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Determination of the depth of penetration of the α subunit of retinal G protein in membranes: A spectroscopic study

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Abstract

This paper reports the fluorescence quenching of the α subunit of retinal rod outer segment G protein ($G_{t\alpha}$) by vesicles of brominated phospholipids. Two different brominated phospholipids with the bromine quencher groups attached at the 6–7 and 9–10 positions in one of the fatty acyl chains have been used to estimate the depth of penetration of the $G_{t\alpha}$ protein in the lipid vesicles using steady-state fluorescence quenching techniques. Our studies provide evidence of the interaction between $G_{t\alpha}$ protein, in its active conformation, with the lipid vesicles mimicking natural membranes. This study demonstrates that in vitro the distance between fluorescent tryptophan site of $G_{t\alpha}$ and the membrane surface is approximately 6.5 Å. © 1998 Elsevier Science B.V.

Keywords: G-protein; Fluorescence quenching; Lipid–protein interaction

1. Introduction

In recent years, there has been considerable interest in G proteins owing to their role in signal transduction processes [1]. It is now well known that in vertebrates, the heterotrimeric G proteins ($G_{\alpha\beta\gamma}$) mediate a variety of cellular responses to hormonal signals, including the hormonal regulation of cAMP level [2] and sensory stimuli such as vision, olfaction and taste [3–5]. All these processes utilize a common molecular mechanism that involves a guanine-nucleotide binding protein performing a three step task. The first step involves stimulation by a signal-detecting protein, which is a hepta-helical transmembrane receptor (R^*); the second step involves the binding of G protein to R^*, releasing GDP and binding GTP. The α subunit (G_α) then separates from R^* and its beta–gamma subunits ($G_{\beta\gamma}$). Finally, G_α in its activated conformation (G_α-GTP) regulates the function of effector elements which are usually an enzyme or an ion channel [6]. Regulation of the effector ceases when G_α hydrolyzes its bound GTP to GDP and returns to its basal inactive state and a conformational change of the G_α allows its reassociation with $G_{\beta\gamma}$. Interestingly, G_α act as molecular switches the 'on' and 'off' state of which is triggered by the binding and hydrolysis of GTP [7].

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In the visual transduction process, the α subunit of G protein ($G_{t\alpha}$) plays a key role involving a sequence of biochemical and electrophysiological events [8]. Two distinctly important features of the visual transduction process is gain and speed, for example, it has been estimated that response to a single photon rises to its maximum in 0.2 s and decays nearly as rapidly in 0.3 s [8,9] and information transfer from one protein to another is brought about by collisional coupling [10]. According to some authors, $G_{t\alpha}$ with its effector, the cGMP phosphodiesterase (PDE), modulates the speed of the transduction process [1,8,10,11]. However, several mechanisms have been suggested by different workers [8,10,12–16]. Chabre and Deterre [8], Chabre [15], and Bruckert et al. [16] have suggested a hopping mechanism where $G_{t\alpha}$ diffuses in an aqueous space with a speed that is two orders of magnitude faster than that in the lipid phase or $G_{t\alpha}$ is located at the surface of the membrane without binding that probably allows a sufficiently rapid encounter between $G_{t\alpha}$ and PDE. Liebman and Sitraramayya [13] and Liebman et al. [14] have proposed a two-dimensional (2D) lateral diffusion model where the diffusion process is fast enough to result in a favourable collision process which is rate limiting. The concept of an increased success rate of collision coupling originates from a decrease in orientational freedom imposed by membrane association which compensates for the slower diffusion speed along the surface [13,14].

Since interaction of the membranes with $G_{t\alpha}$ in its active and inactive conformation may regulate the signal transduction process, it is of utmost importance to achieve a good understanding of the phenomena. Considerable evidence exists that the behaviour of the proteins in vivo and in vitro may not be identical [17]. To have a better understanding of these systems and in an effort to understand better whether or not $G_{t\alpha}$ interact with membranes in vitro, we have studied the quenching of the intrinsic fluorescence of $G_{t\alpha}$ in the presence of brominated lipids that serves as a model system to study such interactions. Several methods exist [18]. One method is to study energy transfer from the tryptophan residues in the protein to an energy acceptor chromophore [18]. Usually the critical radius in such cases is quite large and it is difficult to conclude whether or not the protein has actually penetrated the membrane [18]. An elegant method to bypass these disadvantages is the use of heavy molecular quenchers like bromine which are known to quench through molecular collisional processes [18].

In this study, we have investigated the fluorescence quenching of $G_{t\alpha}$ by brominated lipids incorporated in vesicles mimicking natural membranes to determine whether or not the $G_{t\alpha}$ subunit interacts and penetrates the membrane. Our fluorescence quenching, and polarization anisotropy measurements demonstrate that in vitro the $G_{t\alpha}$ in its active conformation partially penetrates the lipid membrane with one of the tryptophan residues participating in the process.

2. Materials and methods

Highly purified egg yolk L-α-phosphatidylcholine (egg PC), 1-palmitoyl-2-stearoyl (6–7 dibromo)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-stearoyl (9–10 dibromo)-sn-glycero-3-phosphocholine referred to as 6–7 BrPC and 9–10 BrPC, respectively, were obtained from Avanti Polar Lipids and used as received. Rodamine B (laser grade) and 1–2 propanediol were purchased from Kodak and Sigma, respectively.

Highly purified $G_{t\alpha}$-GDP-binding protein from *Rana catesbiana* was obtained as described in details elsewhere [19]. Prior to fluorescence measurements, the protein samples were dialyzed against buffer A to remove glycerol that was used for storage. Buffer A was prepared by mixing Tris 10 mM, NaCl 150 mM, $MgCl_2$ 6 mM, EDTA 1 mM, DTT 1 mM at pH 7.4 (at room temperature). Centrifugation at $100\,000 \times g$ for a period of 1 h using a Beckman L7-80 ultracentrifuge was used to remove insoluble materials. Protein concentration was determined using the Coomassie blue binding method [20].

The structural analogy between PO_4^- and AlF_4^- has led to the proposal that fluoroaluminates mimic the γ-phosphate of GTP at the binding site [21]. Interestingly, the comparison between the crystal structures of $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GTP$_\gamma$S have yielded almost identical protein conformations [22]. In addition, similar levels of intrinsic fluorescence were obtained with $G_{t\alpha}$-GTP and its analogue $G_{t\alpha}$-

GDP + AlF_x where $x = 3–5$ [23,24]. These results thus indicate that $G_{t\alpha}$-GDP + AlF_x may be utilized to simulate $G_{t\alpha}$-GTP. A fluoroaluminate analogue of $G_{t\alpha}$-GTP was thus prepared by adding AlF_x to $G_{t\alpha}$-GDP as described in details elsewhere [21,24–26]. In all the experiments, we have used 2.5 mM NaF and 20 μM $AlCl_3$ in accordance with the method of Kanaho et al. [25].

Vesicles were prepared at room temperature as described elsewhere [27] by sonicating the phospholipids, 6–7 BrPC, 9–10 BrPC or egg PC in buffer A. Briefly, a lipid concentration of 0.3 mM is sonicated at room temperature followed by a centrifugation to remove titanium contaminants and multilamellar structures. After centrifugation, the phospholipid concentration was determined by measuring the phosphorus content in accordance with the method of Miljanich [28], modified by Jacob et al. [29].

Steady-state fluorescence emission was recorded on a Spex Fluorolog 2 spectrophotometer. Rhodamine B was used as a quantum counter in 1–2 propanediol and the detector voltage and amplification factor in all cases were maintained constant. Excitation was provided at 289 nm using a single beam monochromator. Fluorescent grade suprasil quartz cuvettes 5 mm × 5 mm and narrow (5 nm) excitation and emission slits were used for recording all spectra. All experiments were recorded at ambient room temperature. Corrected fluorescence emission spectra were obtained by subtracting the background fluorescence using a software supplied by SPEX.

Anisotropy measurements were made using Glan-Thompson polarizers placed in between the excitation source and the sample while the other one is placed between the sample and detector. Anisotropy (A) was calculated using the formula:

$$A = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$

where I_{vv} is the fluorescence intensity when the excitation and emission polarizers are parallel to the z axis of the laboratory frame while I_{vh} is the fluorescence intensity when the excitation and emission polarizers are parallel to the z and y axes of the laboratory frame. The G factor is obtained from the relation I_{hv}/I_{hh} and is a constant for the apparatus determined at the excitation wavelength of 289 nm.

3. Results and discussion

Addition of lipids to the protein solution resulted in the dilution of the protein. To estimate the correct concentration of proteins, a normalization procedure was used. Inset of Fig. 1 shows the calibration curves of $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GDP that correlates the

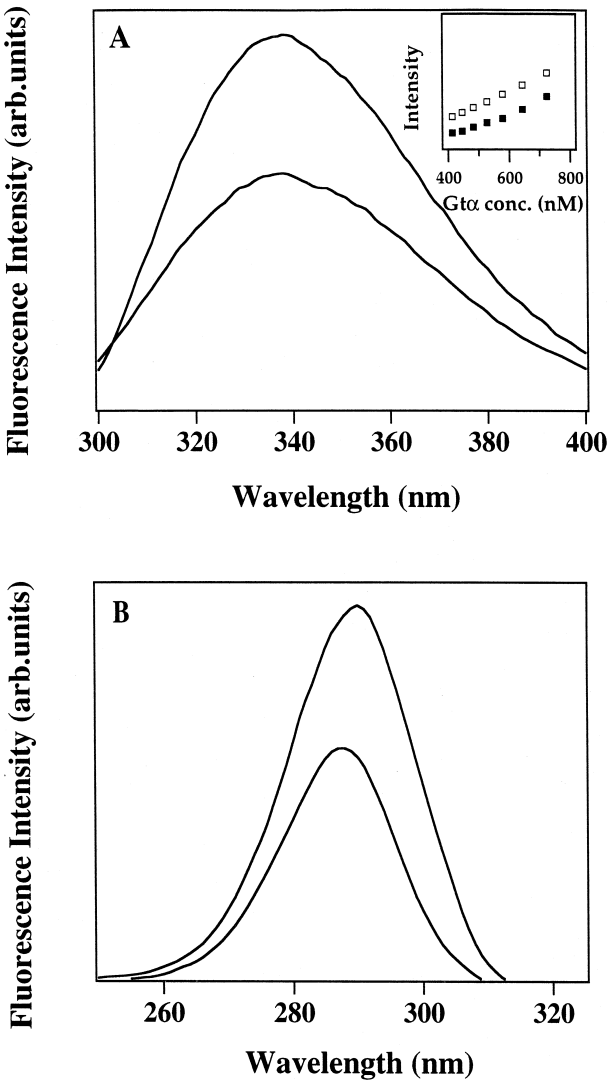

Fig. 1. (A) Emission spectra of $G_{t\alpha}$-GDP + AlF_x (upper curve) and $G_{t\alpha}$-GDP (lower curve), $\lambda_{ex} = 289$ nm. (B) Excitation spectra of $G_{t\alpha}$-GDP + AlF_x (upper curve) and $G_{t\alpha}$-GDP (lower curve), $\lambda_{em} = 337$ nm. Inset: Calibration curve. Plot of the fluorescence intensity of $G_{t\alpha}$-GDP + AlF_x (open squares) and $G_{t\alpha}$-GDP (filled circles) versus the concentration of $G_{t\alpha}$. $\lambda_{em} = 337$ nm; $\lambda_{ex} = 289$ nm.

protein fluorescence intensity to the protein concentration in absence of vesicles. The calibration curves are found to be almost linear in the range of concentrations used in this work.

Fig. 1A shows the fluorescence emission spectra of $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GDP corresponding to excitation provided at 289 nm. The emission profile in the 300–400 nm spectral region is broad, structureless and diffuse with the band maximum for both $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GDP located at approximately 337 nm. This observed broad emission profile originates from the two tryptophan residues W207 and W127 in the protein [30]. The fluorescence emission from $G_{t\alpha}$-GDP + AlF_x is more intense compared to $G_{t\alpha}$-GDP that has been attributed to the presence of the tryptophan residue W207 located near the GTP binding site [23] on the α2 helix compared to W127 located on the αC helix [31]. Fig. 1B shows the excitation spectra of $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GDP with their maximum at approximately 290 and 288 nm, respectively, which are in agreement with reported results [24] obtained on monitoring the fluorescence emission band maximum at 337 nm.

Fig. 2A and B show the plot of the fluorescence intensity of $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GDP, respectively, as a function of the concentrations of the brominated lipids 6–7 BrPC and 9–10 BrPC. To elucidate the effects of the brominated lipids, a control experiment using egg PC instead of the brominated lipids was performed and the results are shown in Fig. 2C. Comparing Fig. 2A and B with Fig. 2C, it is evident that while considerable fluorescence quenching occurs with the brominated lipids, there seems to be no quenching of the tryptophan fluorescence in the absence of the brominated lipids (in the presence of egg PC) that confirm quenching of the tryptophan fluorescence due to the presence of the bromine moieties. It was observed that while approximately 55% of the $G_{t\alpha}$-GDP + AlF_x fluorescence was quenched by 6–7 BrPC, only 15% quenching was observed for 9–10 BrPC (Fig. 2A) under identical experimental conditions. Several possible explanations may exist. A trivial case could be the large excess of protein molecules that may be actually quenched by the added quenchers. To circumvent these difficulties, we have studied the quenching process in large excesses of the brominated lipids. In all cases, 100% quenching of the tryptophan fluorescence was never observed. Such an observation indicates two possibilities: (a) only one of the two tryptophans in the protein participates in the quenching process, and/or (b) the protein is partially embedded in the vesicles such that one or both the tryptophans are located within the radius of interaction with the

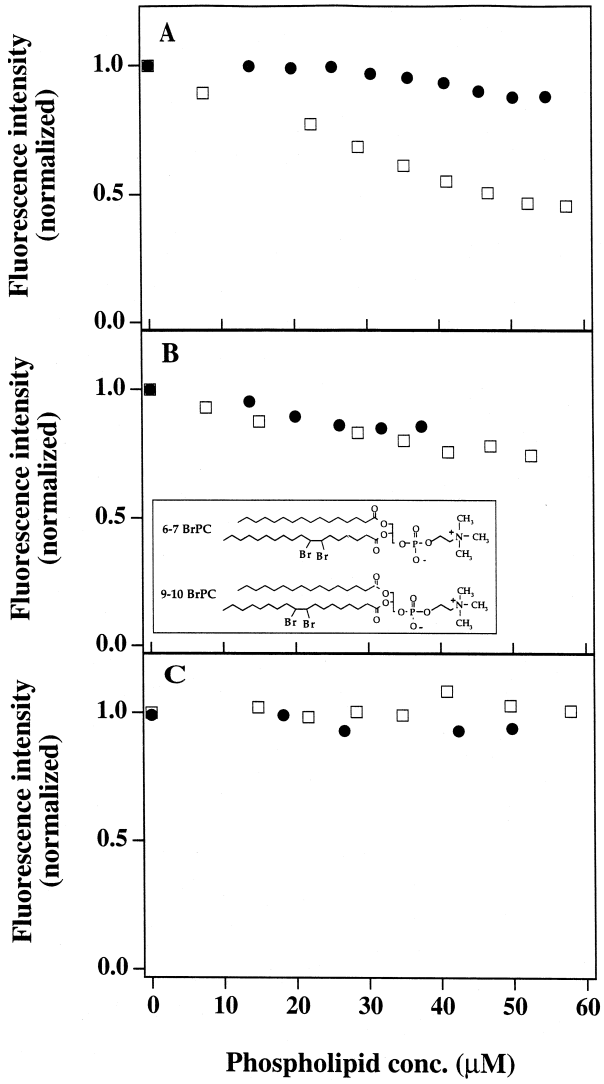

Fig. 2. Fluorescence quenching. (A) Fluorescence intensity of $G_{t\alpha}$-GDP + AlF_x versus the concentration of 6–7 BrPC (open squares) and 9–10 BrPC (filled circles). (B) Fluorescence intensity of $G_{t\alpha}$-GDP versus the concentration of 6–7 BrPC (open squares) and 9–10 BrPC (filled circles). (C) Fluorescence intensity of $G_{t\alpha}$-GDP + AlF_x (open squares) and $G_{t\alpha}$-GDP (filled circles) versus the concentration of egg PC (in the absence of the brominated phospholipids). λ_{em} = 337 nm; λ_{ex} = 289 nm. Inset: Structure of 6–7 and 9–10 BrPC lipids.

bromine atoms. Crystallographic data of $G_{t\alpha}$ [31] show that one of the $G_{t\alpha}$ tryptophans, W127, is more externally located compared to W207. It is observed that 6–7 BrPC quenches the tryptophan fluorescence of $G_{t\alpha}$-GDP + AlF_x by only 55% which probably suggests that only one of the two tryptophans either W127 or W207, participates in the quenching process because bromine, being a collisional quencher with a small atomic radius, is able to quench the tryptophan very close to it, and is probably one of the greatest advantages over using energy transfer probes [32]. Furthermore, the fact that the tryptophan fluorescence is even less quenched in 9–10 BrPC suggests that very likely $G_{t\alpha}$ does not penetrate the membrane deeply.

To evaluate the depth of penetration of the tryptophan residues of $G_{t\alpha}$ in the membrane, we have used Perrin's formula modified for a 2D system [33]. Considering a random distribution of fluorophores and quenchers distributed in a 2D plane as on the surface of a membrane, the three-dimensional Perrin's formula:

$$F/F_0 = \exp V[\mathrm{Q}] \tag{1}$$

is modified to:

$$F/F_0 = \exp\left[-2\pi r C_s(1 - F(r)/F_0)\right] dr \tag{2}$$

where F and F_0 is the fluorescence emission intensity of the fluorophore in the presence and absence of the quencher, respectively. V is the elementary volume surrounding the fluorophore, [Q] is the concentration of the quencher. $F(r)$ corresponds to the intensity of the fluorophore when the quencher is located at a distance of r from it [33–36]. C_s is the surface density of the quenchers. Using a 'hard sphere' approximation, i.e., considering a sphere of critical radius R_c with the fluorophore located at its center and assuming that within this sphere any quencher would quench the fluorophore but not outside this critical radius, this is mathematically equivalent to a step function given by:

$$F(r)/F_0 = 0 \text{ for } r \le R_c \tag{3a}$$

and

$$F(r)/F_0 = 1 \text{ for } r \ge R_c \tag{3b}$$

which when simplified yields:

$$F/F_0 = \exp -\left(\pi C_s R_c^2\right). \tag{4}$$

In Eq. 4, the term $\pi C_s R_c^2$ physically represents a circle of radius R_c around each quencher within which a fluorophore is completely quenched but not outside it [33]. Considering the fluorophore and the quencher to be located on separate planes [34], the above equation modifies to:

$$F/F_0 = \exp -\left[\pi C_s\left(R_c^2 - z^2\right)\right] \quad z \ll R_c^2 \tag{5a}$$

$$F/F_0 = 1 \quad z \ge R_c \tag{5b}$$

In Eq. 5a and 5b, z corresponds to the distance of separation between the fluorophore and the quencher assuming that the origin of the measurement to be located at the center of mass of the quencher which seems justified as the quencher is quite small. Extensive work on the tryptophan fluorescence quenching by brominated lipids have established that R_c is approximately 9 Å [18]. Choosing $R_c = 9$ Å we have fitted our experimental data with Eq. 5a and 5b. Excellent fits have been obtained for $z = 7$ Å (Fig. 3A) in case of the 6–7 BrPC's. This means that only one of the tryptophan residues of $G_{t\alpha}$ is located in the lipid membrane at a distance of approximately 7 Å from the quencher. X-ray and electron density strip model calculations have established that the total length of the 6–7 BrPC lipid is approximately 24.5 Å (i.e., 49 Å for the bilayer thickness) and the Br group is located at a distance of 11 Å approximately from the end of the methylene chain [37]. These results imply that the fluorophore may be located at a depth of 6.5 Å from the membrane surface. Identical results may be expected, assuming the tryptophan fluorophore to be well within the lipid layer at a depth of 4 Å from the centre of the bilayer or 20.5 Å from the top of the polar head. These results simply imply that the tryptophan residue of the $G_{t\alpha}$ are indeed incorporated in the bilayer and are located at a depth of at least 6.5 Å from the membrane surface. This value obtained for the depth of penetration of the tryptophan residue of $G_{t\alpha}$ in the membrane is in excellent agreement with those reported for other proteins using quenching experiments performed with 6–7 BrPC [27,32,36]. In this context, it may however be pointed out that although a precise location of the fluoro-

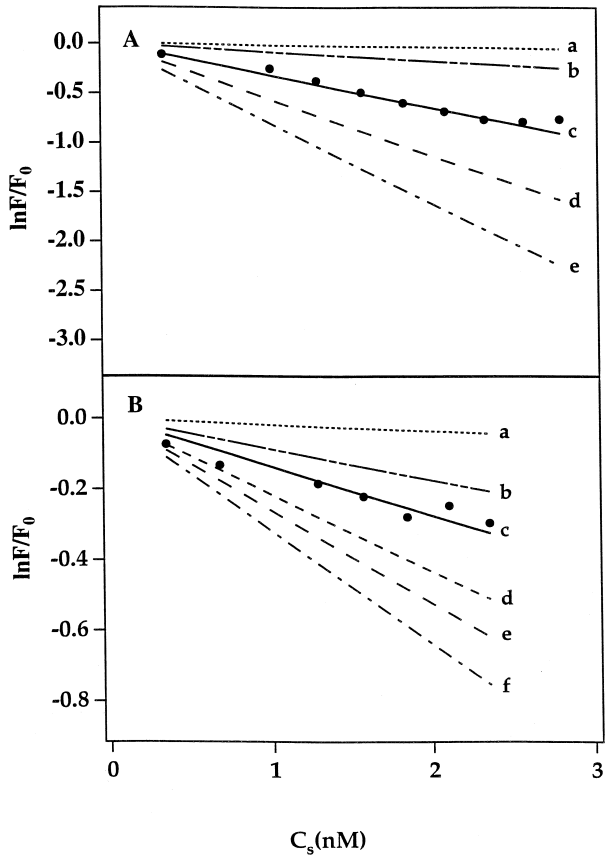

Fig. 3. Estimation of the distance of separation between the fluorophore and quencher. (A) Theoretical fits for the fluorescence quenching curves in 6–7 BrPC vesicles in the presence of $G_{t\alpha}$-GDP + AlF_x: $R_c = 9$ Å and z is (a) 8.9, (b) 8.5, (c) 7.0, (d) 5.0, and (e) 1.0. (B) Theoretical fits for the fluorescence quenching curves in 6–7 BrPC vesicles in presence of $G_{t\alpha}$-GDP: $R_c = 9$ Å while z is (a) 8.9, (b) 8.5, (c) 8.2, (d) 7.7, (e) 7.4, and (f) 7.0.

phores in the membrane is not determinable by the method used, a distribution of the protein molecules located at different depths ranging from 6.5 Å to 20.5 Å measured from the membrane surface also seems plausible.

Electron and X-ray scattering studies have indicated that each CH_2 unit in the lipid is approximately 0.9 Å in length which means that the bromine moieties in 9–10 BrPC is located approximately 2.7 Å below the location of the bromine moiety of 6–7 BrPC that justifies the fact that in 9–10 BrPC's the fluorescence from the tryptophan residues are less quenched compared to that in 6–7 BrPC's and is in good agreement with our data [37]. This conclusion is again based on the assumption that the tryptophan residues of $G_{t\alpha}$-GDP + AlF_x penetrate into the membrane of the 6–7 or 9–10 BrPC's to the same extent that is well supported by X-ray and electron density profile studies that have established these lipid structures to be almost identical [37].

Interestingly, the $G_{t\alpha}$-GDP under identical conditions yielded z equal to 8.2 Å corresponding to $R_c = 9$ Å (Fig. 3B). This value is 1.2 Å larger than that obtained for $G_{t\alpha}$-GDP + AlF_x form. Such a result is not readily explicable. Various possibilities exist. One possibility could be the change in the conformation of the protein [22] that probably makes the tryptophan moiety less accessible to the bromine moieties. The change in the conformation of the protein may allow the other tryptophan residue to participate in the quenching process that gives an impression of a smaller depth of penetration although they may be located at the same depth as its $G_{t\alpha}$-GDP + AlF_x counterpart.

Anisotropy measurements provide an excellent method to determine whether or not a protein is bound to a membrane. Fig. 4A and B show the plot of the anisotropy of $G_{t\alpha}$ versus the concentration of 6–7 BrPC and 9–10 BrPC, respectively. It can be seen that the fluorescence anisotropy of $G_{t\alpha}$ increases with the concentration of brominated lipids. Similar results are obtained with egg PC vesicles (results not shown). However, anisotropy measurements of the protein in the absence of vesicles exhibited a constant value that is independent of protein concentration, that rules out the possibility of protein–protein interaction (Fig. 4C). The large changes observed in Fig. 4A and B in contrast to Fig. 4C indicate strong binding or interaction between brominated lipids and $G_{t\alpha}$-GDP + AlF_x or $G_{t\alpha}$-GDP that are consistent with results reported elsewhere [38,39].

In conclusion, this study reports the depth of penetration of $G_{t\alpha}$-GDP + AlF_x and $G_{t\alpha}$-GDP in lipid membranes prepared from brominated lipids where the bromine moieties are anchored to the lipid at different positions on one of the lipid fatty acyl chain. Using fluorescence quenching of the $G_{t\alpha}$ tryptophan residues, we have assessed the depth of penetration of the $G_{t\alpha}$ in the membrane. Our results clearly demonstrate for the first time that in vitro tryptophan residue of $G_{t\alpha}$-GDP + AlF_x is located at a depth of 6.5 Å from the membrane surface which is consistent with the results and conclusions by Uhl and Ryba [10],

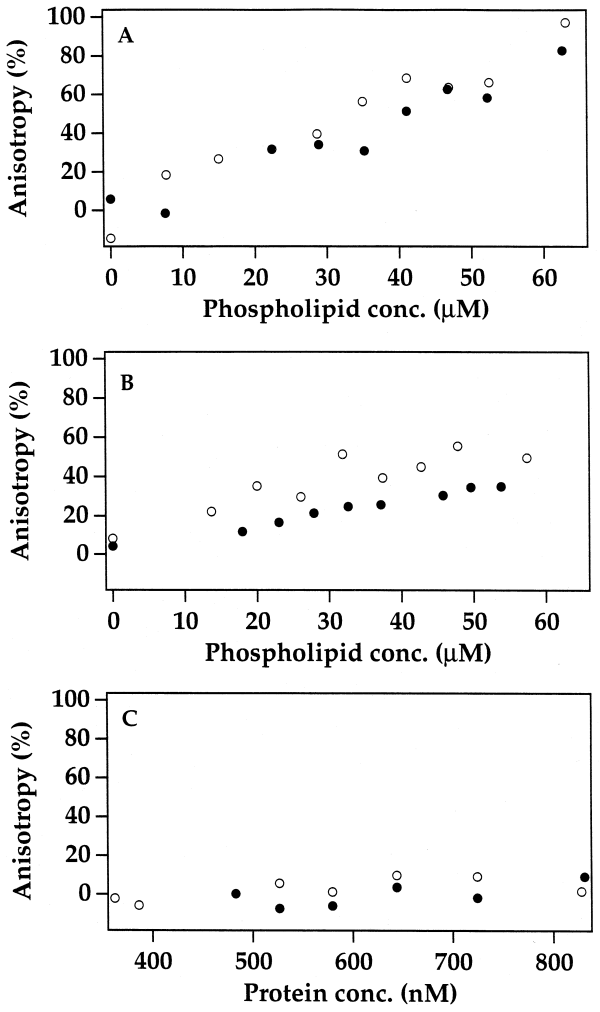

Fig. 4. Anisotropy measurements. Plot of the anisotropy versus the phospholipid concentration. (A) $G_{t\alpha}$-GDP + AlF_x (filled circles) and $G_{t\alpha}$-GDP (open squares) for 6–7 BrPC quenchers. (B) $G_{t\alpha}$-GDP + AlF_x (filled circles) and $G_{t\alpha}$-GDP (open squares) for 9–10 BrPC quenchers. (C) $G_{t\alpha}$-GDP + AlF_x (filled circles) and $G_{t\alpha}$-GDP (open squares) in absence of vesicles.

Liebman and Sitraramayya [13], Liebman et al. [14], and Uhl et al. [17] who have used completely different techniques to show that $G_{t\alpha}$ interacts with the membrane.

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