc Fragments, and by Honey Bee Venom Phospholipase A_2 (Sigma)

expt	substance added to PC	fatty acid (cpm)	phosphatidyl-choline (cpm)	lysophosphatidyl-choline (cpm)	% recovery of radioactivity
1	none	2400 (11)[a]	17100 (81)	1620 (8)	70
2	PC-binding material (100 μg)	8660 (50)	6880 (40)	1700 (10)	57
3	Fc fragment (250 μg)	1540 (8)	16570 (84)	1620 (8)	66
4	PC-binding material (100 μg) + Fc fragment (250 μg)	15870 (90)	1390 (8)	370 (2)	59
5	bee venom phospholipase A_2 (10 μg)	15570 (86)	1260 (7)	1220 (7)	61

[a] Numbers in parentheses represent the percentage of the radioactive counts of the total recovered radioactivity.

250 μg of Fc fragments of pH 9.5 at 37 °C for 30 min in 10 mL of 0.15 M KCl containing 25 mM $CaCl_2$ and 0.5% Triton X-100. The mixtures were then added to 100 μg of phosphatidylcholine containing a trace amount of radioactive substrate (3×10^4 cpm) suspended in 0.1 mL of 0.15 M KCl containing 25 mM $CaCl_2$ and 0.5% Triton X-100. After incubation at 37 °C for 4 h, the lipids were extracted from the reaction mixtures with chloroform/methanol (2:1 v/v).

Fatty acids, phospholipids, and lysolecthin in the lipid extract were fractionated by silicic acid column chromatography as described under Experimental Procedures. The radioactivity in each fraction was measured by a scintillation counter (Searle). The results summarized in Table II show that PC-binding materials (EDTA-eluate) catalyzed the specific hydrolysis at position two of the glycerol backbone of the substrate to give 50% of the recovered radioactivity in the free fatty acid fraction. The preincubation of the PC-binding materials with Fc fragments resulted in the augmentation of their catalytic activity approximately 2-fold. The release of fatty acids from the substrate must have been due to the specific enzymatic cleavage since only 11% of the recovered radioactivity was obtained in the fatty acid fraction when no PC-binding material or enzyme was added to the substrate and since Fc fragment alone also had no enzymatic activity. Bee venom phospholipase A_2 used as a control for the positional specificity clearly cleaved 2-[^{3}H]oleoylphosphatidylcholine to give 86% of the recovered radioactivity in the fatty acid fraction. A small percentage (2–10%) of the recovered radioactivity was found in the lysophosphatidylcholine fractions. This could be due to a small amount of phosphatidylcholine which was not eluted by chloroform/methanol (6:4 v/v) but eluted by methanol since bee venom phospholipase A_2 which does not have phospholipase A_1 activity gave a small amount (7%) of the radioactivity in the lysophosphatidylcholine fraction and since the incubation of the substrate alone without any additional material also gave 8% of the recovered radioactivity in this fraction. However, these data must be cautiously interpreted because of the relatively low recovery of the radioactivity from silicic acid chromatography and the failure of these results to exclude the presence of phospholipase A_1 activity. The use of a substrate such as 1-[^{3}H]palmitic-phosphatidylcholine should definitely clarify the positional specificity of the $Fc_\gamma R$ acylhydrolase activity.

(2) IgG-Binding Property. The specific Fc-binding activity of the PC- and IgG-binding materials (both electrofocused urea eluates) was examined by measuring their capability to inhibit human EA rosette formation. The results summarized in Table III clearly demonstrate that not only IgG-binding materials but also PC-binding materials inhibited EA rosette formation in a dose-dependent manner. This inhibition was due to the specific binding of the inhibitors to antibodies competing against $Fc_\gamma R$ present on the monocular cell surface. As shown in Table IV, the inhibition of EA rosette formation by the PC- and IgG-binding materials was abolished by preincubating them with heat-aggregated IgG or Fc fragments but not with Fab fragments.

Table III: Inhibition of Human EA Rosette Formation by PC- and IgG-Binding Materials[a]

inhibitors added to EA system (μg/mL)		% EA rosette formed	% inhibition
none		28.0 ± 0.8	0
PC-binding proteins	1.6	25.8 ± 4.0	7.9
	3.1	14.3 ± 3.6	49.1
	6.2	6.1 ± 0.6	78.4
	12.5	4.4 ± 0.5	84.2
	25.0	2.7 ± 0.3	90.4
	50.0	1.7 ± 0.3	93.9
IgG-binding proteins	1.6	23.1 ± 1.0	32.9
	3.1	21.8 ± 0.6	36.9
	6.2	18.9 ± 1.4	45.3
	12.5	13.4 ± 1.0	61.1
	25.0	8.2 ± 0.4	76.3
	50.0	9.8 ± 0.2	71.6

[a] Both PC- and IgG-binding materials utilized were the urea eluates from PC- and IgG-Sepharose and were further purified by gel filtration and electrofocusing as described in the text.

Table IV: Effect of Heat-Aggregated IgG and Fc and Fab Fragments on the Inhibition of EA Rosette Formation by PC- and IgG-Binding Materials (Both Urea Eluates)

proteins preincubated with inhibitors (μg/mL)		PC-binding material (25 μg/mL)[a] % EA rosette	% inhibition	IgG-binding material (25 μg/mL)[b] % EA rosette	% inhibition
none		2.7 ± 1.3	90.4	8.2 ± 0.4	76.3
aggregated IgG	10	25.6 ± 1.9	8.9	ND[d]	
	50[c]	27.7 ± 5.0	1.0	27.5 ± 0.6	13.2
Fc fragments	10	17.7 ± 2.1	36.8	ND	
	50[c]	23.8 ± 1.7	15.0	23.3 ± 2.9	26.6
Fab fragment	10	6.2 ± 0.7	78.0	ND	
	50[c]	3.6 ± 0.0	87.3	10.5 ± 1.3	66.8

[a] The percentage of EA rosette formed without PC-binding materials was 28.0 ± 0.8. [b] The percentage of EA rosette formed without IgG-binding materials was 31.7 ± 0.5. [c] Inhibition observed by aggregated IgG and Fc and Fab fragments at a concentration level of 50 μg/mL was 88.2, 83.9, and 0%, respectively. [d] ND, not determined.

(3) Effect of Fc Binding on the Phospholipase A_2 Activity. If the phospholipase A_2 activity is an intrinsic property of $Fc_\gamma R$ proteins as suggested by the data presented above, the enzymatic activity of PC- as well as IgG-binding materials might be altered upon binding to Fc-containing materials. The results of the investigations on the effect of Fc binding on the phospholipase A_2 activity are summarized in Figure 6. It is

clear that the noted enzymatic activity was augmented as much as 6-fold by preincubating with the Fc fragment at a molar ratio of 1:1. Augmentation by the Fc fragment of the enzymatic activity of the urea–eluate from IgG–Sepharose was somewhat lower (about 5-fold), probably due to slight denaturation of proteins during the isolation. Fab fragment had no effect on the enzymatic activity. The decline of the enzymatic activity of PC- and IgG-binding materials by the excess of Fc fragments was possibly due to steric hindrance.

Discussion

The data presented in this paper demonstrate that phospholipase A_2 activity is an intrinsic property of $Fc_\gamma R$ proteins isolated from the surface of human B cells. Evidence for this is (1) the marked homology of the gel filtration patterns, the isoelectric points, the tryptic peptide maps (Figure 4), and the amino acid compositions (Table I) between the PC- and IgG-binding materials isolated from cell lysate of a single CLL patient; (2) the demonstration of the specific inhibition of EA rosette formation by the PC- as well as IgG-binding materials (Tables III and IV); and (3) the demonstration of phospholipase A_2 activity by PC- as well as IgG-binding materials (Table II, Figures 5 and 6) which is augmented by Fc_γ fragments. Furthermore, $NaDodSO_4$–polyacrylamide gel electrophoresis analysis under reducing conditions showed that both PC- and IgG-binding materials moved as a single band essentially in an identical manner with the mobility equivalent to an apparent M_r of 30000 (data not shown). The evidence to show that both PC- and IgG-binding materials represent $Fc_\gamma R$ molecules present on the cell surface is provided by Figures 1 and 2 which demonstrated the radioactivity with a protein peak.

$Fc_\gamma R$ proteins present on the surface of human B cells thus appear to be bifunctional, i.e., endowed with both Fc-binding and phospholipase A_2 activity. Since $Fc_\gamma R$ proteins of human B cells are a single polypeptide chain lipoprotein (Thoenes & Stein, 1979; Suzuki et al., 1980; Suzuki et al., 1981; and Figure 7), the two different biological functions may reside on different parts of the molecule. Efforts to localize the indicated functions to specific regions of the $Fc_\gamma R$ molecule are currently being made in our laboratory.

Characterization of membrane-bound phospholipase A_2 of various immunologically related cells has been reported by a number of investigators. Resch et al. (1971) first showed the modest increase (35%) of phospholipase A_2 activity in the microsomal fraction of rabbit lymphocytes after activation with phytohemagglutinin. The successful isolation of phospholipase A_2 activity from homogenates of rabbit polymorphonuclear leukocytes (Kaplan et al., 1978; Franson & Waite, 1978) and from alveolar macrophages (Lanni & Franson, 1980) has been reported. Our data showed that enzymatic activity inherent in both PC- and IgG-binding materials was strongly dependent on the presence of Ca^{2+} (Figure 5A) and could be inhibited totally by EDTA (Figure 5B). Results presented in Table II suggested the specificity of the PC-binding materials as phospholipase A_2. However, the definite positional specificity of enzymatic activity needs to be confirmed by selectively assaying for phospholipase A_1 activity in these materials. The significant augmentation of the enzymatic activity after the preincubation with Fc fragment at molar ratios below 1:1 (Figure 6) could be due to a conformational change of PC- and IgG-binding materials. The gradual loss of the activation of enzymatic activity by further addition of Fc fragments could be due to steric hindrance. However, it has to be assumed that one molecule of $Fc_\gamma R$ protein will bind more than one molecule of Fc fragments. The mechanism of the activation of phospholipase A_2 activity by Fc fragments is currently being investigated.

The precise functions of $Fc_\gamma R$ at the surface of various cells are to this date not well understood. If $Fc_\gamma R$ is a true cell-surface receptor, it should transmit, by definition, a signal upon specific binding to the Fc portion of IgG. However, the type of signal or the mechanism of signal transmission by $Fc_\gamma R$ is unknown. $Fc_\gamma R$ has been suggested by numerous studies to be involved in the regulation of the immune response (Dickler, 1976), such as suppression of the humoral immune response by immune complexes (Uhr & Möller, 1968), or of B cell differentiation (Kölsch et al., 1980). The finding of the presence of phospholipase A_2 activity within B cell $Fc_\gamma R$ proteins suggests new avenues of investigation on the possible role of cell-surface $Fc_\gamma R$ as a signal transmitter. Phospholipase A_2 could initiate the splitting of phospholipids, major components, of the lipid bilayer of the B cell plasma membrane, and lead to the synthesis of prostaglandins or other arachidonic acid metabolites. These compounds have been shown to play a critical role in the inflammatory process (Vane, 1976; Flower, 1976; Kaliner et al., 1973). Of particular interest is the finding of Passwell et al. (1979), who showed that human monocytes actively synthesize and secrete prostaglandins, especially PGE_2, upon binding of Fc fragments or heat-aggregated IgG to their cell-surface $Fc_\gamma R$. Such a marked increase of prostaglandin synthesis could have been induced by the activation of the phospholipase A_2 activity associated with monocyte $Fc_\gamma R$, if monocyte $Fc_\gamma R$ is structurally and functionally similar to B cell $Fc_\gamma R$. The comparative structural studies aimed to resolve this problem have also been initiated in our laboratory.

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Raman Spectroscopic Study of the Interactions of Dimyristoyl- and 1-Palmitoyl-2-oleoylphosphatidylcholine Liposomes with Myelin Proteolipid Apoprotein†

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ABSTRACT: Recombinants of dimyristoylphosphatidylcholine (DMPC) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) with myelin proteolipid apoprotein prepared in an aqueous medium were investigated by vibrational Raman spectroscopy. Peak height intensity parameters, involving vibrational transitions in both the 3000-cm^{-1} acyl chain methylene carbon–hydrogen (C–H) stretching mode region and the 1100-cm^{-1} carbon–carbon (C–C) stretching mode region, were used to construct temperature profiles reflecting bilayer inter- and intrachain order–disorder processes. For liposomes reconstituted with DMPC, the gel–liquid-crystalline phase transition T_m, monitored by the C–C stretching mode indices, is depressed from 23 °C for the pure system to 12 °C and broadened from less than 1 °C to ~9 °C. On completion of the phase transition at 15 °C, the intramolecular chain disorder is substantially greater compared to that of the pure bilayer form. In addition, no further development of gauche conformers along the chain is apparent as the temperature increases. For the temperature profile derived from the C–H stretching region parameters of DMPC, the phase transition temperature is shifted from 23 to 11 °C. The intermolecular disorder in both the gel and liquid-crystalline states is significantly greater in the recombinant systems in comparison to that in the pure liposomes. Temperature profiles obtained for recombinants prepared with unsaturated phospholipid bilayers (POPC) indicate that the apoprotein only slightly perturbs the inter- and intramolecular parameters describing the lipid matrix. For assessment of the rather different perturbations of the apoprotein on bilayers containing either saturated or unsaturated acyl chains, the pure DMPC and pure POPC temperature profiles are compared by matching the curves to the same relative temperature with respect to their values for T_m. For the liquid-crystalline state, the POPC bilayers possess a greater population of gauche rotamers along the chain, although the lateral chain order is increased in the unsaturated bilayer compared to that of the saturated DMPC system. The increased intermolecular disorder exhibited by the pure and reconstituted DMPC systems is discussed in terms of the diffusion properties exhibited by saturated and unsaturated lipid matrices. Within the precision of the Raman experiment, no evidence on the vibrational time scale exists for a boundary lipid involving the aqueous solution of the apoprotein in either the saturated or unsaturated bilayer assembly. Finally, observed differences in the Raman temperature profiles obtained from reconstituted liposomes involving the apoprotein prepared from either organic or aqueous media are discussed.

For characterization of the response of lipid bilayer structures to intrinsic membrane components, recombinants of the myelin proteolipid apoprotein, or Folch-Pi apoprotein, with lecithin dispersions have provided tractable lipid–protein systems for a variety of spectroscopic, X-ray diffraction, and calorimetric studies (Verma et al., 1980; Curatolo et al., 1978, 1977; Boggs & Moscarello, 1978; Boggs et al., 1977, 1976). For example, recent calorimetric observations indicate that phosphatidic acid, phosphatidylglycerol, and phosphatidylserine exhibit preferential binding of the hydrophobic apoprotein (Boggs et

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