Interaction of a chimeric protein based on the ectodomains of E1 and E2 hepatitis C virus envelope glycoproteins with phospholipid vesicles

Daniel Tello[a] ‡, Mar Rodríguez-Rodríguez [a §], Sara Ortega [a], Laura Lombana [a], Belén Yélamos[a], Julián Gómez-Gutiérrez[a], Darrell L. Peterson[b], and Francisco Gavilanes[a, *]

[a]Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, Madrid 28040 and [b]Department of Biochemistry and Molecular Biology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, 23298

* Corresponding author: F. Gavilanes, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain. Phone: (34) 91 394 42 66. Fax: (34) 91 394 41 59. E-mail: pacog@bbm1.ucm.es

‡ Present address: Hospital Universitario Santa Cristina, C/ Maestro Amadeo Vives, 2 y 3, 28009 Madrid

§ Present address: Hospital Universitario Ramón y Cajal, Ctra. De Colmenar Viejo Km 9,100, 28034 Madrid

Running Title: Fusogenic properties of HCV envelope proteins

Abbreviations

The abbreviations used were: HCV, hepatitis C virus; $E1_{340}E2_{661,}$ chimeric protein containing the ectodomains of E1 and E2 HCV envelope proteins; dimyristoylphosphatidylcholine (DMPC); dimyristoylphosphatidylglycerol (DMPG); egg phosphatidylcholine (PC); egg yolk phosphatidylglycerol (PG); bovine brain phosphatidylserine (PS); N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidyl-ethanolamine (NBD-PE); N-(lissamine rhodamine B sulphonyl) diacylphosphatidyl-ethanolamine (Rh-PE); 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS); *p*-xylenebis-(pyridinium) bromide (DPX); 4,4'-bis(1-anilinonaphtalene 8-sulfonate (bis-ANS). 1,6-diphenyl-1,3,5-hexatriene (DPH).

Keywords: hepatitis C virus, viral envelope proteins, lipid-protein interaction, membrane fusion, protein spectroscopic properties

Abstract

We have used an isolated chimeric protein $E1_{340}E2_{661}$ that includes the ectodomains of the envelope proteins of hepatitis C virus to study its interaction with model membranes. $E1_{340}E2_{661}$ has some of the membrane destabilization properties, vesicle aggregation, lipid mixing and the release of internal aqueous content, which has been previously ascribed to fusion proteins. The effects are preferentially produced on vesicles of acidic phospholipids which would indicate the importance of the electrostatic interactions. In fact, an increase of the ionic strength of the buffer induced a considerable decrease of the destabilizing properties. On the other hand, fluorescence polarization studies show that the recombinant protein reduces the amplitude of the thermal transition of DMPG vesicles and increases the transition temperature at pH 5.0 in a dose-dependent manner. These data would indicate an electrostatic interaction between the phospholipid polar head and the protein which eventually would lead to the insertion of $E1_{340}E2_{661}$ into the bilayer. On the other hand, a decrease of the pH induces a conformational change in the protein structure as evidenced by fluorescence of tryptophan residues and 4,4'-bis(1-anilinonaphtalene 8-sulfonate. A model for the fusion of HCV with the host cell membrane can be postulated. The dissociation of E1E2 dimers would uncover the fusion peptides which can then interact with the polar lipid heads of the outer leaflet of the lipid bilayer and next insert into the hydrophobic moiety producing the destabilization of the bilayer which finally leads to fusion.

Introduction

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family [1, 2]. HCV is the major cause of acute hepatitis and chronic liver disease, including cirrhosis and hepatocellular carcinoma, being the leading cause of liver transplantations in the developed world. According to the World Health Organization, nowadays the HCV infects 180 million people worldwide, and every year 3 to 4 million of new cases emerge. Currently there is no vaccine available against HCV and the present therapy consists of α–interferon and ribavirin. However, only 10-20% of patients respond to interferon treatment and 54-56% to the combined therapy [3].

The HCV envelope glycoproteins, E1 (gp31) and E2 (gp70), are released from the polyprotein coded for by the HCV genome after cleavage by host-cell endoplasmic reticulum proteases in positions 383/384 and 746/747 of the sequence, respectively [4]. The amino acid sequence analysis of E1 and E2 indicates that they are type-I transmembrane proteins, highly glycosylated, with an N-terminal ectodomain and a C-terminal hydrophobic domain anchoring these glycoproteins to the membrane. *In vitro* expression studies have shown that both glycoproteins associate to form a stable non-covalently linked heterodimer which accumulates in the endoplasmic reticulum that has been proposed as the site for HCV assembly and budding [5]. However, recent studies carried out using cell-cultured HCV (HCVcc) show that the envelope glycoproteins E1 and E2 form large covalent complexes stabilized by disulfide bridges in the virion [6].

Several molecules expressed on the cell surface have been implicated in HCV cell attachment. Thus, HCV has been shown to interact with the tetraspanin CD81 and the scavenger receptor class B type I (SR-BI). Also, claudins (claudin-1, claudin-6 and claudin-9) and occludin, proteins that are present in the tight junctions of the cells, have also been identified as key proteins in the HCV infection [7-9]. Other proposed co-factors

involved in the endocytosis of HCV are LDL receptor [10], C-type lectins DC-SIGN and L-SIGN [10, 11], glicosaminoglycans (GAGs) [12], and it has also been observed that the Ewi-2wint protein inhibits HCV infection by blocking the interaction of CD81 with the envelope glycoproteins of the virus [13].

Besides their role in cellular receptor binding, the viral envelope proteins must induce fusion between the viral and host cell membranes. The difficulty of propagating HCV in cell culture has hampered for many years functional studies on HCV infection. The cellular mechanism of HCV entry has been studied using HCV pseudoparticles (HCVpp), infectious retroviral particles with HCV envelope proteins on the surface, and the cell culture model which allows for the production and propagation of virus in cell culture (HCVcc) [14-16]. Reports using both HCVpp and HCVcc models have evidenced the pH sensitivity of HCV entry [17-19]. Several studies suggest that HCV enters cells by clathrin-mediated endocytosis [17] and that fusion occurs in the early endosomes [20]. Furthermore, the acidic pH of endosomes triggers the fusion process probably by inducing conformational changes in the envelope proteins [17, 20-22].

Studies concerning the entry mechanism carried out with HCV isolated envelope proteins are scarce. Based on potential structural homology, E2 has been proposed as a Class II fusion protein [23]. Moreover, E1 has also been proposed as a candidate because sequence analyses suggest that it might contain a putative fusion peptide in its ectodomain [24, 25]. In relation to the location of the regions involved in the fusion process, some E1 and E2 peptides with a potential fusogenic activity have been identified [26, 27]. Although peptides might be a useful model to study the interaction of E2 with cellular membranes, a direct correlation with the fusion process *in vivo* is not always possible because of the lack of properties such as oligomerization or conformational changes. A directed mutagenesis study with HVCpp system has identified three important regions for the fusion with model vesicles [28]. The first one is located in E1 (residues

272 to 287) and the other two region are located in E2 (residues 419 to 433 and 597 to 620). These data suggest that different E1 and E2 regions can cooperate for the fusion process.

In order to shed light into the cell entry mechanism as well as into the protein and amino acid sequences involved on it, we have used a chimeric protein containing the E1 and the E2 ectodomains connected by a small hydrophilic peptide ($E1_{340}E2_{661}$) and study its interaction with phospholipid vesicles. This chimeric protein obtained by baculovirus expression system has been previously shown to have the features of a correctly folded protein and it is recognized by HCV patient sera antibodies [29]. $E1_{340}E2_{661}$ is able to insert into the hydrophobic core of the bilayer inducing aggregation, lipid mixing and destabilization of the bilayers, which are the essential steps required for fusion [30]. Based on the results obtained, a model for the fusion activity of this chimeric protein is proposed.

Results

$E1_{340}E2_{661}$ is able to induce destabilization of phospholipid vesicles

Vesicle aggregation

The chimeric protein used throughout this study is composed of the two ectodomains of E1 and E2 HCV envelope glycoproteins (Fig. 1). This protein is produced in glycosylated form by insect cells infected by recombinant baculovirus, The recombinant protein has an oligomeric nature and is composed mainly of monomers, dimers and trimers [29]. Its ability to induce vesicle aggregation of PG, PS or PC liposomes was monitored by measuring the increment of the optical density at 360 nm (ΔOD_{360}) as a result of the increase in vesicle size upon incubation with different concentrations of $E1_{340}E2_{661}$. The results obtained for the different phospholipids vesicles at various phospholipid/protein ratios at pH 5.0 and 100 mM NaCl are depicted in Fig. 2. In the presence of both PS and PG vesicles, the ΔOD_{360} value increased up to a phospholipid/protein ratio of 0.018, 2.5 μM protein concentration, and then remained nearly constant. However, $E1_{340}E2_{661}$ induced little aggregation of PC vesicles. Therefore, the specificity toward negatively charged phospholipids points to the importance of the electrostatic component on the interaction which is on the other hand not dependent on the nature of the acidic polar head group. In fact when the experiment was performed at high salt concentration, 500 mM, a reduction of almost 60% of the ΔOD_{360} values reached at 100 mM was observed. Moreover, at neutral pH the changes in the OD_{360} observed with either phospholipid were almost negligible. The vesicles employed in these studies constitute a homogeneous population with an average size of 130 ± 40 nm as determined by dynamic light scattering. However, at the highest protein concentration used the vesicles became heterogeneous with an average size of 840 ± 500 nm.

As indicated by kinetic analysis performed at a protein/lipid molar ratio of 3.5 x 10^{-3}, the presence of 20 mol% cholesterol in acidic phospholipid vesicles resulted in an increase of almost 25% in vesicle aggregation at pH 5.0 produced by the recombinant protein (Fig. 3). A similar increase was observed at every protein/lipid molar ratio tested. Moreover, when vesicles composed of PC (36.8%), PE (20%), cholesterol (20%), sphingomyelin (13.6%), phosphatidylinositol (5.6%), PS (3.2%) and phosphatidic acid (0.8%), which resemble the plasma membranes of mouse hepatoma cells, similar results to those obtained with plain negatively charged vesicles were obtained.

Lipid mixing

The ability of $E1_{340}E2_{661}$ to induce vesicle fusion, mixing of phospholipid vesicles was followed by the decrease in fluorescence resonance energy transfer (FRET) between the fluorescent probes NBD-PE and Rh-PE incorporated into a lipid matrix [31]. In this assay, the mixing of phospholipids from labelled and unlabelled liposomes results in an increase in the distance between the donor (NBD) and the acceptor (Rh), with the concomitant decrease in energy transfer (%FRET) which reflects accurately the degree of fusion. As it is observed in Fig. 4, $E1_{340}E2_{661}$ was able to induce lipid mixing of PG vesicles, both at pH 7.0 and 5.0, being the effect produced at neutral pH lower than that observed at acidic pH. At pH 5.0, %FRET decreased from 60%, in the absence of protein, to 7% at a protein/lipid ratio of 7 x 10^{-3}. This value corresponds to a 9 fold dilution in acceptor surface density. This fact together with the considerable increase in vesicle size stated above argue in favour of the complete fusion of the vesicles, since their mere aggregation would not result in such a change in energy transfer [32]. When neutral phospholipids were used, a slight decrease in energy transfer was observed.

At neutral pH the presence of cholesterol has no effect on the lipid mixing of PG vesicles. However, at pH 5.0 and a protein/lipid ratio of 3.5 x 10^{-3} the presence of 20% cholesterol induced an additional decrease in the percentage of FRET, from 18% to 7%. These values correspond to a decrease in the acceptor surface density of 3.5 in the absence of cholesterol and almost 9 in its presence.

Release of aqueous contents

The ability of $E1_{340}E2_{661}$ protein to destabilize the lipid bilayer was assessed by determining the release of aqueous contents of phospholipid vesicles. Liposome leakage was monitored by measuring the increase in ANTS fluorescence at 520 nm [33]. Fig. 5 shows the leakage induced by the recombinant protein when it is added to PG vesicles at pH 5.0 and 7.0. $E1_{340}E2_{661}$ was able to induce the release of internal contents of the vesicles in a dose-dependent manner. At a protein/lipid ratio of 0.45 x 10^{-3}, an 80% of F_{max} is reached in both cases, and the 100% leakage was observed at a protein concentration of 0.33 μM, concentration much lower than that needed to induce vesicle aggregation or lipid mixing (1.4-1.8 μM). Then, there was no a pH-dependence of the lipid destabilization as it was observed in the aggregation and lipid mixing assays. On the other hand, when the experiments were carried out at 500 mM NaCl a reduction of almost 35% of the leakage induced by the recombinant protein was observed.

$E1_{340}E2_{661}$ interacts with phospholipid vesicles

Fluorescence polarization

To ascertain the interaction of $E1_{340}E2_{661}$ protein with acidic and neutral phospholipids, its effect on the thermotropic behaviour of the phospholipids has been studied by measuring the fluorescence polarization of these liposomes labelled in the

hydrophobic core of the bilayer with the fluorescent probe DPH. Fig. 6 shows the fluorescence depolarization of DPH-labelled DMPG vesicles with increasing temperatures in the presence of $E1_{340}E2_{661}$ protein a protein/phospholipid molar ratio of 3.5×10^{-3} at pH 7.0 (Fig. 6A) and 5.0 (Fig. 6B). The addition of the recombinant protein to DMPG vesicles at acidic pH induced a slight decrease in the amplitude of the transition and an increase in the transition temperature. The effect on the fluorescence polarization was observed almost exclusively at temperatures above the transition temperature, indicating that the protein affected mainly the acyl chains in the liquid-crystal phase, inducing a higher order in the chain packing (Fig. 6B). On the other hand, the fact that the amplitude of the phase transition was modified reveals the importance of the hydrophobic component in the interaction of $E1_{340}E2_{661}$ with phospholipids. The shift in the transition temperature indicates that the interaction of the recombinant protein with lipid membranes induces perturbations of the lipid packing due to the relative immobilization of phospholipid molecules around the protein. At temperatures below Tm, $E1_{340}E2_{661}$ is probably excluded from the ordered lipid phases and does not cause substantial effects on the packing of the phospholipid acyl chains. On the other hand, little changes in the transition curve were observed at pH 7.0 (Fig. 6A). Moreover, when neutral phospholipids (DMPC) vesicles were used no changes in the amplitude nor in the temperature of the transition were observed neither at neutral or acidic pH.

Intrinsic and bis-ANS fluorescence spectroscopy studies

The effect of both the pH change and the interaction with phospholipid vesicles on the protein structure was studied both by the use of tryptophan and bis-ANS fluorescence. The later is a compound with a high quantum yield in hydrophobic environments but which almost does not fluoresce in water. Hence it is widely used to detect conformational changes which expose hydrophobic regions of the protein structure [34]. The fluorescence spectrum of $E1_{340}E2_{661}$ at pH 7.0 shows a maximum at 331 nm

characteristic of Trp in a relatively low hydrophobic environment [29]. When the pH is brought to 5.0 a 20% decrease in the Trp quantum yield was observed (Fig. 7A), probably due to a higher exposure of Trp residues. The change is readily reversible since the spectrum obtained after adding NaOH to bring the pH back to 7.0 is indistinguishable of the initial one at that pH. The conformational change is also evidenced by the fluorescence of bis-ANS (Fig. 7B). The increase in fluorescence observed at pH 5.0 is indicative of the existence of new solvent-exposed hydrophobic surfaces in the protein, compatible with tryptophan residues in a more hydrophilic environment. Again, reversing the pH back to 7.0 recovered the bis-ANS fluorescence to its initial value.

Fig. 8 shows kinetic data of the effect of pH and the addition of PG on the fluorescence of bis-ANS when added to $E1_{340}E2_{661}$. The exposed hydrophobic areas which the recombinant protein possesses at pH 5.0 become almost instantaneously labelled by bis-ANS (Fig. 8A). As stated above part of them become buried when the pH is brought to 7.0 (Fig. 8A). However, when the protein in the presence of PG vesicles at pH 5.0 is incubated with bis-ANS, the fluorescence does not reach the value observed when the protein alone is incubated with the probe at pH 5.0 in the absence of phospholipids (Fig. 8B). This result could mean that most of the hydrophobic regions of the protein that become exposed at pH 5.0 are not accessible to bis-ANS in the presence of PG vesicles. In fact, when PG is added to bis-ANS labelled $E1_{340}E2_{661}$ at pH 5.0, the fluorescence reach a value close to that observed at pH 7.0 (Fig. 8C).

Discussion

The knowledge of the HCV viral cycle is scarce and the sequence(s) involved in the fusion step between the host cell and the viral membranes have not yet been identified. By searching for classical features of fusion peptides through the protein sequence, several groups have identified some regions of HCV glycoproteins E1 and E2 that could act as fusion peptides [26-28]. These regions may contribute to merging of viral and cellular membranes either by interacting directly with phospholipid membranes or by assisting the fusion process through their involvement in the conformational changes of E1E2 heterodimer at low pH. In this work, we have used an isolated chimeric protein $E1_{340}E2_{661}$ that includes the ectodomains of E1 and E2 envelope proteins to study the interaction and destabilization of lipid bilayers. This protein has been previously shown to have all the conformational features of a native protein [29]. Aggregation of phospholipid vesicles results showed that both the phospholipid nature and the pH are very important to ascertain the interaction of the recombinant protein with the phospholipids. Thus, $E1_{340}E2_{661}$ interacts preferentially with acidic phospholipids, PG or PS, at pH 5, although it is also able to interact with neutral phospholipid vesicles, PC, at this pH but to a much lower extent. Furthermore, this protein was also able to induce phospholipid mixing of acidic vesicles at pH 5.0 and the release of aqueous content of acidic vesicles at both neutral and acidic pH. The fact that the protein concentration at which $E1_{340}E2_{661}$ induces the last effect is considerably lower than that needed to induce aggregation and lipid mixing indicates that fusion is not necessary to destabilize the bilayer and induce the release of the aqueous contents. A similar behaviour has been described in other systems such as the amino-terminal peptide of HIV [35].

The pH dependence of the fusogenic properties of the protein might indicate that, *in vivo*, infection might proceed through receptor mediated endocytosis. In this case, a

conformational change in the viral envelope glycoprotein induced by the acidic pH might be a prerequisite for membrane insertion and destabilization. Others authors have shown that HCV entry occurs in a pH-dependent manner via endocytosis. Thus, the use of drugs that inhibit endosomal acidification efficiently blocked HCVpp infection [18, 36-38]. Furthermore, low pH treatment of HCVpp led to the exposure of new epitopes in E2 [39], supporting the notion that low pH induces conformational rearrangements in HCV glycoproteins, eventually leading to fusion with the endosome membrane. This would be consistent with a membrane fusion mechanism similar to that described for the glycoproteins of *Flaviviruses*, such as tick-borne encephalitis virus (TBEV) [40], dengue virus [41] and West Nile virus [42].

The dependence of the interaction on the nature of the polar head group indicates the importance of the electrostatic interactions. In fact, when these are diminished by increasing the salt concentration, a considerable reduction of all the destabilizing properties tested was observed. Analogous dependence with bilayer composition has been observed with other fusogenic viral peptides and proteins. In this sense, one of the features of the viral fusion mechanism is the variable requirement for specific lipids in the target cell membrane. For example, for simian immunodeficiency virus and HIV fusion peptides, lipid mixing occurs when there is PE in the lipid bilayer [43, 44]; vesicular stomatitis virus has a preference for PS [45], and influenza virus and Sendai virus peptides interact preferentially with PC membranes [46-48]. However, this dependence with the pH and the lipid composition observed in viral fusion proteins or even with whole viruses does not necessarily have a physiological significance.

The effect of the recombinant protein on the transition temperature of DMPG vesicles at pH 5.0, as indicated by fluorescence polarization studies, is indicative of an electrostatic interaction at surface level, typical of peripheral proteins. Moreover, the decrease in transition amplitude is characteristic of integral membrane proteins [49],

suggesting that some protein regions are able to insert into the membrane interacting in a hydrophobic manner with the hydrocarbon core and restricting the mobility of the acyl chain. Thus, the interaction of $E1_{340}E2_{661}$ protein with the bilayer would take place in two steps: a first one governed by electrostatic interactions between the phospholipid polar head and the protein, and a second step driven by hydrophobic interactions which leads to the insertion of the protein into the bilayer. When the first step does not take place, such as when neutral phospholipids are used, the protein does not insert into the bilayer. Moreover, this mechanism of interaction would explain the differences observed with acidic phospholipids at pH 7.0 and 5.0 since an increment in positive charge as a consequence of the decrease of the pH would favour the initial step and hence the insertion into the bilayer.

Cholesterol is a major component of mammalian membranes. When it incorporates into the bilayer, the main consequence is that it enhances lipid lateral interactions and it is able to locally induce negative membrane curvature [50]. It has been found that there is an important relationship between membrane fusion and cholesterol content for several viruses [51, 52]. In order to determine if this was also the case of HCV, we measured the ability of $E1_{340}E2_{661}$ to induce aggregation, lipid mixing and leakage of liposomes containing 20 mol% cholesterol. In all cases, a lower protein concentration was necessary to reach the highest destabilization when cholesterol-containing vesicles were used. Thus, inclusion of cholesterol in liposomes, although it is not essential, facilitates fusion of HCV, which could indicate the involvement of lipid rafts in HCV entry. These results are consistent with other studies that have been performed with HCV. Thus, it has been described that the presence of cholesterol facilitates the fusion of HCVpp with the target membrane [18, 38] and enhances fusion of HCVcc [19]. On the other hand, it has been described that cholesterol affects the interaction of a peptide derived from the region 314-342 of E1 with liposomes, favouring

that the peptide positions close to the bilayer surface [53]. Also, in other *Flaviviruses*, cholesterol has been shown to enhance fusion [54]. Moreover, cholesterol has also been reported to enhance entry, but not the fusion process itself, of many other pH-dependent viruses [40]. In contrast, for alphaviruses such as the Semliki Forest (SFV) and Sindbis viruses, the presence of cholesterol in the target membrane is an absolute requirement for fusion to occur [51, 55-57].

The data on the conformational change induced by decreasing the pH and by the interaction with phospholipids allow us to propose the following model for the fusion of HCV. As a consequence of the diminution of the pH a shift to a more hydrophilic environment of some of the aromatic residues could have been produced. On the other hand, the acidic pH increases the surface hydrophobic area as monitored by bis-ANS. Taking into account that the interaction with phospholipid vesicles preserves the increase of bis-ANS fluorescence, it can be argued that the hydrophobic regions which become exposed as a consequence of acidification are those which become inserted into the bilayer impairing the interaction with bis-ANS molecules. These changes are compatible with a split of E1 and E2 ectodomains in the $E1_{340}E2_{661}$ chimera which could uncover the fusion peptides allowing their interaction with the polar lipid heads of the outer leaflet of the lipid bilayer and next insert into the hydrophobic moiety producing the destabilization of the bilayer which finally leads to fusion. This model is analogous to that observed with other class II fusion protein in which glycoproteins contact with surface at pH 7.0 exposing aromatic side chains at the outer leaflet of the lipid bilayer [58, 59]. Next, a conformational change is observed at low pH which allows insertion of fusion peptides into the bilayer. This state precedes the opening of the fusion pore [60].

Therefore, the data presented herein indicated that $E1_{340}E2_{661}$ could serve as a good model to study the initial infective steps of HCV, allowing the location of those regions responsible for the interaction and the fusion with the cellular membrane.

Moreover, if this protein is involved in the fusion process it can be a good candidate to develop antiviral strategies to block viral infection.

Materials and methods

Reagents

Synthetic phospholipids, dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), and natural phospholipids, egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS) and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Egg yolk phosphatidylglycerol (PG) was obtained from SIGMA. N-(7-nitro-2,1,3-benzoxadiazol-4-yl) dimyristoylphosphatidyl-ethanolamine (NBD-PE) and N-(lissamine rhodamine B sulphonyl) diacylphosphatidyl-ethanolamine (Rh-PE) were provided by Avanti Polar Lipids. 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) and *p*-xylenebis-(pyridinium) bromide (DPX) and 4,4'-bis(1-anilinonaphtalene 8-sulfonate (bis-ANS) were obtained from Molecular Probes. 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Aldrich.

Expression and purification of $E1_{340}E2_{661}$

$E1_{340}E2_{661}$ was expressed and purified as described in [29]. Briefly, DNA encoding $E1_{340}E2_{661}$ was inserted into a baculovirus transfer vector pAcGP67A with the addition of a six-histidine tag. To produce the recombinant virus that expresses the protein, Sf9 insect cells were cotransfected with the recombinant vector and wild-type viral DNA. In a homologous recombination event, the $E1_{340}E2_{661}$ gene was inserted into the viral genome to generate the recombinant baculovirus. The protein was expressed by infecting High Five cells in Insect X-Press serum-free media with high titer virus (>10^8 pfu/ml) at MOI of 5-10. Medium was collected approximately 120 h postinfection, dialyzed against 20 mM Tris-HCl pH 8.0, 50 mM NaCl and loaded onto a Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen) which had been previously

equilibrated with the same buffer. Once the protein solution had entered the column, it was washed with 10 mM imidazole and later with 30 mM imidazole in dialysis buffer. The recombinant $E1_{340}E2_{661}$ protein was eluted with 200 mM imidazole. The presence of $E1_{340}E2_{661}$ was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which was performed according to Laemmli using 15% polyacrylamide gels [61]. By using this method approximately 2 mg of pure protein from 1 L of culture media were obtained [29].

Vesicle preparation

In all cases a lipid film was obtained by drying a chloroform:methanol (2:1) solution of the lipid under a current of nitrogen. This film was kept under vacuum 6-8 h. The phospholipids were resuspended at a concentration of 1 mg/ml in medium buffer (5 mM Tris-HCl, 100 mM NaCl, 5 mM MES, 5 mM sodium citrate, 1 mM EDTA) at the appropriate pH value for 1 h at 37 ºC and eventually vortexed vigorously. This suspension was sonicated in a bath sonicator (Branson 1200, Bransonic) and was subsequently subjected to 15 cycles of extrusion in an Extruder apparatus (LiposoFast^{TM}-Basic, Avestin) with 100 nm polycarbonate filters (Avestin). The size and size distribution of the vesicles were determined by dynamic light scattering on a Malvern Zetasizer Nano ZS instrument.

Aggregation studies

The aggregation of phospholipid vesicles induced by the recombinant protein was studied by adding different amounts of protein to recently prepared lipid vesicles in medium buffer containing either 100 mM or 500 mM NaCl at the appropriate pH. The change in OD_{360} was measured on a Beckman DU-640 spectrophotometer after incubation for 1 h at 37ºC. The final phospholipid concentration was 0.14 mM. In all

cases, controls of lipid vesicles in the absence of protein and protein in absence of lipid were obtained.

Lipid mixing assay

To follow the adhesion and fusion of lipid membranes induced by the protein we have used the classical fluorescent probe dilution assay [31], in which the decrease in efficiency of the fluorescence energy transfer between NBD-PE (energy donor) and Rh-PE (energy acceptor) incorporated into liposomes, as a consequence of lipid mixing, is monitored. Liposomes, in medium buffer at the appropriate pH, labelled with 1 mol% NBD-PE and 0.6 mol% Rh-PE were mixed in a 1:9 molar ratio with unlabelled liposomes. Lipid mixing was initiated by the addition of the protein from a stock solution in 20 mM Tris pH, 7.5, NaCl 100 mM. The samples were incubated for 1 h at 37ºC and the emission spectra were recorded in a SLM AMINCO 8000C spectrofluorimeter, with the excitation wavelength set at 450 nm. Both the excitation and emission slits were set at 4 nm. The excitation polarizer was kept constant at 90º and the emission polarizer was kept constant at 0º to minimize dispersive interference. The efficiency of fluorescence resonance energy transfer was calculated from the ratio of the intensities at 530 and 590 nm and the appropriate calibration curve which was obtained by using labelled vesicles containing 1% NBD-PE and variable concentrations of Rh-PE (between 0 and 1%). The final phospholipid concentration was 0.14 mM.

Release of aqueous contents

Leakage was determined by the ANTS/DPX assay [32] which is based on the dequenching of ANTS fluorescence caused by its dilution upon release of the aqueous contents of a vesicle population containing both ANTS and DPX. The assay was performed by coencapsulating 12.5 mM ANTS and 45 mM DPX in 10 mM Tris, pH 7.5,

20 mM NaCl or medium buffer, pH 5.0, in either PG or PC phospholipid vesicles. The lipid film was vortexed and the vesicles were sonicated in a bath for 15 min. Afterwards, the vesicles were subjected to five cycles of freeze-thawing in liquid nitrogen and passed at least 15 times through an Extruder apparatus (LiposoFast^{TM}-Basic, Avestin) with 100 nm polycarbonate filters (Avestin). After the vesicles with the coencapsulated probe and quencher had been formed, the whole sample was passed through a Sephadex G-75 column (Pharmacia) to separate the vesicles from the nonencapsulated material using 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, or medium buffer, pH 5.0 for elution. The final phospholipid concentration in the assay was 0.14 mM. Both the excitation and emission slits were set at 4 nm. The excitation polarizer was kept constant at 90º and the emission polarizer was kept constant at 0º to minimize dispersive interference. Leakage was started by addition of the protein and the increase of the fluorescence emission at 520 nm upon excitation at 385 nm was continuously recorded (F_t). The measurements were performed in a thermostated cell holder at 37 ºC. The fluorescence scale was set to 100% by addition of 0.5% Triton X-100 and to 0% by measuring the fluorescence of the control vesicles after the addition of equivalent volumes of buffer.

Fluorescence polarization measurements

Fluorescence measurements were carried out on a SLM AMINCO 8000C spectrofluorimeter. Excitation and emission slit widths were 4 nm. Phospholipid vesicles were labelled with DPH in a probe:phospholipid ratio of 1:500. Emission spectra of DPH were measured at 425 nm with the excitation wavelength set at 365 nm, after equilibration of the sample at the required temperature. DMPG, or DMPC vesicles were incubated in the absence or in the presence of $E1_{340}E2_{661}$ at 37ºC for 30 min, using a lipid concentration of 0.14 mM and protein/lipid molar ratios of $1.75 \cdot 10^{-3}$ and $3.5 \cdot 10^{-3}$.

Intrinsic emission fluorescence

Emission spectra of $E1_{340}E2_{661}$ protein were obtained at 37 ºC on a SLM AMINCO 8000C spectrofluorimeter. Excitation and emission slit widths were 4 nm. The protein concentration was 0.05 $mg \cdot ml^{-1}$ and a 0.4 x 1 cm cuvette was used. In all cases medium buffer at the appropriate pH value was used. Excitation was performed at 275 or 295 nm, and the emission spectra were recorded over the range 285-450 nm. The contribution of the buffer with different concentrations of phospholipid vesicles was always subtracted. The excitation and emission polarizers were kept constant at 90º and 0º to minimize dispersive interference.

Extrinsic emission fluorescence

The fluorescent probe bis-ANS was dissolved in methanol. The fluorescence emission spectra of bis-ANS were obtained on a SLM AMINCO 8000C spectrofluorimeter by using an excitation wavelength of 395 nm, and were recorded over the range 405-600 nm. Samples were prepared by adding 5 µl of 0.2 mM bis-ANS to $E1_{340}E2_{661}$ in 5 mM sodium acetate, 5 mM MES, 5 mM Tris, 150 mM NaCl at the appropriate pH. These samples were incubated at 37 ºC for 5 min before the spectra were taken. Controls without protein and without protein and probe were obtained.

Protein and phospholipid concentrations

The UV-spectrum of the protein was routinely used to calculate the concentration of $E1_{340}E2_{661}$. An extinction coefficient (0.1% w/v, 1 cm, 280 nm) of 1.99 was used [29]. Lipid concentration was calculated based on their phosphorous content determined according to Barlett [62].

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Figure Legends

Fig.1. Amino acid sequence of the chimeric protein $E1_{340}E2_{661}$. Positions 1-16 of the recombinant protein are introduced by the cloning procedure. Positions 17-166 and 181-458 correspond to residues 192-341 and 384-661 of HCV E1 and E2 ectodomains respectively. The FLAG region which connects both ectodomains and which contains the enterokinase recognition sequence (DDDDK) is shown in bold [29]. The putative fusogenic sequences of both ectodomains are underlined [26]. The tryptophan residues of these fusogenic regions are highlighted.

Fig. 2. Aggregation of PS, PG, and PC phospholipid vesicles induced by $E1_{340}E2_{661}$. The increment of the optical density at 360 nm (ΔOD_{360}) was measured after the incubation of vesicles in medium buffer at pH 5.0 with $E1_{340}E2_{661}$ at different protein/lipid ratios. (●) PS (■) PG (▲) PC vesicles. The final phospholipid concentration was 0.14 mM. The results shown are the average ± SD of two different experiments.

Fig. 3. Aggregation kinetic of vesicles induced by $E1_{340}E2_{661}$. PG vesicles in the absence (●, ■) or presence (○, □) of 20 % Chol were incubated with $E1_{340}E2_{661}$ at pH 5.0. (○, ●) and pH 7.0 (□, ■). The optical density at 360 nm (OD_{360}) was measured after the addition of the protein (arrow). The protein/lipid ratio was 3.5×10^{-3}. The results shown are representative of those obtained for two different experiments.

Fig. 4. Lipid mixing of PG vesicles induced by $E1_{340}E2_{661}$. Increasing concentrations of $E1_{340}E_{2661}$ were added to a 1:9 mixture of labelled (NBD-PE 1% and Rh-PE 1%) and unlabelled PG vesicles hydrated in medium buffer at pH 7.0 (○) or 5.0 (●). The

efficiency of fluorescence resonance energy transfer (FRET) was calculated from the ratio of the intensities at 530 and 590 nm and the appropriate calibration curve. The final phospholipid concentration was 0.14 mM. The results shown are the average ± SD of two different experiments.

Fig. 5. Leakage of ANTS/DPX from PG vesicles induced by $E1_{340}E2_{661}$. Increasing concentrations of $E1_{340}E2_{661}$were added to vesicles loaded with ANTS and DPX in medium buffer at pH 7.0 (●) and 5.0 (■). F_{max} was obtained upon addition of 0.5% Triton X-100. The measurements were performed at 37 ºC and the final phospholipid concentration in the assay was 0.14 mM. The results shown are the average ± SD of two different experiments.

Fig. 6. Temperature dependence of fluorescence polarization of DPH-labelled DMPG liposomes. The vesicles were incubated with $E1_{340}E_{2661}$ at a protein/lipid molar ratio of 3.5×10^{-3} in medium buffer at pH 7.0 (A) and 5.0 (B). (●) DMPG vesicles alone (■) vesicles in the presence of the recombinant protein. The results shown are representative of those obtained for two different experiments.

Fig. 7. Fluorescence emission spectra of $E1_{340}E_{2661}$ (A) and bis-ANS-$E1_{340}E_{2661}$ (B). The spectra were taken in medium buffer at pH 7.0 (—) and 5.0 (- - -) upon excitation at 275 (A) and 395 nm (B). The protein concentration was 0.05 mg/ml (A) and 0.025 mg/ml (B). The results shown are representative of those obtained for two different experiments.

Fig. 8. Change of emission fluorescence of bis-ANS. bis-ANS was added to $E1_{340}E_{2661}$ at pH 5.0 in the absence (A) or in the presence (B) of PG vesicles or at pH 7.0 in absence

of lipid (C) and incubated at 37 ºC. The arrows indicate the time at which the corresponding compound was added. The addition of NaOH and HCl bring the pH back to 7.0 and 5.0, respectively. The protein/lipid molar ratio employed was 1.75×10^{-3}. The excitation and emission wavelength were 395 and 495 respectively. The final concentration of bis-ANS was 2.5 μM. The results shown are representative of those obtained for two different experiments.

Figure 1

1 ADPGYLLEFMHHHHHHYQVRNSTGLYHVTNDCPNSSIVYEAADAILHTPGCVPCVHEGNA **60**

61 SRCWVALTPTVATRDGKLPTTQLRRHIDLLVGSATLCSALYVGDLCGSVFLVGQLFTFSP **120**

121 RRHWTTQDCNCSIYPGHITGHRMAWDMMMNWSPTAALVVAQLLRIP**DTKDDDDKAM**GVDP **180**

181 ETHVTGGTAAQTTAGLVSLLSPGAKQDIQLINTNGSWHINSTALNCNDSLYTGWLAGLFY **240**

241 HHKFNSSGCPERFASCRPLTDFAQGWGPISHANGSGPDQRPYCWHYPPKPCGIVPAKSVC **300**

301 GPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFGCTWMNSTGFTKV **360**

361 CGAPPCVIGGVGNNTLHCPTDCFRKHPEATYSRCGSGPWITPRCLVNYPYRLWHYPCTIN **420**

421 YTIFKVRMYVGGVEHRLEAACNWTRGERCNLEDRDRSERSHHHHHH

Figure 2

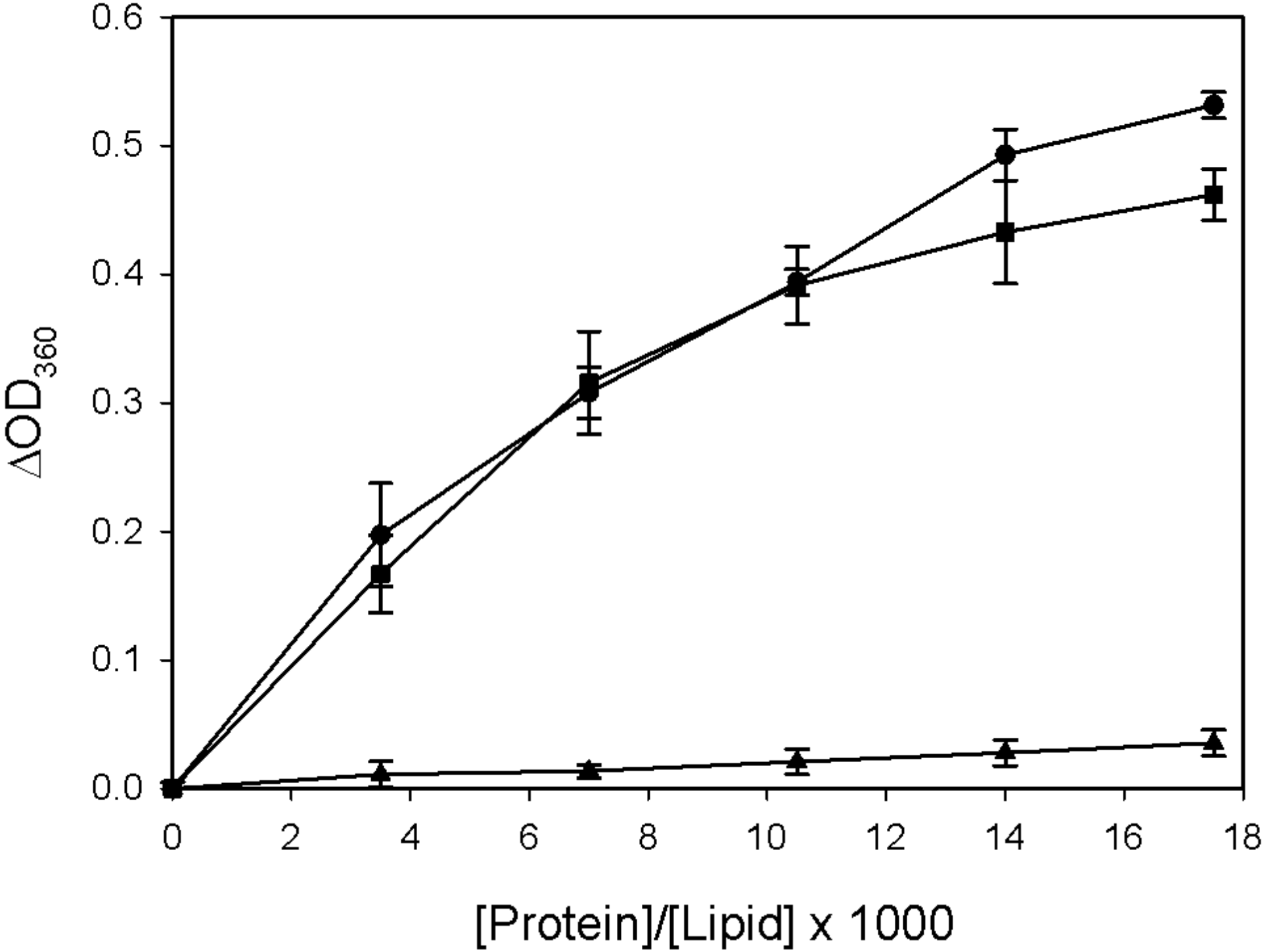
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Figure 3

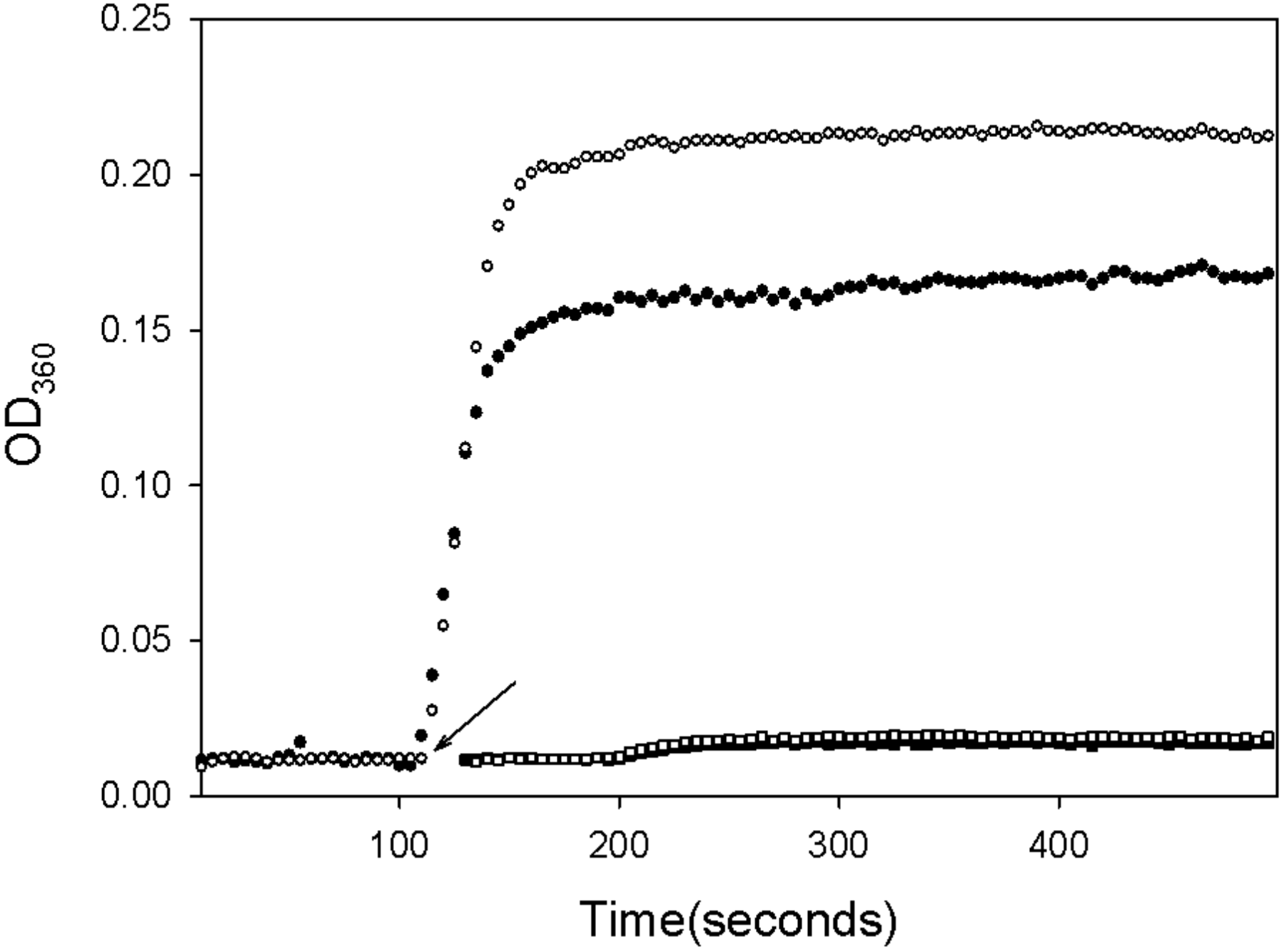

Figure 4

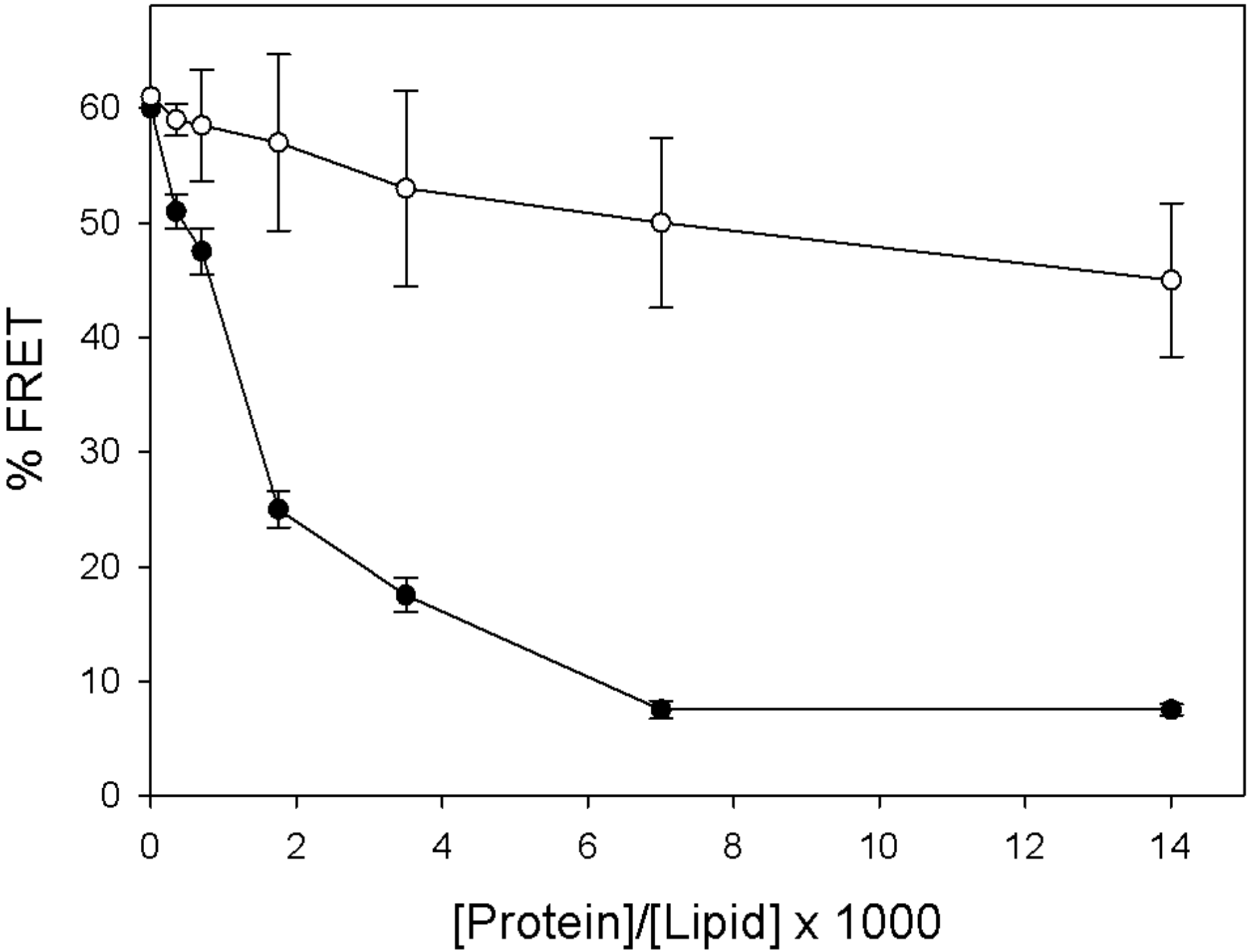

Figure 5

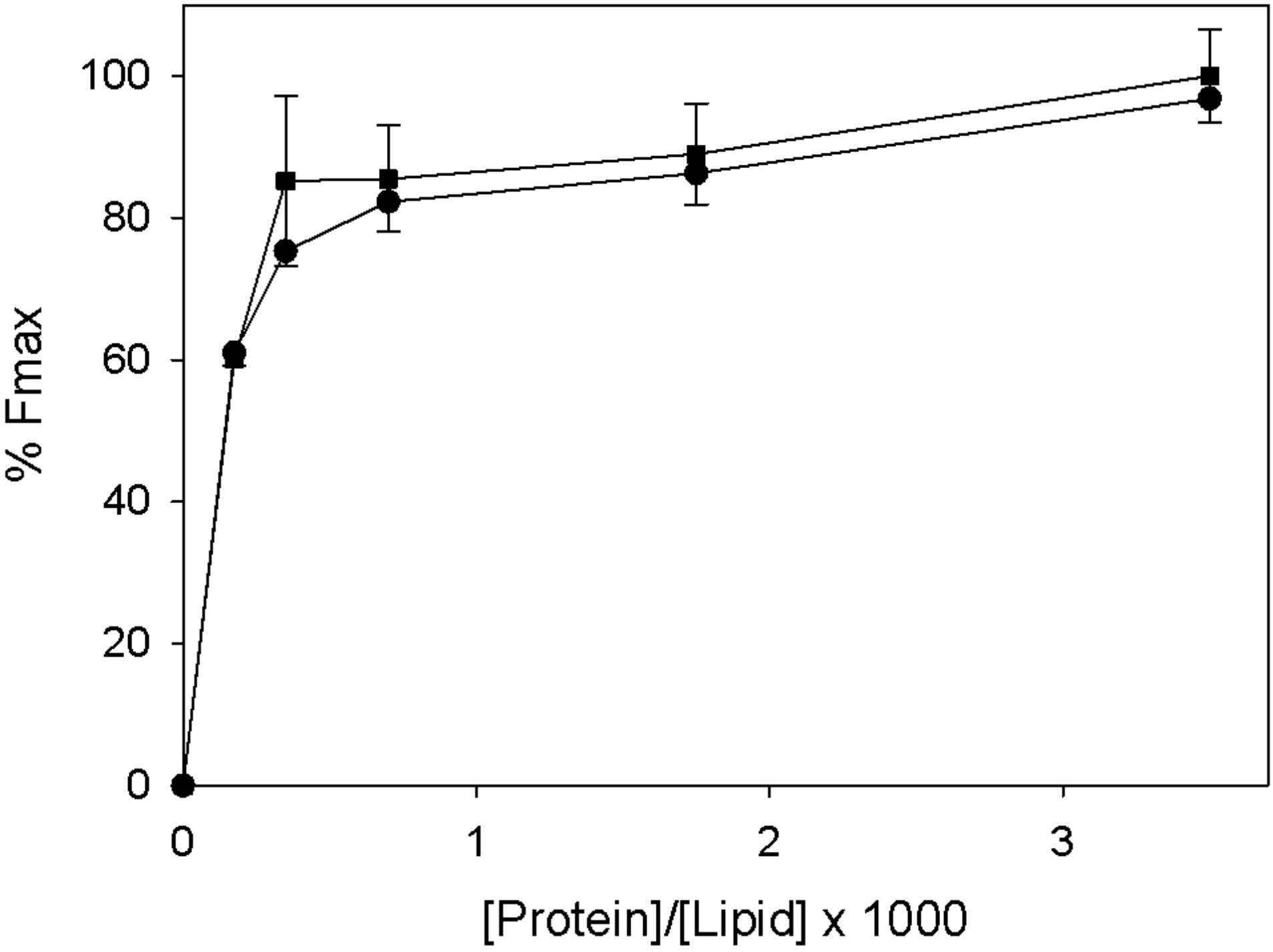

Figure 6

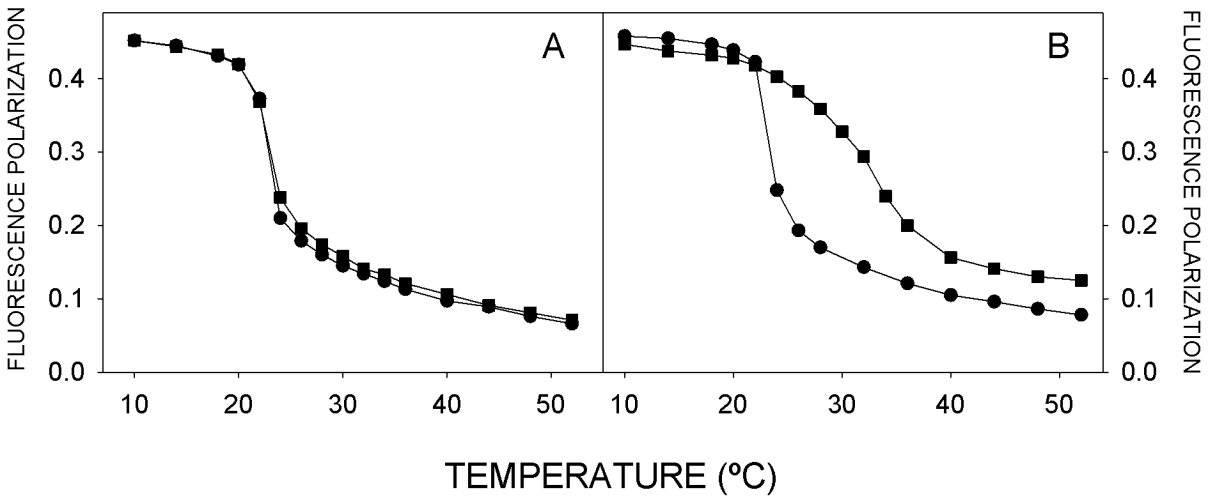
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Figure 7

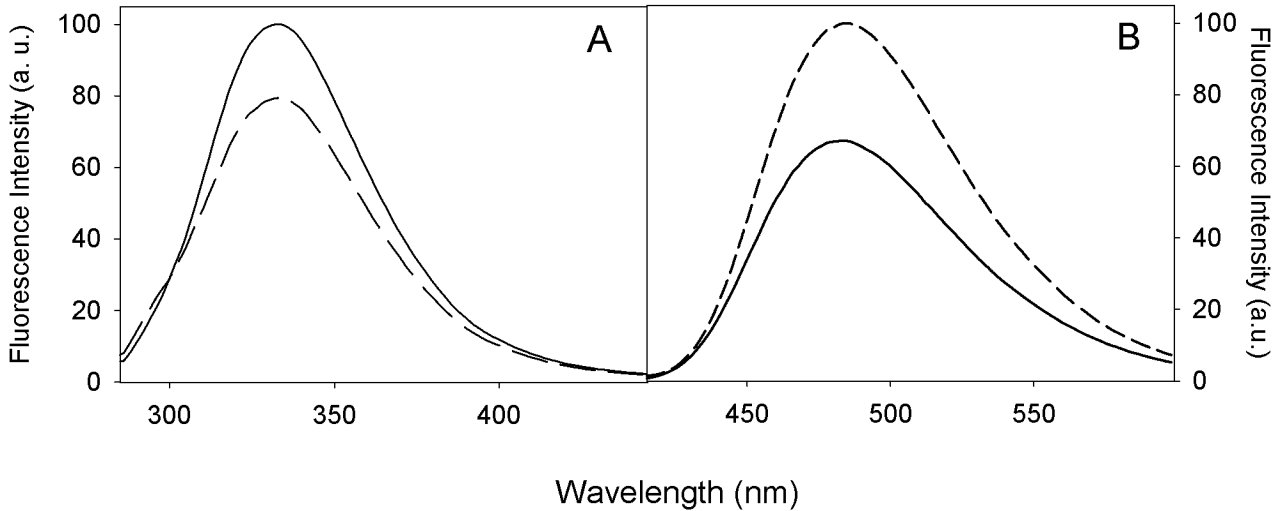
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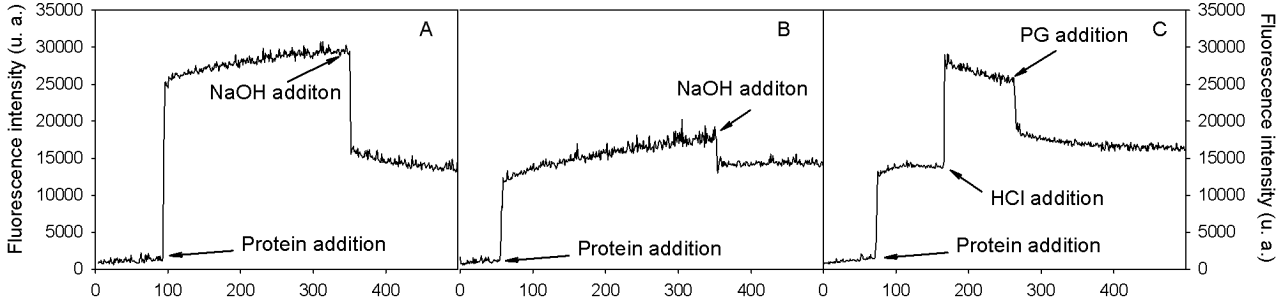
Figure 8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