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THE EFFECTS OF THE MEMBRANE-PENETRATING POLYPEPTIDE SEGMENT OF THE HUMAN ERYTHROCYTE MN-GLYCOPROTEIN ON THE PERMEABILITY OF MODEL LIPID MEMBRANES

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SUMMARY

The insoluble peptide, T(is), prepared by trypsin hydrolysis of the MN-glycoprotein (glycophorin) of the human erythrocyte has been incorporated into phospholipid membranes in the form of liposomes and black lipid membranes. The permeability of liposome membranes to $^{42}K^+$ and of black lipid membranes to water and ions is increased significantly by the presence of the T(is) peptide. Electrophoresis measurements indicate that these effects are not due to the T(is) peptide carrying a net charge. The results suggest that the peptide causes local disordering of the bilayer membrane structures. This is considered in the light of findings published elsewhere: that the MN-glycoprotein penetrates through the cell membrane via a non-polar segment of its polypeptide chain, which is contained intact within the T(is) peptide; that the T(is) peptide is partially helical when associated with phospholipid and forms multimeric 8.0 nm structures within the hydrophobic plane of phospholipid bilayers.

INTRODUCTION

Permeability properties of two lipid membrane systems ((a) phospholipid vesicles (liposomes) [1] and (b) lipid bilayers [2, 3]) have been valuable in interpreting some aspects of natural membrane permeability. The advantages and disadvantages of the two model systems are now well appreciated. Recently, as experimental techniques have improved, attention has focussed on the role of proteins and peptides and their interactions with lipid membranes in controlling natural membrane passive permeability [4, 5]. In using model systems in this context, it is most essential that the membrane protein under study and its association with lipid membranes be well characterized. Unfortunately this situation is realized for few membrane proteins.

The membrane protein whose in situ structure is perhaps most clearly defined is the major surface glycoprotein of the human erythrocyte, the MN-glycoprotein (or by its other name, glycophorin [6]). This glycoprotein has been shown fairly

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convincingly to penetrate the erythrocyte membrane [7–9]. The intramembranous portion of this molecule has a known amino acid sequence [10] with a linear distribution of polar and nonpolar residues with the same polarity as the cross section of a phospholipid bilayer. The 23 residue central hydrophobic portion of nonpolar domain of this sequence belongs to a special class of membrane penetrating protein segments on the basis of degree of hydrophobicity [11, 12]. This non-polar domain is contained intact within an insoluble peptide T(is), produced by trypsin treatment of the solubilized glycoprotein [7, 10]. The T(is) peptide can be incorporated into phospholipid bilayers and under these conditions is largely helical [13]. At appropriate concentrations the T(is) peptide forms multimeric 8 nm structures within the hydrophobic plane of the bilayer as demonstrated by freeze-etch electron microscopy [15]. In the present paper we report some results from permeability studies on phospholipid membranes containing the T(is) peptide. The effect of this peptide on membrane permeability is of particular interest because of the intramembranous location of the nonpolar domain of the MN-glycoprotein.

MATERIALS AND METHODS

The T(is) peptide was prepared by methods described elsewhere [7, 10, 13] and for the permeability studies was used without delipidation. It has been established [13] that undelipidated T(is) peptide contains 50% (w/w) lipid (mainly cholesterol), and in preparing lipid/T(is) peptide mixtures this was taken into account in calculating the lipid: T(is) peptide molar ratios.

Lecithin was prepared from egg yolks by the method of Singleton et al. [14]. Phosphatidyl inositol was prepared as the sodium salt from wheat germ. *n*-Decane, obtained from Hopkin and Williams and from Standard Oil Company, was purified by redistillation and passage through an alumina column. Cholesterol was Fluka puriss grade. The ^{42}KCl (code PES.IP) was obtained from the Radiochemical Centre, Amersham, England. All other chemicals were AR grade.

The solubilization of the T(is) peptide in ethanol

The T(is) peptide is insoluble in water. Therefore to incorporate it into lipid membranes it is necessary to first solubilize it in ethanol. 20 μl trifluoroacetic acid was added to 1 mg T(is) peptide at the bottom of a pear shaped flask. Immediately, 0.4 ml of trifluoroethanol was mixed with it. The resulting solution was evaporated to dryness using a rotary evaporator. The residue was three times dissolved in ethanol to form a solution which was subsequently evaporated to dryness before dessicating in vacuo over P_2O_5 to remove traces of solvents.

Preparation of mixtures of lipids and T(is) peptides

Ethanol soluble T(is) peptide was dissolved with lipids in ethanol, taken to dryness using a rotary evaporator and dessicated in vacuo over P_2O_5.

^{42}K leakage from liposomes

Multilamellar liposomes were prepared from either dry phospholipids alone or dry phospholipids+T(is) peptide (at a molar ratio of 350 to 1) by mechanically shaking the mixtures in 0.15 M KCl containing $^{42}K^+$. The lipid dispersions (9.6–10.1 μmol/ml) were equilibrated at room temperature (23$\pm$1 °C) for 30 min. 0.5 ml

aliquots of each dispersion were then transferred to dialysis tubes (1 cm diameter) and dialysed against 500 ml quantities of 0.15 M KCl at 4 °C until the radioactivity of the diffusates were indistinguishable from background radioactivity. The dialysis tubes were then placed in test tubes (13 mm internal diameter) containing 12 ml 0.15 M KCl at room temperature. At 35 min intervals the bags were transferred to new tubes. The dialysis continued in this way for up to 5 consecutive periods. Throughout, the tubes were shaken mechanically. The radioactivity leaving a bag was measured and expressed as a percentage of the total radioactivity trapped in that bag at the beginning of the relevant period.

Electron microscopy was used to assess whether incorporation of the T(is) peptide into liposomes leads to gross changes in the multilamellar structures. Liposomes were prepared in the same way as for ^{42}K leakage experiments except that they were formed in 0.15 M ammonium acetate (pH 7). The dispersions were negatively stained with a solution containing 2 % ammonium molybdate and ammonium acetate to bring the osmolarity to the same value as the solution used for dispersing the liposomes. Use of ammonium acetate, rather than KCl avoided crystallization of salts on the microscope grid. Liposome surface area: volume ratios were measured on the electron micrographs using the line intersection and point counting stereology methods of Elias et al. [21].

Electrophoretic mobilities of lecithin liposomes containing the T(is) peptide (with a lecithin: T(is) peptide molar ratio of 120 : 1) were measured in 5 mM KCl as a function of pH at 21 ± 1 °C using a cylindrical micro-electrophoresis apparatus [17]. The pH of the liposome dispersions was adjusted by addition of HCl or KOH. Above pH 7 the dispersions were prepared under an atmosphere of nitrogen. The T(is) peptide concentration was greater than that used for the permeability studies, since it was anticipated that if T(is) carried a net charge at pH 5.7, this charge, and hence the mobilities, would be very low.

Permeability of black lipid membranes

Membranes were made by the brush technique [18] on PTFCE supports in 100 mM KCl. Black film areas were measured with a graticule in an eye-piece of a binocular microscope.

Osmotic water permeabilities were measured on horizontal black lipid membranes using an apparatus identical to that of Henson et al. [19] except that in the present work the membrane was illuminated via a fiber optic light pipe placed close to the membrane. Volume changes of 0.005 μl in the closed (lower) compartment could be detected. Experiments were carried out in a constant temperature room at 21 ± 0.05 °C. A thermistor in the open compartment of the apparatus showed that the temperature was constant within 0.01 °C over a 5 h period. The measurements were made following the method of Graham and Lea [20] using either KCl or sucrose to impose an osmotic gradient across the membrane. A small volume flow was often observed in the absence of any osmotic gradient. This was usually in the direction from the closed to the open compartment. This drift has been observed by other workers, and in the present case was attributed to continuous distortion of the plastic forming the closed compartment when clamped under pressure. Before the solution in the open compartment was changed any volume change was measured and subtracted from that measured under an imposed osmotic gradient.

The permeability coefficient, P_{os}, was calculated from the relationship:

$$P_{os} = \frac{J_v}{A\Delta\pi} \cdot \frac{RT}{\bar{V}_w}$$

where J_v = measured volume flow, A = bilayer area, $\Delta\pi$ = osmotic pressure difference, and $\bar{V}_w$ = partial molar volume of water.

For the water permeability measurements, membranes were made from 0.3 % (w/v) solutions of either lecithin and cholesterol (76 : 15 w/w) or lecithin, cholesterol, and peptide (152 : 30 : 1 w/w) in *n*-decane (a molar ratio of peptide to lipid of approximately 1 to 1000).

For measurements of electrical resistance, films were formed from 0.25 % (w/v) solutions of lecithin alone and lecithin with peptide (75 : 1 w/w or a molar ratio of 370 : 1) in *n*-decane, on a vertical 2.5 mm diameter hole. Ag-AgCl electrodes, one on each side of the membrane were used for measurement of the potential difference when a small current was passed between them. Because leakage artifacts can result from applied electric fields, the currents and thus the potential differences were kept as low as possible (of the order 10^{-10} amps and 10 mV, respectively).

RESULTS

Table I shows the results of $^{42}K^+$ leakage from liposomes. The experiments were carried out in pairs, so that liposomes containing the T(is) peptide were treated in the same way as liposomes of the same phospholipid composition without the peptide. Incorporation of peptide causes a marked increase in the rate of $^{42}K^+$ leakage. The effect is larger for liposomes without phosphatidyl inositol.

The cation permeability of lipid membranes depends among other factors on the surface charge of the membranes [21]. It was confirmed that from pH 4 to pH 8 pure lecithin liposomes had zero electrophoretic mobility and hence no net surface charge. Fig. 1 shows the electrophoretic mobilities of lecithin - T(is) peptide liposomes in 5 mM KCl as a function of pH. These measurements were made with a lipidated T(is) peptide sample. At pH 5.7 the mobility, and hence the surface charge density,

TABLE I

LEAKAGE OF $^{42}K^+$ FROM LIPOSOMES DISPERSED IN 0.15 M KCl

Interlamellar spacing is measured in arbitrary units, and given as mean ± S.D. The number in brackets gives the number of measurements.

Lecithin (μmol/ml)	Phosphatidyl-inositol (μmol/ml)	T(is) (μmol/ml)	$^{42}K^+$ leakage expressed as % of total $^{42}K^+$ trapped at start of each period				Electron microscopy data	
			1 st 35 min	2 nd 35 min	3 rd 35 min	4th 35 min	Interlamellar spacing	$\frac{S}{V}$
9.56	–	–	0.62	0.47	0.34	0.32	4.38 ± 0.35 (60)	0.72
9.56	–	0.027	8.6	9.2	7.8	7.3	4.97 ± 0.24 (35)	0.79
9.56	0.50	–	1.15	0.80	0.58	0.48	4.33 ± 0.42 (15)	0.80
9.56	0.50	0.027	9.26	9.92	8.56	7.87	4.60 ± 0.43 (57)	0.82

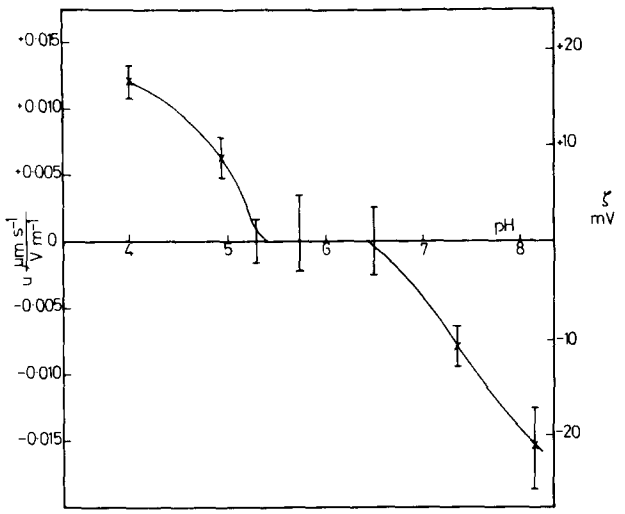


Fig. 1. The electrophoretic mobilities, u, (left hand ordinate) and ζ potentials (right hand ordinate) of lecithin/T(is) peptide liposomes as a function of pH. The molar composition of the liposomes was 1 mol T(is) peptide: 120 mol lecithin. Potentials in mV were calculated using the relationship $\zeta = 1365\,u$, where u is expressed in $\mu m\ s^{-1}/(Vm^{-1})$. The results are given as mean and S.D., except between pH 5 and pH 6.5. In this range the bars represent the maximum and minimum observed values. This method of presenting the results was used because of shortage of material, and when the mobility was very low, insufficient particles moved into the field of view to allow calculation of S.D. values comparable with those of other pH values.

are indistinguishable from zero. However, subsequent measurements with a delipidated T(is) peptide sample gave the electrophoretic mobility at pH 5.8 as 0.008 ± 0.004 $\mu m\ s^{-1}/V\ m^{-1}$. It seems, therefore, that there is some variation between T(is) peptide samples and that this variation may be associated with whether the T(is) peptide has been delipidated.

The rate of $^{42}K^+$ leakage from multilamellar liposomes depends also on the interlamellar spacings and the surface area: volume ratio, S/V. The results (summarised in Table I, Columns 8 and 9) of measurements on electron micrographs show that neither the interlamellar spacings nor the surface: volume ratios of the liposomes are significantly altered in the presence of the T(is) peptide. This is in accord with the electrophoresis results which indicate that in 150 mM salt at pH 7 the ζ potential would be $\leqslant 1$ mV and hence interlamellar repulsion would be very low.

At pH 5.7 ± 0.3 any net charge is even less significant. The observed differences in $^{42}K^+$ leakage from liposomes can therefore be considered in terms of the effect of the T(is) peptide on the bilayer structure.

Fig. 2 shows results of a typical experiment to measure black lipid membrane osmotic water permeability. Either KCl or sucrose was used to impose an osmotic gradient across the membrane. P_{os} values for both solutes fell within the same range. However, as Table II shows, the results for membranes containing T(is) peptide were more variable than those without. This could be due to different amounts of peptide being incorporated into the bilayer part of the membrane. The average value for P_{os} for membrane without peptide was 30.1 ± 2.6 $\mu m\ s^{-1}$ compared to 41.4 ± 5.7 $\mu m\ s^{-1}$

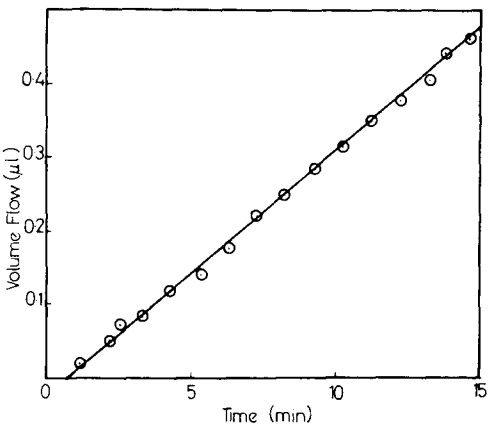


Fig. 2. Volume flow in μl as a function of time for a black lipid membrane containing T(is) peptide. The osmotic pressure difference was 277 mosm, and film area 2.4 mm^2. For this experiment P_{os} = 47.0 μm s^{-1}.

with peptide. Student's t test gives the difference as significant at the 1 % confidence level.

Table III shows that the mean resistance of black lipid membranes formed from solutions of lecithin in *n*-decane is $(7.8\pm1.2)\times10^8$ Ω cm^2 (mean and S.E. for 10 films) and the mean resistance of membranes formed from solutions of lecithin with peptide (75 : 1 w/w) is $(6.3\pm0.9)\times10^6$ Ω cm^2 (for 11 films).

TABLE II

OSMOTIC WATER PERMEABILITY COEFFICIENTS

p = 0.01.

	$P_{os}\pm$S.D. μm · s^{-1}	No. of experiments	Range of values μm · s^{-1}
No T(is)	30.1±2.6	11	24.4–33.6
+ T(is)	41.4±5.7	5	35.5–47.3

TABLE III

RESISTANCE OF BLACK LIPID MEMBRANES

	Resistance (Ω cm^2) (Mean ±S.E.)	No. of films
No T(is)	$(7.8\pm1.2)\cdot10^8$	10
+ T(is)	$(6.3\pm0.9)\cdot10^6$	11

DISCUSSION

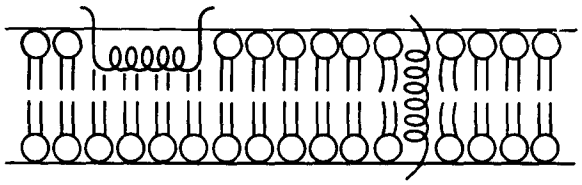
The trypsin-insoluble peptide T(is) of the MN-glycoprotein has a molecular weight of about 3750 and is composed of 35 residues with a 23 residue run of non-polar residues [7, 10]. From measurements of circular dichroism the peptide has been shown to be 75 % helix in lecithin liposomes [12, 13]. In this form the length of the peptide would be sufficient to span the hydrocarbon part of the bilayer. Freeze-etch studies of lecithin/T(is) peptide/water mixtures show that at a peptide: lecithin molar ratio greater than 1 : 90–200 (the critical multimer concentration) intramembranous particles are present in the hydrocarbon "middle" of the bilayer. These particles appear as pore-like structures with a doughnut shape. Their number increases with the T(is) peptide concentration in such a way as to suggest they represent multimeric aggregates of peptides [15]. Below the critical multimer concentration the peptide seems to be incorporated monomerically into the lecithin bilayer [15].

In the present work we report that incorporation of the T(is) peptide into phospholipid membranes causes an increase in the membrane permeability to water and cations. The water permeability measurements on membranes were made at a T(is) peptide concentration well below the critical multimer concentration. Hence we can assume the 30 % increase in the value of P_{os} when the peptide is present is due to the effect of single peptide molecules on the permeability barrier. Previous workers [20, 22, 24] have shown that the water permeability of black lipid membranes is consistent with a simple solubility-diffusion mechanism. That is, the rate of water permeation is determined by the water diffusion coefficient and solubility in the hydrocarbon region of the bilayer. If this picture is correct then single T(is) peptide molecules must increase the water permeability by causing local increases in either the diffusion coefficient, or the solubility, or as seems most probable, both these parameters. The T(is) peptide helices although unusually hydrophobic are more polar than the paraffin chains of the lecithin. Thus, the water solubility in the hydrocarbon region should increase in the presence of T(is) peptide. Moreover, whether the peptide molecules are incorporated perpendicular or parallel to the plane of the membrane (see Fig. 3) they are likely to induce some local disordering of the bilayer structure. Trauble [22] has suggested that the diffusion of water in a lipid bilayer is controlled by movements of "kinks" or "free spaces" through the hydrocarbon phase. Disordering of the paraffin chains by T(is) peptide molecules would increase the kink concentration and hence increase the water diffusion rate. It should be pointed out that the P_{os} measurements were made with some cholesterol in the membranes. Cholesterol has a condensing effect on the lecithin paraffin chains [25, 26], and may act to damp out any disordering effect of the T(is) peptide [27].

Measurements of $^{42}K^+$ leakage from liposomes gave average ratios for:

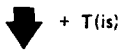
$$\frac{^{42}K^+ \text{ released in the presence of the T(is) peptide}}{^{42}K^+ \text{ released without the T(is) peptide}}$$

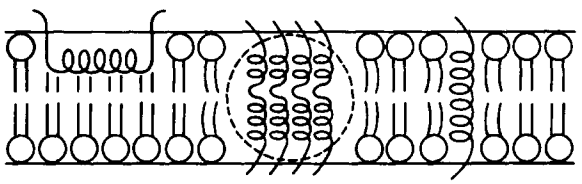
of 20 and 12.9 in the systems containing lecithin alone, and lecithin with phosphatidyl inositol respectively. We have presented some evidence based on electron microscopy indicating that incorporation of T(is) peptide into liposomes does not cause any drastic changes in liposome structure. Therefore, it seems that the peptide increases $^{42}K^+$ leakage rates by, either giving the lipid membranes a net negative charge, or as



LEC/T(is) **120**

BELOW CRITICAL MULTIMER CONCENTRATION





LEC/T(is) **40**

ABOVE CRITICAL MULTIMER CONCENTRATION

Fig. 3. Schematic diagram of proposed interactions of the nonpolar domain containing the T(is) peptide with liposome membranes and/or black films. The peptide monomer which is partially helical when associated with lecithin [14, 16] could be either oriented perpendicular or parallel to the paraffin chains of the phospholipid bilayer when present below a certain concentration (the critical multimer concentration). This is shown in the upper diagram. When the concentration of peptide exceeds the critical multimer concentration, multimeric units are formed as shown in the lower diagram.

postulated earlier, inducing some local disordering and increase in polarity of the bilayer structure.

The T(is) peptide has four ionizable groups: two glutamates ($pK_a = 4.5$ [28]) and two histidines ($pK_a = 6.5$ [29]). The net surface charge density and surface potential of lecithin/T(is) peptide membranes at pH 5.7 should be extremely low. This is confirmed by the electrophoresis results (see Fig. 1).

Therefore, the main effect of the T(is) peptide on $^{42}K^+$ leakage from liposomes is likely to be due to some change in organisation of the bilayer structure. For ions and polar solutes there is some evidence [30, 31] to suggest that the rate determining step for permeation across lipid membranes is penetration from the aqueous solution into the hydrocarbon region. It seems probable that, if the peptide disturbs the "tight" barrier [32] close to the lipid polar groups, cation permeability would be enhanced.

Recently attention has been drawn to the possible importance of dipole potentials in affecting in permeation rates through lipid membranes [33]. The T(is) peptide may well alter the potential profile at the membrane surface and hence alter the cation permeation rates.

The fact that the T(is) peptide has a smaller effect on $^{42}K^+$ leakage rates when phosphatidyl inositol is present in a reflection of the higher intrinsic cation

permeability of negatively charged phospholipid membranes. The negative charges increase the area per lipid molecule by charged head group repulsion, leading to a decrease in thickness and increase in disorder of the bilayer. In addition, the local cation concentration at the membrane is increased.

The effect of the peptide on black lipid membrane conductance is an order of magnitude larger than its effect on $^{42}K^+$ leakage from liposomes. This observation may reflect the importance of *n*-decane in determining the nature of the permeability barrier. However, it is also possible that the T(is) peptide increases anion permeability.

In all these permeability studies the molar ratio of T(is) peptide: lipid was below the critical multimer concentration for the peptide. Experiments to examine the effect of T(is) peptide at concentrations greater than its critical multimer concentration, when multimeric units are known to be present (see Fig. 3) are currently under way.

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