The Binding of Calcium to Phospholipid–Protein Complexes[1] (34325)

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Lipoproteins are a major substructure in many biosystems whose molecular structure remains to be determined. A key problem concerns the bond between phospholipids and proteins (1). Investigations of this interaction have shown that proteins with isoelectric points above pH 9 form water-insoluble complexes with net negatively charged phospholipid micelles at neutral pH (2). The composition of these complexes varies with pH, ionic strength, relative proportions of components and the basic groups on proteins in a manner consistent with an electrostatic interaction (2–6). Stoichiometric complexes are formed between the net basic groups in proteins (those not internally neutralized) and the phosphate groups in acidic phospholipids. Studies using X-ray diffraction (7) or monomolecular film techniques (8, 9) also suggest that the primary interaction between proteins and phospholipids results from electrostatic forces.

We have previously shown that calcium binding is a sensitive indicator of the electrostatic binding capacity of phospholipids for cations and is capable of detecting interactions in solution (10). It was therefore considered that the ability of proteins to mask calcium binding sites on phospholipids might provide a method for quantitative studies of the interactions of proteins with phospholipids.

In addition, the ternary complex between divalent cations, phospholipids and proteins is significant in many biological processes and deserves study (11). The present studies of calcium binding on phospholipid–protein complexes demonstrated the reversible, stoichiometric binding of phospholipids to proteins, provided indications of their affinity, and showed the advantages of binding methods. The data are consistent with an electrostatic interaction between the negative charges on phospholipids and positive charges on proteins. The strength of this interaction suggests that electrostatic bonding should occur in most lipoproteins.

Methods and Materials. The binding of calcium with mixtures of protein and phospholipid was studied by equilibrium dialysis. The details of our procedure were previously reported (10). The protein and phospholipid studied were placed inside a cellophane dialyzing bag and dialyzed to equilibrium against a known solution of $CaCl_2$. The binding results are expressed as the moles of calcium bound per mole of phospholipid phosphorus in the inside compartment (Ca/P).

Two samples of animal cephalin (10), crystalline lysozyme, protamine sulfate, and bovine serum albumin, fraction V, were obtained from Nutritional Biochemicals Co. Quantitative thin-layer analysis indicated the cephalins were mainly phosphatidylserine, phosphatidylethanolamine, and phosphoinositides (10).

The protamine sulfate was converted to protamine chloride by passage through the chloride form of Dowex 1. The arginine content of the protamine chloride was 77% when assayed by a modified Sakaguchi reaction following a digestion in 6*N* HCl (12). The diffusibility of protamine complicated the calcium color assay; however, the addition of one drop of a 0.4% solution of undenatured deoxyribonucleic acid before the EDTA titration neutralized protamine interference. The binding of amino acids was studied with similar equilibrium dialysis techniques. Free ar-

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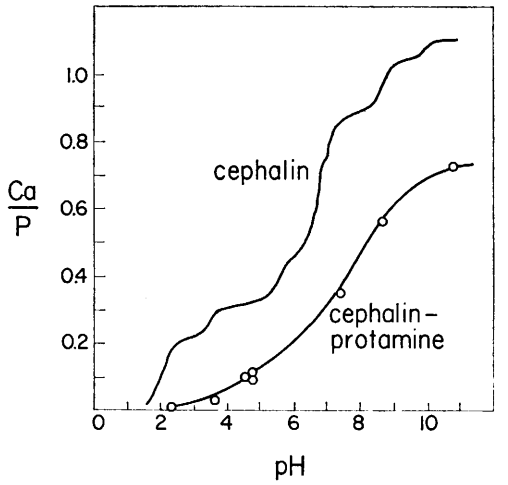

FIG. 1. Binding of calcium with animal cephalin in the presence with and absence of protamine; 2.6 m*M* cephalin-P, 2.4 meq protamine, 5 m*M* acetate and ammonium buffers.

ginine was assayed by the Sakaguchi reaction (12), and other amino acids were assayed by a Ninhydrin reaction (13).

Results. Figure 1 demonstrates the effect of pH on calcium binding to cephalin and to a cephalin–protamine complex. The complex binding curve seen with cephalin alone reflects stoichiometric binding of calcium to the various charged groups in this phospholipid mixture (10). Because this binding is dependent on the presence of a net negative charge, the pH dependence of binding reflects the acid dissociation constant of the binding group, or the dissociation constant of the cationic partner of a phospholipid zwitterion. The results in Fig. 1 indicate that protamine represses the calcium binding to cephalin in the pH range from 2 to 11. Above pH 7 this calcium binding competition by protamine approached a stoichiometric displacement of calcium by the positive arginine residues in protamine. Because at low pH there are fewer negative charges presumably more widely separated on the phospholipid micelle, the lack of stoichiometric binding below pH 7 could reflect these steric hindrances preventing binding of all the charge groups on the protamine. There is no calcium binding to protamine alone.

Figure 2 demonstrates that lysozyme competition for calcium binding by cephalins becomes less as the pH increases. This effect reflects the decrease in the net positive charge of lysozyme with higher pH. The different cephalin–calcium binding profile in Fig. 2 results from a somewhat different phospholipid composition. The overshoot in the binding above pH 10 reflects the binding of calcium to lysozyme which occurs only at these pH values (14).

The competition of lysozyme and protamine for calcium binding to animal cephalin is shown to be a linear function of protein concentration in Fig. 3. A straight line indicates a proportional displacement of calcium by the proteins. When calculated on the basis of the net positive charge on the proteins, the competition was stoichiometric at pH 8.8 for protamine and at pH 4.6 for lysozyme. The net positive charge was calculated for protamine from its arginine content and for lysozyme from the data of Jolles *et al.* (15).

The results in Fig. 3 show that the decrease in calcium binding upon addition of 0.71 meq of lysozyme is 0.14. If Ca/P = 0.14 and P = 2.6 m*M*, the calcium displaced = $0.14 \times 2.6 = 0.36$ m*M* = 0.72 meq. Comparison of 0.72 meq of Ca displaced by addition of 0.71 meq of lysozyme indicates stoichiometry. A similar calculation showed stoichiometric binding to protamine. At higher protamine and lysozyme concentrations there was less displacement of calcium, again suggesting steric factors hindering protein

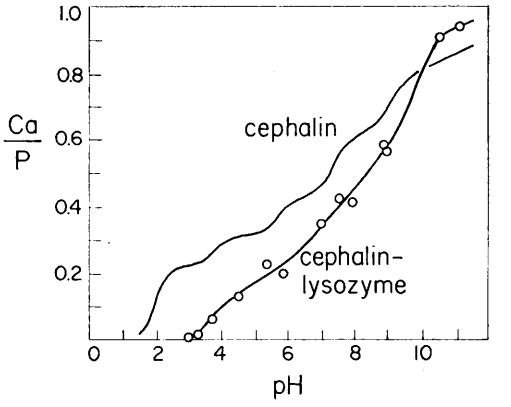

FIG. 2. Binding of calcium with animal cephalin in the presence and absence of lysozyme; 2.8 m*M* cephalin-P, 0.17 m*M* lysozyme.

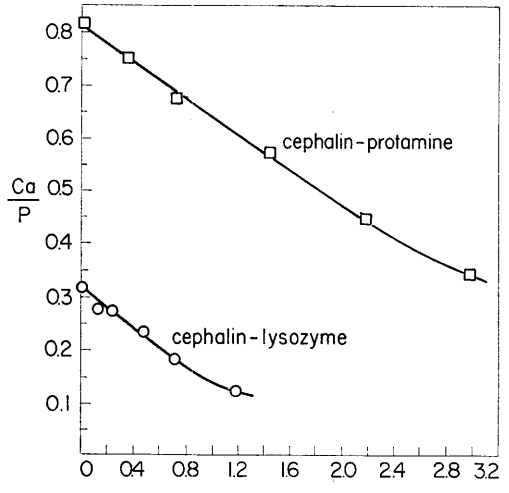

FIG. 3. Competition of increasing amounts of lysozyme and protamine for calcium binding sites on phospholipids; (□), protamine, pH 8.8, 2.45 m*M* cephalin-P, 5 m*M* ammonia buffer; (○), lysozyme, pH 4.6, 2.6 m*M* cephalin-P.

competition. These results suggest that the net positive protein charges of the protein will bind to phospholipids at proper pH and ionic environment when there is a large excess of phospholipid anionic groups. Similarly, all negative charges on the phospholipid will be bound to proteins in the presence of a sufficient excess of protein.

The ability of higher concentrations of free calcium ion to compete with protamine for cephalin binding sites is shown in Fig. 4. There was no calcium displacement below approximately 16 m*M* free calcium concentration where there was a stoichiometric displacement of calcium by protamine. However, above 16 m*M* calcium concentration, protamine was displaced from cephalin by calcium. Competition studies of this type enable comparison between the relative binding affinities of various substances. The data indicate a 30 times higher concentration of calcium was needed to displace protamine from cephalin than was needed to bind maximally to phospholipid alone.

A similar study which shows the calcium binding to cephalin in the presence and absence of lysozyme at two pH values and 4.7 and 12.0 m*M* free calcium is presented in Table I. Two controls with water in the inside compartment assayed 4.95 and 12.38 m*M* calcium. These binding data show that lysozyme competition was reversed approximately 50% with 12 m*M* free calcium.

TABLE I. The Binding of Calcium with Lysozyme–Cephalin Complexes.

Experimental conditions	Ca/P	
	Without lysozyme	With lysozyme
pH 4.7		
4.7 m*M* Ca^{2+}	0.24	0.084
12.0 m*M* Ca^{2+}	0.24	0.16
pH 8.8		
4.7 m*M* Ca^{2+}	0.64	0.55
12.0 m*M* Ca^{2+}	0.65	0.62

Some data on calcium binding to albumin–cephalin complexes at pH 8.8 are presented in Table II. The experimental format is similar to that of previous studies with the exception that in nearly all instances protamine and lysozyme did not require a correction for protein binding. The composition of the 10 ml of solution in the inside compartments is in the first column. Column 3 shows the equilibrium concentration of bound calcium in the inside. In column 4 is given the concentration of phospholipid bound calcium obtained by applying a correction for calcium binding to albumin which was determined in an experiment with-

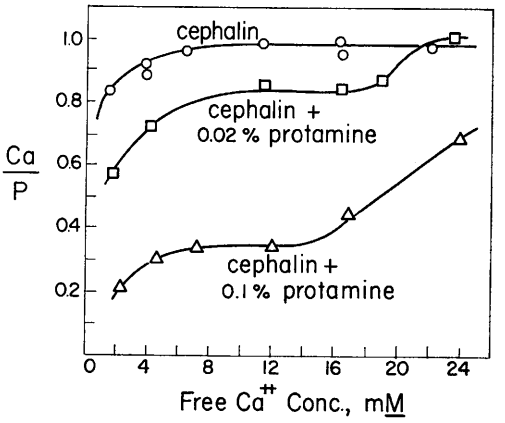

FIG. 4. Effect of calcium concentration on protamine binding cephalin; 2.3 m*M* cephalin-P.

TABLE II. The Binding of Calcium with Albumin-Cephalin Complexes at pH 8.8.

Composition of inside compartment	Calcium conc outside (m*M*)	Total calcium bound (m*M*)	Phospholipid bound calcium (m*M*)	Ca/P	Moles of Ca displaced/mole of albumin
Control, $CaCl_2$ only	4.90				
0.074 m*M* BSA + $CaCl_2$	4.48	0.84			
2.36 m*M* P + $CaCl_2$	3.83	2.14	2.14	0.91	
P, 2.36 m*M*; BSA, 0.015 m*M*	3.78	2.24	2.07	0.88	5
0.044	3.72	2.36	1.85	0.78	7
0.059	3.66	2.48	1.81	0.76	6
0.074	3.64	2.52	1.68	0.71	6

out phospholipid. The binding in terms of Ca/P) was calculated by dividing this value by the concentration of phospholipid phosphorus in the inside compartment.

The data indicate a linear relationship between the added albumin and the decrease in calcium binding at pH 8.8. The calculated values in column 6 of Table II indicate that one molecule of albumin released 12 equivalents of calcium from binding sites on cephalin. This suggests that under these conditions albumin was able to bind 12 molecules of phospholipid at pH 8.8. In another experiment at pH 4.0, there were 37 equivalents of calcium displaced at two different cephalin–albumin ratios.

Protamine binding to phospholipids was interpreted in terms of charge interactions through the arginine residues. As a check on this model, the binding of free arginine to animal cephalin was studied by single analysis, equilibrium dialysis techniques. Data obtained in absence of added cations indicate an appreciable binding of arginine. An animal cephalin sample (1 m*M* P) equilibrated with 0.03 m*M* arginine at pH 6.7 bound 0.03 $\pm$ 0.01 moles of arginine/mole of P. At pH 8.1 the same sample in 0.3 m*M* arginine bound 0.18 $\pm$ 0.02 moles of arginine/ mole of phosphorus. The addition of 10 m*M* sodium acetate at the same pH lowered binding 80%. A desalted cephalin sample (0.5 m*M* P) at pH 7.5 in 0.3 m*M* arginine bound 0.34 $\pm$ 0.05 moles of arginine/ mole of phosphorus. Donnan corrections were between 20 and 30% when measured potentiometrically across the membrane. It was concluded that a single arginine molecule will bind to cephalins but with considerably less affinity than protamine. The strong binding affinities found with proteins are likely the result of concerted attractions of several positive amino acids acting cooperatively.

Because many models for native lipoprotein systems suggest that the attraction between the hydrophobic regions of the lipid and protein results in stability, a series of equilibrium binding studies was conducted comparing the attraction of phospholipids for amino acids of varying hydrophobic character. The binding of glycine, phenylalanine, and leucine to commercial vegetable lecithin and animal cephalin samples was studied. Only about 0.05 moles of leucine and phenylalanine were bound per mole phosphorus at neutral pH and 6 m*M* amino acid concentration. There was no binding of 5 m*M* glycine at neutral pH. While it was reasonable to assume that neutral amino acid zwitterions cannot bind to phospholipid micelles through electrostatic interactions, the above data demonstrate that the hydrophobic character of individual amino acids does not favor interaction either.

Discussion. Three different proteins were shown to compete reversibly for calcium binding sites on phospholipids. When there were appreciably more cephalin charges than protein charges, one equivalent of (net) added lysozyme or protamine positive charge reduced the calcium binding capacity of cephalins by 1 equivalent. Because calcium has been shown to bind stoichiometrically to cephalins (10), it is concluded that proteins also bind to cephalins by an electrostatic stoichiometric interaction of the net positive

charges on the proteins and the net negative charged groups in the phospholipid micelles.

The ability of albumin to bind with cephalins at pH values basic to its isoelectric point was confirmed, a characteristic which is quite similar to fatty acid and detergent binding to albumin at pH 7 (16, 17). Because the availability of chemically pure detergents has resulted in more and better studies on the detergent–protein complexes, it is useful to make other comparisons of the binding characteristics of this group of ionic lipid–protein complexes to phospholipid–protein complexes. Mixtures of protein and detergents form insoluble electrostatic complexes at pH values acid to the protein isoelectric point and soluble complexes at pH values above this value. Once all the positive groups of the proteins are bound to detergent, additional detergent is bound through secondary bonds between the hydrophobic regions of the protein-bound detergent and those of the added detergent. This secondarily bound detergent may equal 10 times the total protein positive charge and may be removed by common lipid solvents and by freezing (18). Some reports in the literature and our own work indicate that phospholipid–protein complexes have characteristics similar to these described for detergent–protein complexes (2).

There has been little indication in the literature, however, that electrostatic models comparable to those used to explain detergent–protein binding are suitable for natural lipoproteins (1). This is mainly because natural lipoproteins are composed largely of phosphatidylcholine which is incapable of strong electrostatic binding (2) and because the ability of lipids and proteins to form complexes when both components are negatively charged has not been recognized generally. In view of the binding of negatively charged proteins with anionic phospholipids and the analogy to detergent binding, we suggest that even a few electrostatically bound anionic phospholipids could provide sites for hydrophobic binding of large amounts of phosphatidylcholine. The characteristics of many lipoprotein complexes appear consistent with this model. Furthermore, the strong affinity between ionic lipids and proteins has been attributed to the presence of binding sites with both hydrophobic and ionic character. Our studies show that phospholipids bind arginine but have little affinity for hydrophobic amino acids. While our hydrophobic amino acid models may not be representative of the hydrophobic regions on proteins, their lack of binding to phospholipids and the strong binding of protamine which has little hydrophobic character suggests that hydrophobic regions on proteins are not required for binding phospholipids.

The observation that the concentrations of calcium required to reverse phospholipid–protein complexes become less for proteins possessing less basic character suggests that there may be complexes with some proteins which are reversible in the physiological range of calcium concentration. Because the binding of calcium with certain phospholipids is sensitive to pH changes near the physiological range (10), it is possible that, indirectly through calcium competition, the binding of phospholipids with proteins is dependent upon small pH changes *in vivo*. There can also be a direct effect of pH as shown with cephalin–histones (5), phosphoinositides–brain protein (19) and phosphatidylserine–albumin (20). The ubiquitous nature of phospholipids in membranes suggests that one or more biological functions may be controlled by a coupling of changes in pH and calcium ion concentration which affects phospholipid–protein binding.

Summary. The ability of protamine, lysozyme, and bovine serum albumin to compete for calcium binding sites on acidic phospholipids provides a method for quantitative studies of the electrostatic interactions of proteins with phospholipids. The results demonstrate the reversible, stoichiometric binding of proteins with phospholipids. Crude cephalin binds arginine but not glycine, leucine, or phenylalanine. The data suggest a strong electrostatic interaction between negatively charged phospholipids and positively charged proteins and indicate that electrostatic bonding may occur in lipoproteins.

1. Gurd, F. R. N., *in* "Lipide Chemistry" (D. J.

Hanahan, ed.), Chaps. 8 and 9. Wiley, New York (1960).

2. Green, D. E. and Fleischer, S., *in* "Metabolism and Physiological Significance of Lipids," (R. M. C. Dawson and D. N. Rhodes, eds.) Wiley, New York (1964).

3. Reich, M. and Wainio, W. W., J. Biol. Chem. **236**, 3058 (1961).

4. Das, M. L. and Crane, F. L., Biochemistry **3**, 696 (1964).

5. Chargaff, E. and Ziff, M., J. Biol. Chem. **131**, 25 (1939).

6. Chargaff, E., J. Biol. Chem. **125**, 661 (1938).

7. Palmer, K. J., Schmitt, F. O., and Chargaff, E., J. Cell. Comp. Physiol. **18**, 43 (1941).

8. Fraser, M. J., Kaplan, J. G., and Schulman, J. H., Discussions Faraday Soc. **20**, 44 (1955).

9. Payens, T. A. S., Biochim. Biophys. Acta **38**, 539 (1960).

10. Joos, R. and Carr, C. W., Proc. Soc. Exptl. Biol. Med. **124**, 1268(1967).

11. Hendrickson, H. S. and Fullington, J. G., J. Biol. Chem. **241**, 4098 (1966).

12. Van Pilsum, J., Martin, R. P., Kito, E., and Hess, J., J. Biol. Chem. **222**, 225 (1956).

13. Rosen, H., Arch. Biochem. Biophys. **67**, 10 (1957).

14. Carr, C. W., Arch. Biochem. Biophys. **46**, 424 (1953).

15. Jolles, J., Jangregui, J., Bernier, I., and Jolles P., Biochim. Biophys. Acta **78**, 668 (1963).

16. Putnam, F. W., Advan. Protein Chem. **4**, 79 (1948).

17. Goodman, D. S., J. Am. Chem. Soc. **80**, 3892 (1958).

18. Putnam, F. W. and Neurath, H., J. Biol. Chem. **159**, 195 (1945).

19. Lebaron, F. N., Biochim. Biophys. Acta **70**, 658 (1963).

20. Terriault, D. G. and Taylor, J. F., J. Am. Oil Chemists' Soc. **41**, 490 (1964).

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