COMPARISON OF THE EFFECTS OF INORGANIC AND ORGANIC (HYDROPHOBIC) CATIONS ON THE PHYSICAL STATES OF PROTEINS AND PHOSPHOLIPID BILAYER MEMBRANES

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Received 25 May 1977

1. Introduction

Earlier studies [1,2] have been shown that hydrophobically hydrated organic cations such as tetraethylammonium and acetylcholine have quite different effects to sodium and potassium ions (which are electrostrictively hydrated) on the dispersion properties of acidic phospholipids. The organic ions salt-in single bilayer vesicles of phosphatidylserine whereas sodium and calcium ions promote bilayer fusion and formation of multilamellar precipitates (i.e., they salt-out). Quite apart from this modification of inter-bilayer interactions, addition of acetylcholine can perturb the equilibrium between lamellar and hexagonal phospholipid phases [3] and the question arises as to whether the intra-bilayer equilibrium between gel and liquid–crystalline phases [4,5] can be displaced also. Although these consequences of phospholipid–acetylcholine interactions may be pertinent to the function of acetylcholine in the synapse where vesicles contain concentrations of up to ~ 0.1 M, it is clear that interactions with the membrane proteins must be considered as well. Therefore, it is important to establish whether parallel effects to those observed with phospholipids also occur with proteins.

The objective of this report is to present a comparison of the effects of sodium, tetraethylammonium and acetylcholine halides on (1) the aggregation properties of proteins and (2) the thermally induced order–disorder transitions of globular proteins and phospholipid bilayers (i.e., heat denaturation and gel–liquid crystal transition, respectively). In agreement with the earlier results for phosphatidylserine vesicles [1,2], we find that 1 M potassium chloride precipitates (i.e., salts-out) 50% of an acidic protein such as ovalbumin, whereas acetylcholine chloride has no effect at concentrations up to 2 M (i.e., it salts-in). These inorganic and organic cations also have opposite effects on the temperatures of the thermal denaturation of proteins and gel–liquid crystal transition of neutral (isoelectric) phosphatidylcholine bilayers; sodium ions tend to increase both temperatures whereas tetraethylammonium ions tend to decrease both temperatures via a reduction in the hydrophobic free energy which stabilises the ordered phases. The effect of acetylcholine is smaller than that of tetraethylammonium, but in the same direction.

2. Experimental

2.1. *Chemicals*

Salt-free, crystallised and lyophilised ovalbumin (Grade V, Sigma Chemical Co., London, England)

was used without further purification. 5-Doxylstearic acid was obtained from Syva (Palo Alto, USA). The remaining chemicals have been described before [1,2].

2.2. *Methods*

The heat denaturation of ovalbumin was monitored by determining the amount of protein precipitated over the temperature range 40–100°C. Solutions (0.2% w/w) of protein in 0.015 M phosphate buffer (pH 7.9) plus added solute were incubated in a Haake water bath at a specified temperature (± 0.2°C) for 20 min. After cooling to room temperature and filtering at 100–150 p.s.i. through a 200 Å or 1000 Å pore-size Sartorius filter, the concentration of protein remaining undenatured and in solution was determined from the absorbance at 280 nm. The temperature at which 50% of the protein was precipitated was taken as the mid-point temperature (T_m). The aggregation of ovalbumin caused by addition of solute at room temperature was monitored by a similar procedure; the precipitate was removed by filtration after a 20 min period.

The gel to liquid crystal transition temperature (T_c) of dispersions of phospholipid in excess water was determined by a spin-label procedure [6]. 5-Doxylstearic acid was codispersed with phospholipid in salt solutions containing the appropriate amount of solute to give 0.5–1% dispersions (lipid/spin-label mol ratio, 200/1). The ESR spectra were recorded on a Varian E-104A spectrometer at about 3° increments over the temperature range 10–70°C. T_c was derived to ± 1°C from the maximum slope of the plots of the hyperfine splittings $2T_{||}$ against temperature.

3. Results

The effects of increasing concentrations of potassium chloride and acetylcholine chloride on the precipitation of ovalbumin molecules and phosphatidylserine vesicles are compared in fig.1. It is apparent that potassium at concentrations of 1.5 M precipitates or salts-out ~ 70% and 60% of the ovalbumin and phosphatidylserine, respectively, whereas acetylcholine does not cause precipitation at concentrations up to 2 M (cf. [1,2]).

Quite apart from the above variations in aggregation behaviour, alkali metal ions and hydrophobic cations can have quite different effects on the physical state of individual protein molecules and phospholipid bilayers. Thus, both T_m for the thermal denaturation of ovalbumin and ribonuclease (cf. [8]) and T_c for phosphatidylcholine bilayers can be affected by these materials. The data in table 1 show that increasing the concentration of sodium chloride up to 1 M leads to a small increase in T_m and T_c for ribonuclease and L-dipalmitoyl phosphatidylcholine, respectively. A similar effect was observed with 0.2% ovalbumin solutions, at pH 7.9 (cf. [7]) in that T_m was 78°C in the presence of 0.1 M and 0.5 M sodium chloride. However, 0.5 M acetylcholine had a different effect in this system because T_m was decreased to 72°C. As can be seen from table 1, the transition temperatures of ribonuclease and dipalmitoyl phosphatidylcholine are not very sensitive to acetylcholine whereas increasing concentrations of tetraethylammonium bromide decrease both temperatures. Essentially the same T_c was observed for phosphatidylcholine with tetraethylammonium bromide and chloride.

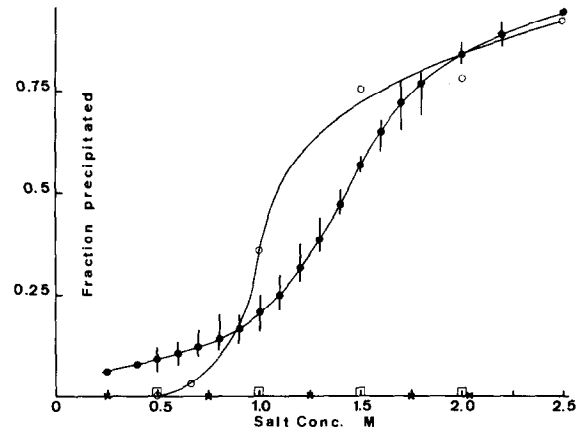

Fig.1. The precipitation of ovalbumin and phosphatidylserine as a function of added electrolyte concentration. The ovalbumin solution contained 0.2 w/w protein at pH 7.9 and either potassium chloride (○) or acetylcholine chloride (□) was added. Potassium chloride (●) and acetylcholine chloride (×) were added to sonicated phosphatidylserine dispersions prepared as described before [1,2]. The bars represent the spread of 3 experiments.

Table 1

Solute (M)		Protein T_m (°C)	Phospholipid T_c (°C)	
		Ribonuclease (0.4%, pH 7, 0.15 M KCl	Phosphatidylcholine (Excess aqueous phase, pH 6)	
		a	c	b
NaCl	0.1	61	39[d]	(23.7)
	0.5	61	39	–
	1.0	62	40.8	(24.8)
$(C_2H_5)_4NBr$	0.1	a	c	
	0.1	60	39	
	0.5	57	–	
	1.0	53	35	
			c	
Acetyl-Choline-Chloride	0.1	61	39	
	0.5	61	39	
	1.0	60	37	

[a] Data from [8]
[b] Data for L-dimyristoyl phosphatidylcholine from [9]
[c] Data obtained for L-dipalmitoyl phosphatidylcholine using the spin label method
[d] This value is ~ 2°C lower than the value determined by differential scanning calorimetry. The lower values obtained by the spin probe technique probably reflect local perturbations induced by the spin probe

4. Discussion

As mentioned before [2], the aggregation and precipitation of phosphatidylserine vesicles on addition of sodium or potassium ions can be explained in terms of a decrease in the electrostatic repulsion between particles. Tetraalkylammonium ions have a similar effect on the surface charge of phosphatidylserine vesicles but do not lead to precipitation. The reasons for this relative reduction in net attraction between vesicles are not entirely clear but could involve (1) a decrease in any hydrophobic contribution to aggregation [10] and (2) an added repulsive potential due to the bulkiness of bound tetraalkylammonium ions and/or retention of an increased hydration shell. Tetraalkylammonium and acetylcholine ions are hydrophobically hydrated [2,11]; the perturbation of water structure by acetylcholine is intermediate to that caused by tetramethylammonium and tetraethylammonium ions [2]. Indeed, the general physical properties of acetylcholine, such as surface activity, which depend upon its hydrophobicity, are intermediate to those of tetramethylammonium and tetraethylammonium cations.

Since the forces governing the aggregation of globular ovalbumin molecules [12] and phosphatidylserine vesicles are the same, the above considerations apply directly to the protein system. Thus, the salting-out of ovalbumin by sodium and the salting-in by acetylcholine (fig.1) is to be expected. These results suggest that membrane fusion and any concomitant exchange processes may be inhibited by the replacement of bound sodium ions by hydrophobically hydrated cations such as acetylcholine.

4.1. *Order–disorder transitions in proteins and phospholipid bilayers*

Since conformational transitions in either phospholipid molecules or associated protein molecules in a bilayer have an obvious significance with respect to membrane functions such as transport processes, it is of interest to know whether the exchange of ions with different hydration properties (e.g., sodium and acetylcholine) could perturb membrane structures. Because of its surface activity, acetylcholine may

reduce the hydrophobic contribution to the free energy of stabilisation of proteins (cf. [10]) or bilayers (cf. [13]). This means that in the presence of organic cations transfer of apolar groups from the interior of a protein molecule or phospholipid bilayer into contact with the aqueous phase becomes less unfavourable, as compared to the situation with only sodium ions present. Since both the transition of a globular protein from native to denatured state and the hydrocarbon chain-melting within a bilayer involve such a transfer, it is to be expected that these changes of state will respond similarly to addition of the solutes under consideration. Because the electrostatic effects are similar [1,2], ions such as sodium should stabilise the ordered form whereas acetylcholine and tetraalkylammonium ions should destabilise it. We have checked this by monitoring T_m and T_c as a function of solute concentration.

It has been established previously [10] that the action of hydrophobically hydrated solutes such as tetraalkylammonium or acetylcholine on the T_m of the two-state heat denaturation of proteins such as lysozyme and ribonuclease can be described semi-quantitatively by eq. (1).

$$\delta T = \frac{}{\Delta H_0}\,\Delta A \cdot \delta\gamma_h \qquad (1)$$

$\delta T = (T_s - T_o)$ where the subscripts 's' and 'o' refer to the system with and without solute, T_o and ΔH_o are the midpoint and enthalpy of denaturation, respectively, ΔA is the net change in surface area of hydrophobic residues exposed to solvent on denaturation, and $\delta\gamma_h$ is the change in hydrophobic interfacial free energy due to addition of solute. Since, to a first approximation, T_o and ΔH_o for lysozyme, ribonuclease and ovalbumin are similar (cf. data in [7,14,15]), for a given concentration of solute (i.e., a fixed $\delta\gamma_h$), δT is proportional to ΔA for these proteins. Now Chothia [16] and Teller [17] have given expressions for ΔA for the transition from native to random coil state as a function of molecular weight; from this approach, if all three proteins undergo the same degree of unfolding on denaturation, δT for ovalbumin should be larger by a factor of ~ 3.5 than the value for lysozyme or ribonuclease. Bearing in mind that $\delta\gamma_h$ for acetylcholine will be intermediate to that for tetramethylammonium and tetraethylammonium, the δT data seem to agree qualitatively, at least, with eq. (1). Quantitative treatment requires knowledge of the conformation of the heat denatured states of proteins so that ΔA can be calculated precisely.

Since the above considerations apply in general to the T_c of phosphatidylcholine bilayers, eq. (1) can be used to predict the changes in T_c expected on addition of solutes such as sodium, tetraethylammonium and acetylcholine. T_o and ΔH_o for phosphatidylcholine gel to liquid crystal transitions are known [4,5,20] and typically have values of about 320°K and 30 kJ. mol^{-1}, respectively. If the net changes in hydrophobic surface exposed to the aqueous phase (ΔA) can be expressed as the change in molecular area on chain-melting [21] then $\Delta A \sim 20$ Å^2. Substitution of the above values into eq. (1) indicates that with $\delta\gamma_h = 1$ mNm^{-1}, $\delta T \simeq 1$°C. In practice, 1 M sodium chloride gives $\delta\gamma_h = 1.6$ mNm^{-1} (ref. [18]) so that δT calculated from eq. (1) is ~ 2°C; this agrees with data in table 1. In contrast to sodium chloride, tetraethylammonium halides reduce the surface tension of water and $\delta\gamma_h = -3.5$ mNm^{-1} for the bromide [10,19]. Substitution of this value in eq. (1) gives $\delta T \simeq -3.5$°C as compared to the experimental value of −3°C (table 1). We can conclude that eq. (1) predicts satisfactorily the effects of the added solutes on the T_c of isoelectric phosphatidylcholine. However, the situation is more complicated with acidic phospholipids such as phosphatidylserine where changes in ionic strength can cause large alterations in the electrostatic free energy of the bilayer. Nonetheless, for a constant degree of dissociation of the phospholipid, eq. (1) should give the difference in T_c in the presence of the same concentration of sodium chloride and, say, tetraethylammonium chloride.

In summary, inorganic cations tend to stabilise the native form of globular proteins while promoting their aggregation, whereas organic (hydrophobic) cations such as acetylcholine tend to destabilise the native form although promoting the solubility of proteins in water. The two classes of cations give parallel effects on the aggregation properties of bilayers and the stability of the gel phase in phospholipid systems. The above effects may have biological

significance for molecules which are as apolar as tetraethylammonium halides, but it seems unlikely that the hydrophobicity of acetylcholine contributes significantly to its biological function.

Acknowledgements

We are indebted to J. Dickson for technical assistance. H.H. wishes to acknowledge financial support by the Swiss National Science Foundation under Grant No. 3.0090.73.

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