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A quantitative electrophoretic migration shift assay for analyzing the specific binding of proteins to lipid ligands in vesicles or micelles

Marc Arnold, Philippe Ringler, Alain Brisson *

Laboratoire de Génétique Moléculaire des Eucaryotes, CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique, INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg, France

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Abstract

We present a new assay for analyzing the specific binding of proteins to lipid ligands contained within vesicles or micelles. This assay, referred to as the electrophoretic migration shift assay, was developed using a model system composed of cholera toxin and of its physiological receptor, monosialoganglioside G_{M1}. Using polyacrylamide gel electrophoresis in non-denaturing conditions, the migration of toxin components known to interact with G_{M1} was retarded when G_{M1} was present in either lipid vesicles or micelles. This effect was specific, as the migration of proteins not interacting with G_{M1} was not modified. The localization of retarded proteins and of lipids on gels was further determined by autoradiography. The stoichiometry of binding between cholera toxin and G_{M1} was determined, giving a value of five G_{M1} per one pentameric assembly of cholera toxin B-subunits, in agreement with previous studies. The general applicability of this assay was further established using both streptavidin and annexin V together with specific lipid ligands. This assay is fast, simple, quantitative, and requires only microgram quantities of protein.

Keywords: Protein-lipid binding; Polyacrylamide gel electrophoresis; Lipid vesicle; Micelle; Cholera toxin; Ganglioside; Streptavidin; Annexin

1. Introduction

Many biological processes involve interactions between macromolecules, such as protein–protein, protein–nucleic acid, protein–lipid interactions. This explains the general interest in analytical methods for characterizing the formation of such macromolecular complexes. Our interest in protein–lipid interactions is related to the fields of two-dimensional crystallization of soluble proteins on planar lipid films [1] and of protein structure determination by electron crystallography [2]. Knowledge of protein–lipid binding properties would be very helpful for designing crystallization experiments in a rational manner. Our objective was therefore to develop an assay fulfilling the requirements of simplicity regarding the necessary equipment and of sensitivity due to the general difficulty of protein purification.

Lipid vesicles have been extensively used as membrane model systems for analyzing protein–lipid interactions [3]. Many biochemical and biophysical methods are available for characterizing the binding of proteins to liposomes, such as gel filtration [4], sedimentation [5], fluorescence spectroscopy [6] or microscopy [7], surface pressure measurements [8], electron microscopy [9], neutron scattering techniques [10], etc. Although these methods present valuable individual advantages, none of them fulfills the above requirements.

We present here a simple, sensitive and quantitative method for characterizing protein–lipid interactions based on polyacrylamide gel electrophoresis (PAGE) in non-denaturing conditions. We used the system composed of cholera toxin and of monosialoganglioside G_{M1} as a model system, due to our interest in the structure of this complex [11]. Cholera toxin, a protein secreted by the bacterium *Vibrio cholerae*, is composed of two subunits, A ($M_r \sim 27\,000$) and B ($M_r \sim 11\,600$), assembled with the stoichiometry AB_5 [12,13]. The B_5 pentameric assembly is responsible for binding of the toxin to its cellular receptor

Abbreviations: PAGE, polyacrylamide gel electrophoresis; G_{M1}, monosialoganglioside; CHAPS, (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate); DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; DODA-EO_2-biotin, dioctadecylamine-ethyloxy2-biotin; NEM, *N*-ethylmaleimide; SDS, sodium dodecylsulfate; DTT, dithiothreitol; c.m.c., critical micellar concentration.

* Corresponding author. Fax: +33 88 370148.

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of lipidic nature, the monosialoganglioside G_{M1}. The A-subunit is made of two fragments A1 ($M_r \sim 22000$) and A2 ($M_r \sim 5000$) linked through a disulfide bond (review in [14]). The A1 fragment catalyzes the ADP-ribosylation of the $G_{s\alpha}$ regulatory component of the adenylate cyclase complex, which effect ultimately results in an extensive fluid loss characteristic of cholera disease (review in [15]).

The assay presented here, referred to as the electrophoretic migration shift assay, is likely to be of general interest in domains involving protein–lipid interactions, as well as for other applications of liposomes, such as in cell targeting [16] or for designing biosensors [17].

2. Materials and methods

2.1. Chemicals

G_{M1}, n-octyl β-D-glucopyranoside, (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate) (CHAPS), sodium cholate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) were from Avanti-Polar Lipids (Alabaster, AL, USA). Dioctadecylamine-ethyloxy2-biotin (DODA-EO_2-biotin) [18] was a gift from Prof. Ringsdorf (Mainz, Germany). The purity of G_{M1} and phospholipid solutions was checked and all lipids were used without further purification. *N*-[1-^{14}C]Ethylmaleimide (^{14}C-NEM) was from NEN (Wilmington, DE, USA) and 1,2-di[1-^{14}C]oleoylphosphatidylcholine (^{14}C-DOPC) was from Amersham (Amersham, UK). Acrylamide, bis-acrylamide and glycine were of electrophoresis purity grade and other chemicals were the best available commercial grade.

2.2. Protein solutions

Cholera toxin AB_5 and B_5 pentamer were purchased from either Sigma or List Biological Laboratories (Campbell, CA, USA). Lyophilized products were reconstituted at 2 mg protein/ml for AB_5 and 1 mg protein/ml for B_5, in a final buffer made of 200 mM NaCl, 1 mM EDTA, 3 mM NaN_3, 50 mM Tris (pH 7.5).

For labeling of the free thiol group of $A2B_5$ with ^{14}C-NEM, cholera toxin was first reduced to $A2B_5$ and A1 with dithiothreitol (DTT) [12,13] under optimized conditions (Arnold, M., Brisson, A., unpublished data). Briefly, four volumes of AB_5 at 2 mg/ml were incubated with one volume of a freshly prepared 2.4 M DTT solution in distilled water at room temperature for 10 min. Reduced cholera toxin was then incubated at 37°C for 30 min, a treatment which is known to induce the aggregation of A1 [12,19]. A1 was discarded by centrifugation at $134000 \times g$ for 15 min in a Beckman airfuge (Palo Alto, CA, USA). DTT was subsequently eliminated by gel filtration chromatography using G10-Sephadex columns (Pharmacia, Uppsala, Sweden). Fractions of the void volume containing $A2B_5$ were pooled and ^{14}C-NEM was added at a 10-fold molar excess for 30 min. ^{14}C-labeled $A2B_5$ was separated from excess ^{14}C-NEM by another step of filtration.

Streptavidin was purchased from Pierce Europe (Amsterdam, The Netherlands). Annexin V was a gift from Dr. Freyssinet (Strasbourg, France) [10].

2.3. Preparation of lipid vesicles

Large unilamellar phospholipid vesicles were prepared by detergent dialysis [20] as follows. One ml of a 2 mg G_{M1}/ml chloroform-methanol (2:1, v/v) solution was mixed with 250 μl of 40 mg DOPC/ml chloroform and 300 μl of 200 mg n-octyl β-D-glucopyranoside/ml chloroform, and solvents were evaporated under reduced pressure in a rotary evaporator. 1 ml of a buffer made of 100 mM NaCl, 1 mM EDTA, 3 mM NaN_3, 20 mM sodium phosphate, pH 6.5, was added, the mixture was stirred and allowed to stand at room temperature for 1 h. The solution was then poured into Spectra/Por No. 1 dialysis tubing (Medicell, London, UK) and was dialyzed against the same buffer at room temperature. Three bath changes were performed over two days. Concentrations of both G_{M1} and DOPC were verified after dialysis to ensure that no lipid loss had occurred. Lipid vesicle stock solutions containing 1.3 mg G_{M1} and 6.7 mg DOPC /ml buffer were used throughout this study and are thereafter referred to as lipid vesicle solution L. Lipid vesicles were stored at 4°C until use.

A similar procedure was used for preparing both DOPC/DOPS vesicles (molar ratio: 10:1) in a buffer made of 150 mM NaCl, 3 mM NaN_3, 10 mM Hepes, pH 7.4, and DOPC/DODA-EO_2-biotin vesicles (molar ratio: 9:1) in 100 mM NaCl, 1 mM EDTA, 3 mM NaN_3, 20 mM sodium phosphate, pH 6.5.

For autoradiography experiments, 1 μl of a 0.75 μCi/μl solution of ^{14}C-DOPC was added to one ml of lipid vesicle solution L, in order that several thousand cpm were ultimately deposited on gels.

2.4. Preparation of lipid micelles

Lipid micelles [21] containing G_{M1} were prepared by either one of the following methods: (a) G_{M1}/CHAPS micelles were obtained by evaporating an aliquot of a G_{M1} solution (2 mg G_{M1}/ml in chloroform-methanol) which was solubilized at a final G_{M1} concentration of 0.5 mM by addition of an aqueous solution of 1% CHAPS (the critical micellar concentration (c.m.c.) of CHAPS is about 0.5% [22]); (b) G_{M1}/DOPC/CHAPS micelles were obtained by mixing one volume of lipid vesicle solution L with one volume of 50% CHAPS; (c) proteins were preincubated with lipid vesicle solution L, then solubilization was performed by adding a volume of 50% CHAPS equal to the

volume of lipid vesicle solution L. The solubilization of lipids was instantaneous in all three methods.

Similar procedures were used with cholate (c.m.c.: 0.36%) and Triton X-100 (c.m.c.: 0.02%).

2.5. Incubation of proteins with lipid solutions

Incubation of proteins with lipid vesicle or micelle solutions was carried out at room temperature for 10 min. The total lipid-protein mixture was then deposited on the gel.

2.6. PAGE

PAGE was performed in 0.8 mm thick slab gels. No stacking gels were used unless otherwise stated. For experiments with lipid vesicles, separating gels contained 10% acrylamide and 0.05% bis-acrylamide in 400 mM Tris (pH 8.8). Before deposition on the gel, the samples (25 μl maximal volume) were mixed with 5 μl of a solution containing Bromophenol blue in 30% glycerol. The migration buffer was 25 mM Tris, 192 mM glycine (pH 8.3).

For experiments with micelles, buffers used for the preparation of separating gels and for migration were supplemented with either CHAPS, cholate or Triton X-100 at detergent concentrations twice their respective c.m.c.

Gels were stained with Coomassie brilliant blue according to standard procedures.

2.7. Cryo-electron microscopy

Lipid vesicles were observed in the frozen-hydrated state by cryo-electron microscopy [23]. A drop (5 μl) of a lipid vesicle solution at 0.1 to 0.2 mg phospholipid/ml was adsorbed onto carbon-coated grids rendered hydrophilic by glow discharge in air under reduced pressure. The grids were quickly plunged into liquid propane or ethane, and then observed at about -170°C using a Gatan-626 (Warrendale, PA, USA) cryotransfer specimen holder. Electron microscopy was performed with a Philips CM12 operating at 100 kV under low electron dose conditions. Images were recorded on Kodak SO163 films.

3. Results

3.1. Binding of cholera toxin and derived molecular species to G_{M1}-containing lipid vesicles

Cholera toxin AB_5, B_5 pentamer, and the two molecular species, $A2B_5$ and A1, formed upon reduction of AB_5, migrated as single bands when analyzed after PAGE in non-denaturing conditions (Fig. 1, lanes 1,3,5), as previously described [19]. These bands were broad and sometimes mutiple, which is likely to reflect a charge heterogeneity within protein species [24], as migration in non-denaturing conditions is mainly dependent on charge characteristics.

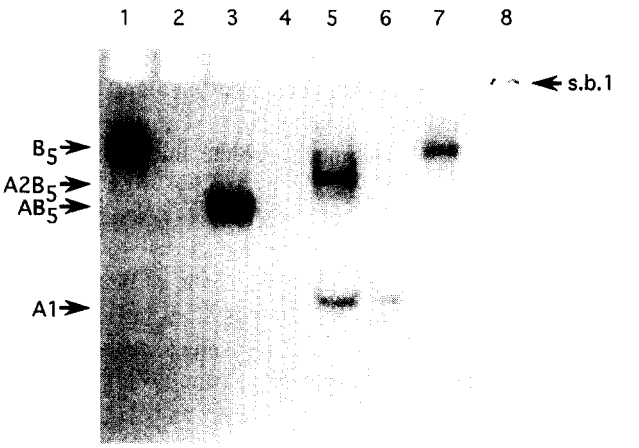

Fig. 1. Electrophoretic migration shift assay of the binding of cholera toxin components to G_{M1}-containing lipid vesicles. (1) 5 μg B_5 (86 pmol); (2) 5 μg B_5 mixed with 5 μl lipid vesicle solution L (4.3 nmol G_{M1}); (3) 6 μg AB_5; (4) 6 μg AB_5 mixed with 5 μl solution L; (5) 8 μg AB_5 in the presence of 0.47 M β-mercaptoethanol; (6) same sample as in (5) mixed with 6 μl solution L; (7) 5 μg B_5 mixed with DODA-EO_2-biotin/DOPC vesicles (37 nmol total lipid); (8) same as (2) after drying the gel. s.b. 1 stands for shifted band-1.

When solutions of either B_5, AB_5, or reduced AB_5 were incubated with vesicles made of G_{M1} and DOPC (molar ratio: 1:10) and the mixtures analyzed by PAGE in non-denaturing conditions, the bands corresponding to B_5-containing species entirely disappeared (Fig. 1, lanes 2,4,6). A faintly stained band was observed at the uppermost level of the gel after drying the gel (Fig. 1, lane 8). This band is thereafter referred to as shifted band-1. On the other hand, the migration of A1 (Fig. 1, lane 6) and of proteins non-related to cholera toxin (data not shown) was not affected by the presence of G_{M1}-containing lipid vesicles. Furthermore, addition of vesicles devoid of G_{M1} did not affect the migration of either B_5 (Fig. 1, lane 7) or AB_5 (data not shown).

3.2. Binding of cholera toxin and derived molecular species to G_{M1}-containing micelles

When AB_5 was mixed with micelles prepared by solubilizing G_{M1}/DOPC vesicles with CHAPS (method b in Materials and methods) and the mixture analyzed by non-denaturing PAGE in the presence of CHAPS, the band corresponding to AB_5 was totally absent, while a new band was observed near the top of the gel (Fig. 2, lane 2). This band is referred to as shifted band-2. The same result was observed upon mixing AB_5 with micelles made of G_{M1} and CHAPS (Fig. 2, lane 3) and when G_{M1}/DOPC vesicles were preincubated with AB_5 before solubilization with CHAPS (Fig. 2, lane 4). Similar features were observed with B5 (data not shown) and with $A2B_5$ (Fig. 2, lane 6). On the other hand, the migration of A1 (Fig. 2,

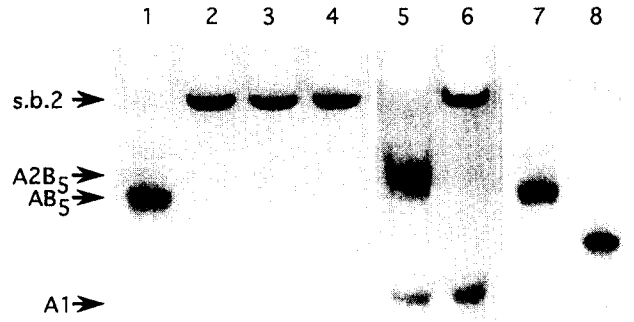

Fig. 2. Electrophoretic migration shift assay of the binding of cholera toxin to G_{M1}-containing micelles. (1) 6 μg AB_5; (2) 6 μg AB_5 mixed with micelles of G_{M1} /DOPC/CHAPS (2.15 nmol G_{M1}); (3) 6 μg AB_5 mixed with G_{M1} /CHAPS micelles (1.5 nmol G_{M1}); (4) 6 μg AB_5 incubated with 2.5 μl lipid vesicle solution L and then solubilized with CHAPS at a 17.5% final concentration; (5) 8 μg AB_5 in the presence of 0.47 M β-mercaptoethanol; (6) same as (5) mixed with G_{M1} /CHAPS micelles (1.5 nmol G_{M1}); (7) 6 μg AB_5 mixed with micelles of DODA-EO_2-biotin/DOPC/CHAPS (18 nmol total lipid); (8) 6 μg streptavidin mixed with G_{M1} /CHAPS micelles. s.b. 2 stands for shifted band-2.

lane 6) and of other proteins which do not bind to G_{M1}, such as streptavidin (Fig. 2, lane 8), was not affected by the presence of G_{M1}-containing micelles. In addition, the presence of lipid micelles devoid of G_{M1} did not affect the migration of AB_5 (Fig. 2, lane 7) or of other B_5-containing species (data not shown).

We observed identical patterns when Triton X-100 (see Fig. 4b) or cholate (data not shown) were used as detergents instead of CHAPS.

3.3. Characterization of the shifted bands by autoradiography

The positions of phospholipids and of G_{M1}-binding proteins on gels were further analyzed by autoradiography, using both ^{14}C-labeled DOPC and $A2B_5$ labeled with ^{14}C-NEM on a single cysteine residue of the A2 fragment. When ^{14}C-labeled $A2B_5$ was mixed with G_{M1}-containing vesicles, all the radioactivity was found at the uppermost level of the gel, overlapping the faintly stained shifted band-1 (Fig. 3a, lanes 2,2*). When reduced AB_5 was mixed with G_{M1}/DOPC vesicles containing trace amounts of ^{14}C-DOPC, all the radioactivity was also found at the level of shifted band-1 (Fig. 3a, lane 4*). A similar pattern was observed with pure lipid vesicles in the absence of toxin (Fig. 3a, lane 5*), indicating that lipid vesicles do not penetrate the gel.

When ^{14}C-labeled $A2B_5$ was mixed with G_{M1}-containing micelles, all the radioactivity was found at the top of the gel, overlapping the shifted band-2 (Fig. 3b, lanes 2,2*). When AB_5 was mixed with G_{M1}/DOPC micelles containing ^{14}C-DOPC, all the radioactivity was found at the uppermost level of the gel, above the Coomassie stained shifted band-2 (Fig. 3b, lanes 4,4*). A similar pattern was observed with G_{M1}/DOPC/CHAPS micelles in the absence of toxin (Fig. 3b, lane 5*), and also when cholate or Triton X-100 were used as detergents instead of CHAPS (data not shown).

3.4. Quantitative analysis

Titration experiments were performed by incubating a fixed amount of B_5-containing molecular species in the presence of varying amounts of G_{M1} in lipid vesicles or micelles. A typical example is presented in Fig. 4, in

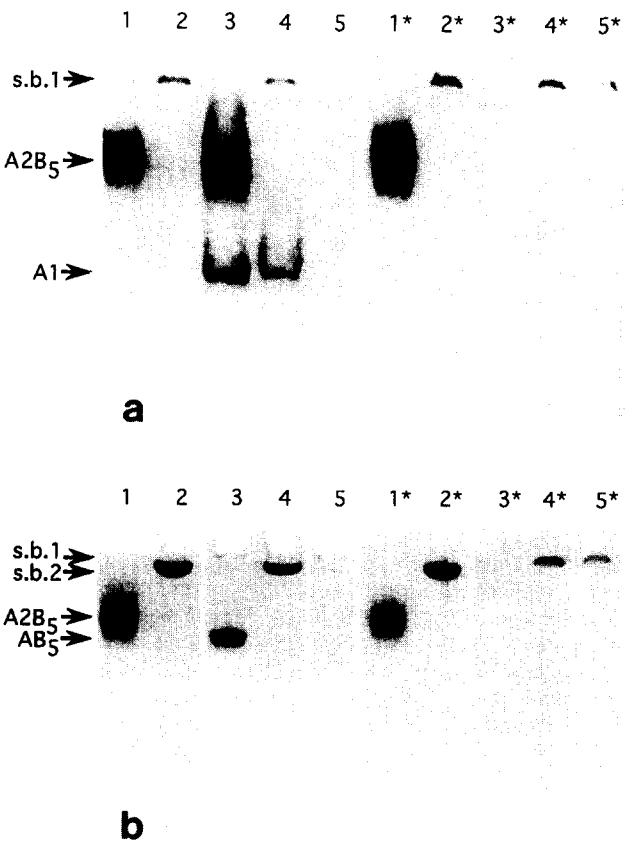

Fig. 3. Autoradiography analysis of shifted bands using lipid vesicles (a) and micelles (b). (a) (1) 7 μg ^{14}C-labeled $A2B_5$; (2) same sample mixed with 5 μl lipid vesicle solution L; the shifted band-1 is clearly visible on this dried gel; (3) 20 μg AB_5 in the presence of 0.23 M β-mercaptoethanol; (4) same sample as in (3) mixed with 5 μl solution L containing trace amounts of ^{14}C-DOPC; (5) 5 μl solution L containing ^{14}C-DOPC. (1*), (2*), (3*), (4*) and (5*) are the corresponding autoradiograms. (b) (1) 7 μg ^{14}C-labeled $A2B_5$; (2) same sample mixed with 5 μl G_{M1} /CHAPS micelles (2.5 nmol G_{M1}); (3) 6 μg AB_5; (4) 6 μg AB_5 mixed with micelles of G_{M1} /DOPC/CHAPS containing ^{14}C-DOPC (2.15 nmol G_{M1}); (5) micelles of G_{M1} /DOPC/CHAPS containing ^{14}C-DOPC. (1*), (2*), (3*), (4*) and (5*) are the corresponding autoradiograms.

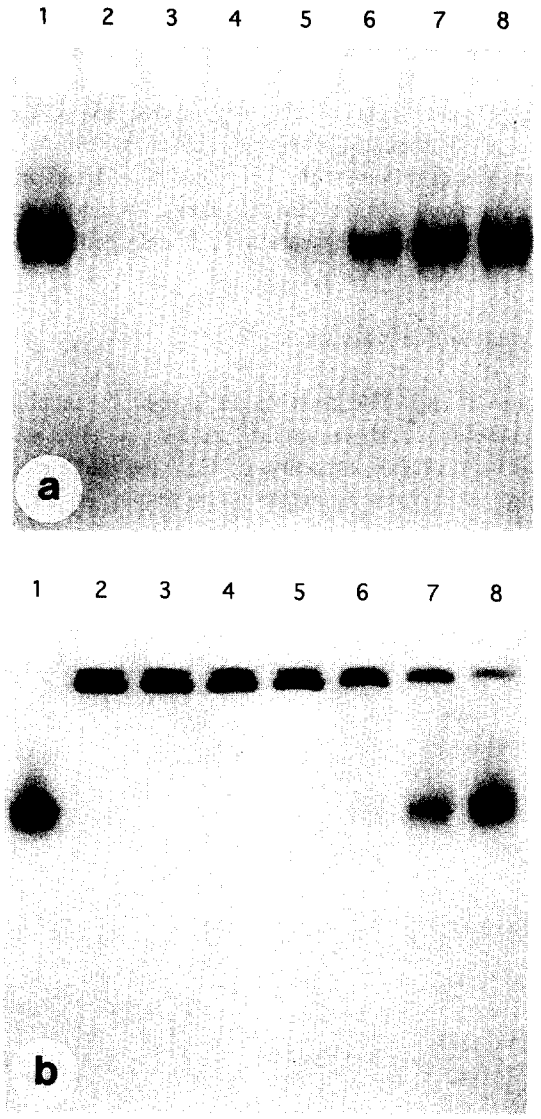

Fig. 4. Titration of cholera toxin binding to G_{M1} incorporated into lipid vesicles (a) and micelles (b). 12 μg of AB_5 (140 pmol) were mixed with varying amounts of G_{M1} /DOPC vesicles. After 10 min incubation, each sample was divided into two equal volumes. One volume was analyzed directly by PAGE (a). The other was mixed with Triton X-100 at a final concentration of 0.66%, further incubated for 10 min, and finally analyzed by PAGE in the presence of 0.04% Triton X-100 (b). The amounts of G_{M1} are, from lane 1 to 8: 0; 5760; 2880; 1440; 720; 360; 180; 90 pmol.

which the same solutions made of AB_5 and G_{M1}/DOPC vesicles are examined in the vesicular state (Fig. 4a) and in the micellar state after vesicle solubilization with Triton X-100 (Fig. 4b). The AB_5 band was completely shifted by lipid vesicle suspensions containing a total amount of G_{M1} larger than 10 G_{M1} per AB_5 (Fig. 4a, lanes 2–4). Almost no shift was observed when the amount of G_{M1} was smaller than 0.6 G_{M1} per AB_5 (Fig. 4a, lane 8). A mixed pattern was visible for intermediate conditions (Fig. 4a, lanes 5–7).

After solubilization of the lipid vesicle-protein mixture with Triton X-100, a similar pattern was observed, except that the AB_5 band was completely shifted when the total amount of G_{M1} was larger than 5 G_{M1} per AB_5 (Fig. 4b, lane 5).

A stoichiometric ratio $[G_{M1}]_{acc}/[AB_5]$, defined as the ratio of the concentration of accessible G_{M1}, $[G_{M1}]_{acc}$, to the concentration of AB_5, was deduced from these experiments. We verified by cryo-electron microscopy [23] that the lipid vesicles used in this study were unilamellar (Fig. 5). Therefore, $[G_{M1}]_{acc}$ in lipid vesicle solutions was taken as half the total concentration of G_{M1} in the sample. For micellar solutions, $[G_{M1}]_{acc}$ was taken as the total concentration of G_{M1} in the sample. The values of $[G_{M1}]_{acc}/[AB_5]$ obtained are 5 in both conditions. These values are in agreement with the accepted idea that five G_{M1} molecules bind to one B_5 oligomer [25], and provide further evidence that the vesicles were indeed unilamellar.

3.5. Application of the electrophoretic migration shift assay to two other protein-lipid complexes: streptavidin, annexin V

We further investigated the use of this assay with two other proteins, streptavidin and annexin V, known to bind specifically to biotinylated lipids and DOPS, respectively [7,26]. Qualitative results are presented in Fig. 6. In the presence of lipid vesicles containing biotinylated lipids, the streptavidin band was totally absent, with no significantly visible shifted band (Fig. 6a, lane 2). In the presence of biotinylated lipids in the micellar state, the streptavidin band was retarded and located near the top of the gel (Fig. 6b, lane 4).

Similarly, the annexin V band disappeared in the pres-

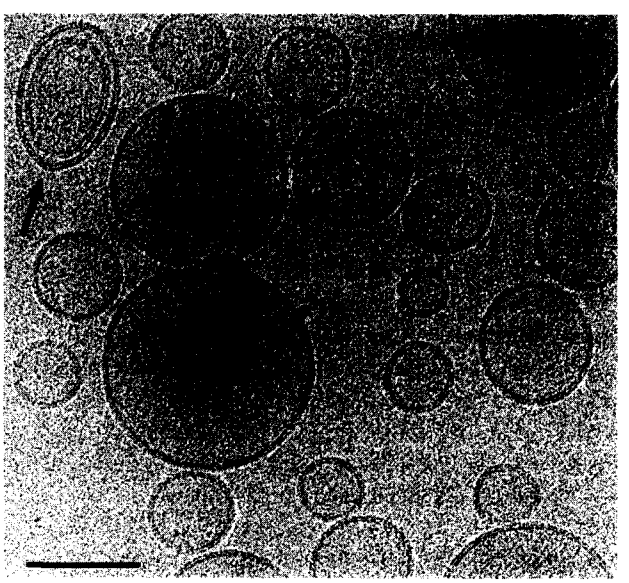

Fig. 5. Electron micrograph of frozen-hydrated lipid vesicles. Most of the lipid vesicles (> 90%) are unilamellar. One bilamellar vesicle is observed (arrow), constituted of one small vesicle enclosed within a larger, outer vesicle. Scale bar represents 100 nm.

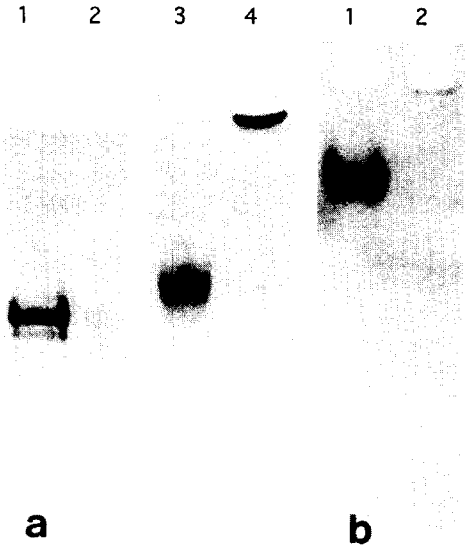

Fig. 6. Electrophoretic migration shift assay applied to streptavidin (a) and annexin V (b). (a) (1) 6 μg streptavidin (100 pmol); (2) 6 μg streptavidin mixed with DODA-EO_2-biotin/DOPC vesicles (3 nmol biotinylated lipid); a stacking gel was present in this experiment; (3) same sample as in (1) on a gel containing 1% CHAPS; (4) 6 μg streptavidin mixed with DODA-EO_2-biotin/DOPC/CHAPS micelles (1.5 nmol biotinylated lipid). (b) (1) 5 μg annexin V (140 pmol); (2) 5 μg annexin V mixed with DOPS/DOPC vesicles (12 nmol DOPS); 100 mM Ca^{2+} was added in the gel and in the electrophoresis buffer.

ence of DOPS-containing vesicles (Fig. 6b, lane 2). This only occurred when Ca^{2+} ions were present in the gel and in the elution buffer, in agreement with the fact that annexin V binding to liposomes is strictly dependent on the presence of Ca^{2+} ions [26]. After solubilization of the lipid vesicles, no shift in annexin V migration was noticed (data not shown), as expected because dispersion of the vesicles with detergents induces a complete dissociation of annexin V-DOPS-Ca^{2+} ternary complexes [27].

4. Discussion

We present here a novel assay enabling the analysis of the specific binding of proteins to lipid vesicles and lipid micelles by means of standard PAGE techniques in non-denaturing conditions. Using cholera toxin as a model system, we show that the molecular species carrying a B_5-moiety, which contains the binding sites for G_{M1} [14], are shifted in the presence of G_{M1} molecules contained either in vesicles or in micelles. This effect is highly specific, as the migration of molecular species not associated with B_5, such as the A1 fragment formed after reduction of AB_5, is not modified by the presence of G_{M1}, and the migration of B_5 is neither affected by lipid vesicles or micelles devoid of G_{M1}. This assay is simply based on a change of migration properties of a protein induced by its affinity binding to a lipidic ligand. The present assay is therefore analogous in its principle to gel retardation assays which are commonly used for studying interactions between nucleic acids and proteins [28,29].

A simple interpretation of our results obtained with lipid vesicles is that the vesicles are intact and too large to enter the gel, and that the proteins which are specifically bound to their lipid ligands remain associated with the vesicles during electrophoresis. The fact that shifted band-1 is only detected after drying is certainly due to a restricted stain accessibility within the compact protein-vesicle deposit or to a loss of material in staining baths. Drying is responsible for a concentration of the stain within a thin band and thus of a strengthening of the signal. With micelles, shifted bands-2 penetrate the gel and present a typical staining aspect. The fact that, after non-denaturing PAGE, B_5, AB_5, $A2B_5$ and streptavidin are well separated and present migration properties non directly related to their respective mass indicates that the charge of the protein plays a major role in these conditions. On the other hand, the fact that these proteins migrate to identical positions when complexed to their lipid ligands suggests that shifted bands-2 correspond to protein-lipid complexes in a micellar state rather than free protein-ligand complexes.

The assay uses standard laboratory equipment, is fast and highly sensitive. Its sensitivity is that of PAGE for proteins, namely in the microgram range when combined with Coomassie brilliant blue staining. Identical results were obtained with 0.1 μg protein when silver staining was used to reveal proteins (data not shown). One major advantage of this assay is that the complete protein-lipid mixture can be analyzed by simple deposition on the gel, thus allowing characterization of both bound and free components. A preliminary step of separation between bound and free protein is not required, which frequently results in sample dilution and/or sample loss by adsorption, particularly noticeable with protein amounts in the microgram range. For comparison, analyzing protein binding to lipid vesicles by gel filtration requires about 10 μg of protein. In addition, dilution and loss of material render difficult a quantitative analysis of protein-liposome binding. Sedimentation experiments require a similar amount of material, and face the variable propensity of lipid vesicles to sediment, which is frequently incomplete with large unilamellar vesicles. Biophysical methods, such as fluorescence techniques [27] or monomolecular film techniques [30] require even larger amounts of protein. Electron microscopy constitutes an exception as it is less demanding in material, but the results are difficult to interpret on an overall quantitative basis.

The present assay is quantitative, as demonstrated by the determination of the binding stoichiometry between cholera toxin and G_{M1}, leading to a value strongly supported by previous studies [25].

The general applicability of this assay is attested by our results with streptavidin, and particularly with annexin V

which only binds to phosphatidylserine in the presence of Ca^{2+} ions [31]. The fact that it is performed in non-denaturing conditions constitutes a significant advantage, as most proteins submitted to non-denaturing PAGE provide well-defined bands. One common parameter of the three protein-lipid systems analyzed here is the high affinity of the protein–lipid interactions, all characterized by association constants larger than 10^9 M^{-1} [14,32,33]. However, it is interesting to note that the presence of large lipid quantities does not induce artefacts of gel electrophoresis, even up to 100 μg deposited per slot (data not shown), which could enable the analysis of protein–lipid interactions of lower affinity.

We postulate that this assay will be useful not only for analyzing interactions between proteins and lipids, but more generally for analyzing interactions between membranes or large particles and soluble ligands.

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