

# Transferrins selectively cause ion flux through bacterial and artificial membranes

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Received 23 April 2003; revised 13 June 2003; accepted 13 June 2003

First published online 1 July 2003

Edited by Stuart Ferguson

**Abstract** Serum transferrin, ovotransferrin and lactoferrin constitute the most notable members of the transferrin family. Among their multiple biological functions, they possess an important antibacterial activity. These proteins can permeate the *Escherichia coli* outer membrane, reaching the inner membrane where they selectively cause permeation of ions, resulting in dissipation of the electrical potential without affecting the pH gradient. Similar results were obtained using artificial liposomes, suggesting a direct action of the proteins on the lipid bilayer, which was mediated by detectable conformational changes in their structures.

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**Key words:** Transferrin; Lactoferrin; Ovotransferrin; Serum transferrin; Antibacterial mechanism; Proton-motive force

## 1. Introduction

Transferrins are an extended family of metal-binding transport proteins with an in vivo preference for ferric iron. They are single-chain glycoproteins with a molecular weight of about 80 kDa widely distributed in physiological fluids and cells of vertebrates. This family includes four subtypes: serum transferrin, the iron transport protein in the blood of vertebrates; lactoferrin, first discovered in milk; ovotransferrin, from egg white; and melanotransferrin, first identified in malignant melanoma cells. All these proteins are soluble molecules except melanotransferrin, which is bound to the cell membrane by a glycosyl phosphatidylinositol anchor, although it can also be secreted [1].

These molecules have very frequently been considered transport proteins because of their high affinity for iron. However, they are also implicated in many other important physiological processes. Serum transferrin plays a role in the stimulation of cell proliferation [2]; it can also act as an insulin

antagonist, producing acute hyperglycemia in normoglycemic rats and ketonuria in diabetic rats [3]; it is also involved in tumoral processes, correlating with severity of disease [4] and promoting endothelial cell migration and invasion [5]. Ovotransferrin is a chicken serum protein whose levels increase in the acute phase of inflammation and infection [6]; its expression appears correlated with some differentiation processes [7], and shows antiviral [8] and antibacterial activities [9]. Lactoferrin has been detected at very high concentrations in human colostrum [10], tears [11] and different mucosal secretions [12]. Lactoferrin is involved in many aspects of the host defence. It is released by neutrophils in response to inflammatory stimuli that promote the aggregation of these cells [13]; it is able to inhibit the growth of solid tumors and the development of metastases [14]; it shows antiviral effects [15]; finally, lactoferrin is able to produce bactericidal or bacteriostatic effects against a wide variety of Gram-positive and Gram-negative pathogens [16,17].

Up until now, in spite of the relevance of these glycoproteins as part of the first barrier against pathogen microorganisms, the molecular basis of their antibacterial mechanisms remains unknown. It has been proposed that some peptides (lactoferricins, OTAP-92) obtained by hydrolysis could be responsible for the antimicrobial properties. Nevertheless, those peptides show different activities and a wider antimicrobial spectrum than the native proteins [18–20]. Moreover, in previous work we described that Lfpep, a synthetic peptide derived from lactoferrin, is bactericidal against *Escherichia coli* [21], in contrast to the bacteriostatic activity reported for the native protein [16,17]. In this report, lactoferrin, serum transferrin and ovotransferrin are shown to produce analogous effects when acting either on *E. coli* cells or on synthetic vesicles, carrying out selective permeation of ions. The resulting alteration of the pre-existing electrochemical gradients could constitute a molecular explanation for the antibacterial effects of transferrins.

## 2. Materials and methods

### 2.1. Materials

Iron-free lactoferrin, ovotransferrin, serum transferrin, actinomycin D, nigericin and valinomycin were purchased from Sigma Chemicals. [<sup>14</sup>C]Phenylalanine (450 mCi/mmol), [<sup>3</sup>H]tetraphenylphosphonium (34 Ci/mmol), [<sup>3</sup>H]uridine (27 mCi/mmol), [<sup>14</sup>C]acetic acid (62 mCi/mmol), [<sup>14</sup>C]methylinulin (20 mCi/mmol) and tritiated water (5 mCi/ml) were purchased from Amersham. *E. coli* phospholipids were from Avanti Polar Lipids. 3,3'-Dipropylthiadicarbocyanine iodide (diS-C<sub>3</sub>-

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**Abbreviations:** BHI, brain heart infusion; CD, circular dichroism; diS-C<sub>3</sub>-(5), 3,3'-dipropylthiadicarbocyanine iodide; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid; SUV, small unilamellar vesicle

(5) and 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) were purchased from Molecular Probes Europe BV. Brain heart infusion (BHI) was supplied by Difco.

Purity of transferrin solutions was confirmed by gel filtration (Superose 12 HR 10/30, Pharmacia) and Western blotting with goat anti-lactoferrin (Immunoresearch Laboratories), rabbit anti-lysozyme and anti-transferrin antibodies (Sigma).

## 2.2. Outer membrane permeability assays

*E. coli* was obtained as described [21]. Alterations to the outer membrane permeability were tested as previously reported [25]. Cells were resuspended in the minimal medium M9 and adjusted to  $A_{600} = 0.8$ . Experiments were performed at 37°C and under continuous shaking. Changes in the outer membrane permeability were determined in transferrin-treated (20  $\mu$ M) and in non-treated cells, measuring the incorporation of [<sup>14</sup>C]phenylalanine (10  $\mu$ Ci/ml) into proteins in the presence or absence of a subinhibitory concentration of actinomycin D (80  $\mu$ g/ml). Aliquots of 0.5 ml were extracted, precipitated with 5 ml 5% trichloroacetic acid on ice, filtered through cellulose nitrate filters (0.45 nm pore size) and the radioactivity measured.

## 2.3. Inner membrane permeability assays

Permeabilization of the bacterial inner membrane was studied determining the efflux of [<sup>3</sup>H]uridine previously accumulated into *E. coli* cells. A bacterial suspension ( $A_{600} = 0.5$ ) in BHI was incubated with rifampicin (50  $\mu$ g/ml) at 37°C for 5 min. [<sup>3</sup>H]Uridine (1  $\mu$ Ci/ml) was added and the incubation continued for 15 min. Cells were then washed twice and resuspended in the same initial volume of medium. 0.5 ml aliquots of transferrin-treated (20  $\mu$ M) and non-treated bacterial suspensions were extracted at different times, filtered through cellulose nitrate filters (0.45 nm), and the radioactivity measured.

## 2.4. Atomic emission spectrometry

*E. coli* cells were harvested, washed three times and resuspended ( $A_{600} = 2$ ) in deionized Milli-Q water. Cells were incubated at 37°C for 20 min in the presence or absence of transferrins (20  $\mu$ M). Aliquots (2 ml) were centrifuged at 8000  $\times g$  for 1 min, the supernatants collected, and [K<sup>+</sup>] and [Na<sup>+</sup>] quantified using an atomic emission spectrometry detector (UNICAM 929, Unicam Limited, Cambridge, UK). Controls containing only Milli-Q water or lactoferrin were performed independently.

## 2.5. Determination of *E. coli* $\Delta\Psi$ , $\Delta$ pH and proton-motive force ( $\Delta p$ )

*E. coli* cells were harvested, washed three times and resuspended ( $A_{600} = 0.8$ ).  $\Delta\Psi$  and  $\Delta$ pH of glucose-energized cells were measured from the distributions of [<sup>3</sup>H]tetraphenylphosphonium bromide and [<sup>14</sup>C]acetic acid, respectively, as described [21]. *E. coli*  $\Delta p$  was calculated using the general ion electrochemical equation:  $\Delta p = \Delta\Psi - (RT/F)\Delta$ pH.

## 2.6. Preparation of liposomes

Small unilamellar vesicles (SUVs) from *E. coli* phospholipids were prepared as described [21]. Total lipids from *E. coli* were mixed in chloroform:methanol (9:1 v/v) and extensively evaporated. For binding and transmembrane electrical potential assays, dried phospholipids were resuspended in 50 mM potassium phosphate buffer (pH 6.0)

to obtain a final lipid concentration of 20 mg/ml. The suspensions were sonicated, kept in an ice bath, using a titanium tip ultrasonicator to obtain SUVs. Titanium debris was removed by centrifugation. To study  $\Delta$ pH changes, pyranine-containing SUVs were prepared using a pyranine buffer solution (20 mM potassium phosphate, pH 6.0; 100 mM potassium acetate; 100  $\mu$ M pyranine). Non-encapsulated fluorescent probe was separated from the vesicle suspension using a Sephadex G-50 gel filtration column (25  $\times$  0.5 cm) and eluted with 20 mM potassium phosphate buffer (pH 6.0) containing 100 mM K-PIPES. When required, strictly isotonic conditions were ensured by checking the osmotic pressure of intra- and extravesicular solutions in a Osmomat 30 osmometer (Gonotec, Berlin, Germany). Phospholipid concentrations were determined by inorganic phosphorus analysis.

## 2.7. Monitoring of transmembrane $\Delta\Psi$ and $\Delta$ pH in liposomes

Changes in pre-established  $\Delta\Psi$  and  $\Delta$ pH in liposomes were monitored at 25°C as previously described [22]. The reaction mixtures (1 ml) were prepared by diluting liposomes (1/100) in 50 mM potassium phosphate buffer (pH 7.2) and adding the fluorescent potential sensitive dye diS-C<sub>3</sub>-(5) at a final concentration of 1  $\mu$ M. When required, a transmembrane K<sup>+</sup> diffusion potential (negative inside) was generated in the phospholipid vesicles by addition of valinomycin (1  $\mu$ M). Alterations in the electrical potential of *E. coli* liposomes directly treated and not treated with lactoferrin (ranging from 0.01 to 40  $\mu$ M) were continuously monitored by the fluorescence quenching of diS-C<sub>3</sub>-(5) using a Perkin Elmer LS 50 spectrofluorometer. Experiments were performed at 25°C at excitation and emission wavelengths of 616 nm and 676 nm respectively.

The internal pH (pH<sub>i</sub>) of liposomes was monitored from the fluorescence of pyranine entrapped within the liposomes. Liposomes were previously 100-fold diluted in 20 mM potassium phosphate containing 100 mM K-PIPES (pH 7.8) for the generation of a pH gradient. Fluorescence of vesicles treated and not treated with lactoferrin (ranging from 0.01 to 40  $\mu$ M) was monitored at excitation and emission wavelengths of 450 nm and 508 nm respectively. The assays were carried out at 25°C.

## 2.8. Far-UV circular dichroism (CD) spectroscopy

Determinations of transferrins CD spectra were performed in 50 mM potassium phosphate buffer (pH 7.2) in the presence or absence of liposomes using a Jasco J-500A spectropolarimeter. The spectra were the average of eight scans obtained using a quartz optical cell with pathlength of 0.1 mm at room temperature. When required, compensations for the scattering were performed by subtracting the CD spectra measured for liposomes. The data were expressed as mean residue ellipticity (degrees cm<sup>2</sup>/dmol). Concentrations of protein and phospholipids were 50  $\mu$ M and 4 mM, respectively.

## 2.9. Intrinsic tryptophan fluorescence measurements

Transferrins were added to SUVs at a fixed phospholipid:protein ratio (15:1, mol/mol). No corrections were made for changes in osmolarity as little change in 90° light scattering indicated little change in vesicle structure. Small aliquots of the stock solutions of quenchers (4 M KI, 3 M acrylamide) were added and fluorescence measured as described [21]. Since excitation at 280 and 295 nm showed very similar results, 295 nm was used in order to minimize tyrosine effects. The

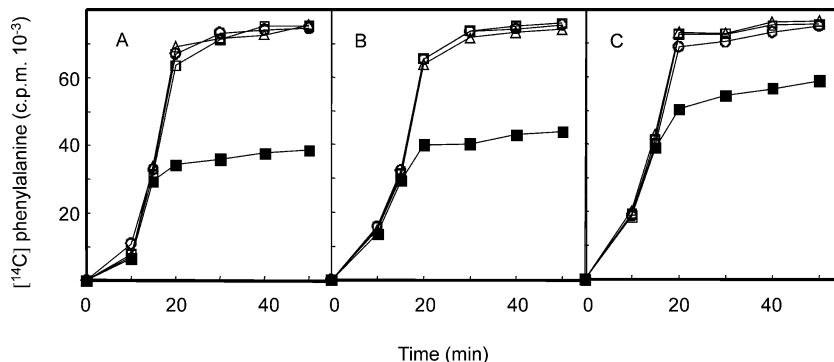


Fig. 1. Effect of transferrins on [<sup>14</sup>C]phenylalanine incorporation into proteins in the presence or absence of actinomycin D. Control (○); actinomycin D (▲); transferrins (□); actinomycin D+transferrins (■). A: Lactoferrin. B: Ovotransferrin. C: Serum transferrin.

fluorescence was recorded in a range from 310 to 380 nm, and intensity at the  $\lambda_{\text{max}}$  used for Stern–Volmer plots.  $F$  and  $F_0$  are the fluorescence intensities with and without quencher respectively.

### 3. Results

#### 3.1. Transferrins alter *E. coli* outer and inner membrane permeabilities

The ability of serum transferrin, ovotransferrin and lactoferrin to disturb the *E. coli* outer membrane permeability was assayed using actinomycin D. This hydrophobic antibiotic, bactericidal for Gram-positive bacteria, possesses a limited action against Gram-negative bacteria due to the outer membrane charged lipopolysaccharides. No effect on protein biosynthesis was observed in assays performed in the presence of a subinhibitory concentration of actinomycin D or serum transferrin, ovotransferrin and lactoferrin in the absence of actinomycin D, in separate assays. Nevertheless, simultaneous addition of actinomycin D and any of the transferrins inhibited about 50% of the protein synthesis, indicating an increased outer membrane permeabilization for the drug (Fig. 1).

Permeabilization of the inner membrane was assessed by determining the leakage of previously accumulated [ $^3\text{H}$ ]uridine in *E. coli* cells. No efflux of the previously accumulated [ $^3\text{H}$ ]uridine in the presence of any of the transferrin molecules could be measured (data not shown). Interestingly, atomic emission spectrometry determinations showed that the addition of serum transferrin, ovotransferrin or lactoferrin to the cells increased the extracellular  $\text{K}^+$  concentration to  $0.6 \pm 0.1$  ppm without any significant effect on  $\text{Na}^+$ . These changes in ion concentration had no effect on *E. coli* viability.

#### 3.2. Transferrins dissipate *E. coli* electrical potential ( $\Delta\Psi$ )

Ion concentration changes should have consequences on the transmembrane  $\Delta\Psi$ . Addition of serum transferrin, ovotransferrin or lactoferrin to the cell suspensions resulted in a rapid reduction and even abolition of  $\Delta\Psi$  (Table 1). The  $\Delta\Psi$  dissipation was dependent on the protein concentration. Above 50% reduction of  $\Delta\Psi$  was achieved at 0.13  $\mu\text{M}$  lactoferrin and 1.8  $\mu\text{M}$  serum transferrin and ovotransferrin, while the abolition was reached at 6.4  $\mu\text{M}$  lactoferrin, 13  $\mu\text{M}$  ovotransferrin and 26  $\mu\text{M}$  serum transferrin. On the other hand, determinations of the transmembrane pH gradient ( $\Delta\text{pH}$ ) showed that transferrins produced only slight internal alkalization ( $\leq 0.15$  units). After applying these data to the general ion electrochemical equation, the proton motive force (estimated to be  $-198$  mV in control assays) was decreased significantly

Table 1  
Effect of transferrins on *E. coli*  $\Delta\Psi$

[Protein] ( $\mu\text{M}$ )	$\Delta\Psi$ (mV)		
	Lactoferrin	Ovo-transferrin	Serum transferrin
0	$-110 \pm 11$	$-110 \pm 11$	$-110 \pm 11$
0.013	$-71 \pm 5$	$-88 \pm 4$	$-97 \pm 5$
0.13	$-50 \pm 4$	$-79 \pm 8$	$-95 \pm 5$
0.64	$-31 \pm 5$	$-68 \pm 6$	$-75 \pm 4$
1.8	$-15 \pm 2$	$-30 \pm 3$	$-40 \pm 3$
3.2	$-7 \pm 3$	$-20 \pm 2$	$-25 \pm 3$
6.4	0	$-6 \pm 1$	$-13 \pm 3$
13	0	0	$-8 \pm 2$
26	0	0	0
38.4	0	0	0

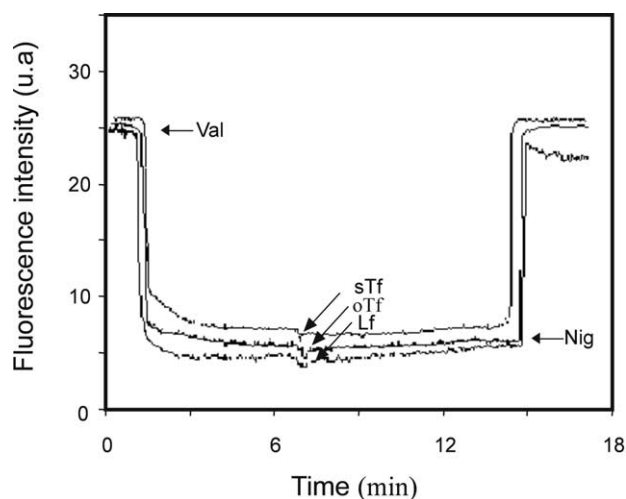


Fig. 2. Effect of transferrins on valinomycin-hyperpolarized liposomes. Serum transferrin, ovotransferrin or lactoferrin at 40  $\mu\text{M}$  were added at indicated times. 1  $\mu\text{M}$  nigericin (Nig) was used to abolish the electrical potential created by transferrins. Under the experimental conditions assayed, nigericin acts as a general cation ionophore (see Section 4).

to  $-56$  mV after the addition of the proteins at an external pH of 7.5 and at 25°C.

#### 3.3. Transferrins produce selective ion permeabilization on liposomes

Differential ion concentration changes and  $\Delta\Psi$  dissipation without significant alteration of  $\Delta\text{pH}$  suggested a selective inner membrane permeabilization mediated by transferrins. This hypothesis was verified using artificial vesicles with an imposed  $\Delta\Psi$  (negative inside).  $\Delta\Psi$  was generated by diluting  $\text{K}^+$ -loaded liposomes in  $\text{Na}^+$ -phosphate buffer, and then hyperpolarizing them by addition of the  $\text{K}^+$  ionophore valinomycin (1  $\mu\text{M}$ ). The effect of transferrins on this  $\Delta\Psi$  was monitored using the fluorescent potential-sensitive dye diS-C<sub>3</sub>-(5). In separate experiments, additions of serum transferrin, ovotransferrin or lactoferrin at concentrations from 0.01 to 40  $\mu\text{M}$  did not dissipate  $\Delta\Psi$ . However, later addition of the cation ionophore nigericin (1  $\mu\text{M}$ ) shifted the fluorescence signal, indicating the abolition of  $\Delta\Psi$  (Fig. 2).

Interestingly, addition of any of the transferrins to liposome suspensions in which  $\Delta\Psi$  was not previously created by valinomycin gave rise to immediate fluorescence quenching (Fig. 3), indicating the generation of  $\Delta\Psi$  across the lipid bilayer. The minimal concentrations of serum transferrin, ovotransferrin or lactoferrin necessary to reach complete bilayer hyperpolarization were 1.6, 1.2 and 0.6  $\mu\text{M}$  respectively. Later addition of valinomycin to the transferrin-hyperpolarized vesicles had no effect on  $\Delta\Psi$ , although  $\Delta\Psi$  could be abolished by addition of nigericin.

It is noteworthy that fluorescence quenching was not observed after the addition of lactoferrin, ovotransferrin or serum transferrin to liposomes in which the location of ions was exchanged ( $\text{Na}^+$  inside,  $\text{K}^+$  outside), indicating that transferrins were not able to establish a  $\Delta\Psi$  in these conditions (data not shown).

$\text{H}^+$  permeation by the effect of transferrins was determined using liposomes in which a  $\Delta\text{pH}$  was preformed and containing pyranine as internal pH indicator. None of the molecules

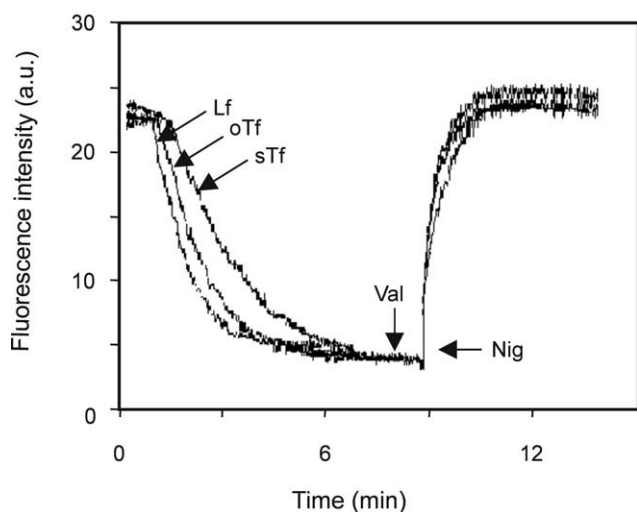


Fig. 3. Hyperpolarization of liposomes by effect of transferrins. Serum transferrin, ovotransferrin or lactoferrin at 0.6  $\mu\text{M}$  and 1  $\mu\text{M}$  nigericin (Nig) were added at indicated times.

was able to alter  $\Delta\text{pH}$ , which could be abolished by nigericin (data not shown).

### 3.4. Transferrin-mediated ion permeation is accompanied by conformational changes

Changes in transferrin secondary structure induced by binding to liposomes were analyzed by far-UV CD. Lactoferrin exhibited the most marked differences, mainly at 200 and 212 nm, while very similar spectra were recorded for serum transferrin both in soluble and in bound form. Ovotransferrin showed intermediate differences (Fig. 4).

Tryptophan intrinsic fluorescence was also used to determine binding-induced alterations in transferrin structures. Exposure of tryptophanyl residues from serum transferrin, ovotransferrin or lactoferrin to low molecular weight quenchers in the presence and absence of vesicles was examined. The effi-

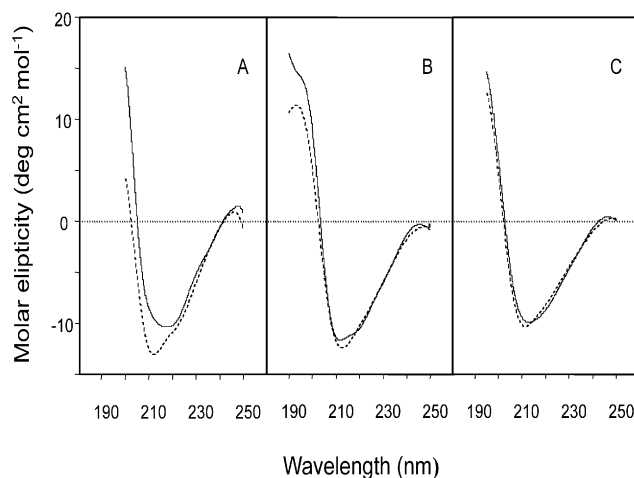


Fig. 4. CD spectra of transferrins in aqueous solution (continuous line) and in the presence of liposomes (dotted line). A: Lactoferrin. B: Ovotransferrin. C: Serum transferrin.

ciency of the ionic quencher  $\text{I}^-$  varied in aqueous suspensions; it was notably high for serum transferrin and, especially, for lactoferrin. However, the efficiency of the quencher was lower for ovotransferrin (Fig. 5). In all cases, addition of liposomes resulted in a decrease of the  $\text{I}^-$  quenching effect. In different experiments, we used the polar (non-ionic) molecule acrylamide as the quenching agent. The accessibility of tryptophanyl residues to acrylamide displayed remarkable changes in the presence of *E. coli* liposomes (Fig. 5). Exposure of the tryptophan residues to the quenching agent was considerably increased.

## 4. Discussion

The transferrin family includes proteins that perform many biological activities, including a physiological role in the de-

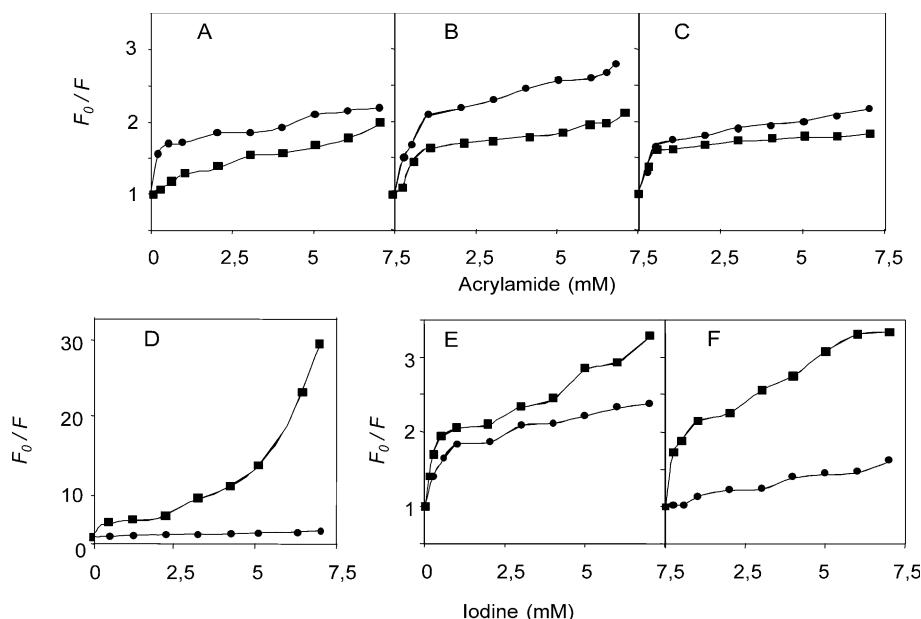


Fig. 5. Stern-Volmer plots for transferrins in the presence (●) or absence (■) of liposomes using acrylamide (A–C) or iodine (D–F) as quenchers. A,D: Lactoferrin. B,E: Ovotransferrin. C,F: Serum transferrin.

fence against microbial infections. This report shows that the most significant members of this family are able to act on biological membranes in a closely related manner. This mechanism could constitute the molecular basis of the antibacterial activity of the transferrin family.

Interaction of the transferrins with the anionic *E. coli* outer membrane can be hypothesized due to the remarkable cationic nature of these molecules. This subject was studied using the hydrophobic antibiotic actinomycin D, able to inhibit RNA synthesis, bactericidal against Gram-positive bacteria but with a restricted effect against Gram-negative bacteria due to the charged lipopolysaccharides. Neither the addition of any of the transferrins nor the antibiotic produced any detectable change in protein biosynthesis. However, their simultaneous addition allowed the permeation of actinomycin D through the outer membrane, so inhibiting RNA and protein synthesis, showing a permeabilization effect on the outer membrane mediated by transferrins.

Assays to detect inner membrane permeabilization were also performed. Exposure to serum transferrin, ovotransferrin or lactoferrin did not allow the efflux of previously accumulated [ $^3\text{H}$ ]uridine, indicating the inability of these proteins to cause extensive damage. Nevertheless, spectrometric determinations showed a marked increase in extracellular [ $\text{K}^+$ ] induced by these proteins, without any detectable changes in [ $\text{Na}^+$ ]. These results could indicate a permeabilization of ions that would have an important effect on the transmembrane ionic gradients. In fact, *in vivo* determinations of  $\Delta\Psi$  on *E. coli* cells showed that transferrins were able to produce a rapid membrane depolarization. Nevertheless, contrary to our expectations, these proteins were not able to dissipate the transmembrane  $\Delta\text{pH}$ .

Actually, a slight internal cell alkalinization was detected resulting in a small increase in  $\Delta\text{pH}$ . Our results point to a selective permeation of some ions through the inner membrane mediated by transferrins. This event produced a bacteriostatic effect without affecting *E. coli* viability in agreement with previous reports [16].

To demonstrate the selective ion permeation, we used artificial membrane systems. Addition of serum transferrin, ovotransferrin or lactoferrin to  $\text{K}^+$ -loaded liposomes diluted in  $\text{Na}^+$  buffer had no effect when a previous  $\Delta\Psi$  was generated by the  $\text{K}^+$  ionophore valinomycin. However,  $\Delta\Psi$  could be dissipated by  $\text{Na}^+$  permeation mediated by the cation ionophore nigericin. Nigericin is a water-insoluble polyether antibiotic and mainly, but not strictly, a potassium/proton exchanger. Nigericin can also complex (although with less affinity) rubidium, sodium, cesium and lithium. In a situation when a single cation predominates, the cation is exchanged for  $\text{H}^+$ . Under the conditions tested, sodium is the cation that predominates outside the liposome.

Addition of valinomycin to the system allowed  $\text{K}^+$  efflux from the liposome, therefore creating a  $\Delta\Psi$  negative inside the liposome. Under the assayed conditions, following addition of nigericin should lead to an electroneutral  $\text{Na}^+/\text{H}^+$  exchange. However, according to Donnan equilibrium, movement of ions through a membrane is ruled by the charge distribution across the membrane and the concentration. Both factors contribute to a favorable release of  $\text{Na}^+$  ions inside the liposome. However, the movement of  $\text{H}^+$  from the inside to the outside of the liposome against the concentration gradient (pH is the same at both sides of the membrane) and charge (negative

inside the liposome) is thermodynamically less favorable. Under these conditions, the observable effect upon addition of nigericin is a dissipation of  $\Delta\Psi$  created by valinomycin [22].

Interestingly, transferrins were able to generate a  $\Delta\Psi$  when directly added to  $\text{K}^+$ -loaded liposomes. Later addition of valinomycin produced no further effects; however, this  $\Delta\Psi$  could be dissipated by nigericin. Moreover, none of the transferrins was capable of establishing a  $\Delta\Psi$  after their addition to  $\text{Na}^+$ -loaded liposomes diluted in  $\text{K}^+$  buffer, nor to modify a previously generated  $\Delta\text{pH}$ . These results show that transferrins act directly on a lipid bilayer selectively permeating ions such as  $\text{K}^+$  and excluding others such as  $\text{Na}^+$  or  $\text{H}^+$ . These data correlate with those obtained for living cells, and support a molecular explanation for them.

Serum transferrin, ovotransferrin and lactoferrin are soluble proteins. Interaction with the cell inner membrane should involve some sort of conformational changes. Alterations in the secondary structure were determined by far-UV CD. An increase in the  $\alpha$ -helical content for the liposome-bound lactoferrin and, to a lesser extent, for ovotransferrin was observed.

Lactoferrin, ovotransferrin and serum transferrin contain 10 conserved tryptophan residues. The presence of an aromatic indole group in this amino acid allows for the use of its intrinsic fluorescence to observe protein structural changes. Modifications of the intrinsic tryptophan fluorescence intensity and of the maximum emission wavelength evidence changes in the amino acid's physico-chemical surroundings due to the protein-liposome binding, protein structural changes or both phenomena [23]. Protein folding alterations result in the burial of certain amino acid residues from the external aqueous environment and/or exposure of other amino acids previously buried. It is possible to check this sort of modifications by registering differences of tryptophan accessibility to low molecular weight fluorescence quenching agents. In proteins and peptides having only one tryptophanyl residue in their amino acid sequence, the fluorescence emission of the molecule is decreased in a linear way upon increasing the concentration of the quenching agent. Quenching patterns for most multi-tryptophan-containing proteins are difficult to analyze, but qualitative information can, nevertheless, be extracted [23]. All the transferrins analyzed produced changes upon binding to liposomes, as shown by a clear decrease of the quenching mediated by the ionic iodine. Since a protein is a polyelectrolyte, electrostatic effects, due to the presence of the basic residues arginine and lysine in the lactoferrin amino acid sequence flanking the tryptophan molecule, might influence the potassium iodide quenching effect [24]. Acrylamide is a polar, non-ionic molecule and does not share the features of KI. It is able to sense the exposure of residues in a purely random fashion [23]. Stern-Volmer plots carried out in the presence of the polar, non-ionic acrylamide showed an increased fluorescence quenching in all cases, which eliminates the possibility that the observed effects could be exclusively mediated by binding to liposomes.

In summary, in this report we describe a molecular mechanism common to all transferrins studied that can constitute the basis of their antimicrobial effect. Serum transferrin, ovotransferrin and lactoferrin are able to permeate the *E. coli* outer membrane and to access the inner membrane, where they cause permeation of ions in a selective manner. This effect can be measured both in cells and in artificial membranes, and allows the selective permeabilization of at least

K<sup>+</sup>, without any action on Na<sup>+</sup> or H<sup>+</sup>, although action on other ions must be considered and would need future analysis. The consequence is an in vivo dissipation of  $\Delta\Psi$  without a significant alteration of  $\Delta\text{pH}$  and a decrease of the electrochemical potential from  $-198$  mV to  $-56$  mV. As a result of the uncoupling of the respiration-dependent energy production, bacteria enter bacteriostasis.

*Acknowledgements:* We would like to thank Dr. Carmen Herranz and Prof. Arnold J.M. Driessen for critically reading the manuscript. The Instituto Universitario de Oncología is supported by Obra Social Cajastur-Asturias.

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