Interaction of phospholipid–metal complexes with water-soluble wheat protein

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ABSTRACT Insoluble lipid–protein complexes are formed in the presence of Ni(II), Ca(II), or Mg(II) by specific components of the water-soluble proteins of wheat flour and either triphosphoinositide or phosphatidyl serine. The pattern of protein species bound by the lipid–metal complex is dependent upon the metal and the phospholipid used. A group of proteins, containing carbohydrate, may be solubilized and recovered by washing the precipitate with acidic chloroform–methanol–water.

Analyses of reactive and nonreactive protein species have shown no differences which clearly account for their behavior. Methylation of protein increases binding to lipid; acetylation decreases the interaction. Weak interaction has been observed between certain components of flour proteins and phospholipid in the absence of metal ions, but the components differ from those bound in the presence of metal ions.

It is suggested that properly oriented groups of the protein molecules are chelating onto available coordination positions of metal ions already bound to phospholipid.

KEY WORDS complexes · Ni(II), Ca(II), Mg(II) · soluble proteins · triphosphoinositide · phosphatidyl serine · oriented groups · coordination

PREVIOUS STUDIES at this and other laboratories have demonstrated metal ion involvement in lipid–protein interaction (1–4) and suggest the possibility of mixed chelation upon metal ions as a type of interaction in lipoproteins (5, 6). The most commonly accepted theory of orientation of polar lipids and protein in biological membranes (7, 8) focuses interaction possibilities on the polar portion of the phospholipid molecules, the same region of the phospholipid known to form stable metal ion complexes (5, 9, 10). With coordination positions then still available on the metal, binding of additional molecules to the phospholipid–metal complex should be possible. Such metal ion coordination has been suggested by Maas and Colburn (11) in explanation of the function of ATP in transport through membranes and maintenance of membrane integrity. Protein interaction by similar coordination seemed possible and was demonstrated by model systems at this laboratory (6). Similar lipid–protein interaction has now been observed for a naturally occurring protein mixture from wheat flour. The striking aspect of this observation has been the specificity for certain of the protein components in forming complexes with phospholipids in the presence of metal ions.

Doubts have been expressed from time to time concerning the interpretation of data that has led to the unit membrane theory (12), and there is no question that the nonpolar interactions that do occur between lipid and protein (13) are difficult to reconcile with the unit membrane theory. The present paper presents a description of the effect of metal ions upon phospholipid–protein systems in vitro. The study was actually undertaken in an attempt to understand the effects of metal ions and metal-containing additives in various wheat flour products on the formation of a dough, but the observations may provide insight into natural lipid–protein interactions and structures.

MATERIALS AND METHODS

Materials

Wheat flour water-soluble protein was prepared from an unbleached spring wheat long patent flour, which was slurried with twice its weight of ice water by mixing for 1 min in a Servall Omni-mixer.[1] The mixture was centrifuged for 40 min at 17,000 *g* at 4°C, and the supernatant solution was dialyzed in the cold against several

[1] Reference to a company or the name of a product does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

changes of deionized distilled water. After centrifugation as before, the clear supernate was made 2% in disodium EDTA and again dialyzed against water. The product was kept in frozen solution until use. Such preparations from wheat flour consist of proteins, glycoproteins, and carbohydrate. The carbohydrate, comprising 67% of this preparation, is known to be polymers of arabinose, xylose, and galactose (14).

Phospholipids were obtained by the Folch fractionation (15, 16) of bovine brain that had been frozen within 30 min of death. DEAE-cellulose chromatography was used for purification (17), with 0.005% 4-methyl-2,6-di-*tert*-butylphenol included as an antioxidant in the solvent mixture (18). Lipid purity was determined by thin-layer silicic acid chromatography, paper chromatography of the deacylated products, and analysis of ester: phosphorus ratio as previously described (6). For use in binding studies, phospholipids were dispersed in 0.05 M *N*-ethylmorpholine buffer (pH 8.0) by ultrasonic irradiation at 20 kc for 3–4 min. In dispersions of phosphatidyl serine of this type, the molecular weight averages 4×10^6, with all hydrophilic groups exposed to the aqueous medium (19, 20).

N-Ethylmorpholine from Matheson, Coleman, & Bell (Norwood, Ohio) was diluted and titrated to pH 8.0 to give a 0.05 *M* stock solution. Stock 0.5 M solutions of Ni(II), Co(II), Ca(II), and Mg(II) were prepared from analytical grade nitrates or from chloride in the case of Mg(II). For acetylation of protein, acetic anhydride and sodium acetate from Baker and Adamson (Allied Chemical, Morristown N. J.) were used.

All other solvents and chemicals used were of reagent grade. Water used was distilled and deionized by passage through a research model resin deionizer from the Illinois Water Treatment Co., Rockford, Ill.

Complex Formation

Fractions for gel electrophoresis were prepared by mixing 2 ml of solution containing 20 mg of protein with 20 mg of phospholipid dispersed in 3 ml of buffer. Previous studies showed that phospholipid and polyamino acid bind in the presence of metal ions in a 1:1 weight ratio (6). 40 μmoles of metal ion was added slowly with stirring. The resulting precipitate was centrifuged down in a few minutes in a small clinical centrifuge, shaken once with water to remove contaminating soluble protein, and again centrifuged. The proteins left in the first supernate were considered to be unbound, since all phospholipid was precipitated from the mixture. The "acid-solubilized fraction" was made by shaking the precipitated material with 5 ml of water and 3 ml of chloroform–methanol 2:1 to which 0.1 ml of 10 N HCl had been added (2). All samples were dialyzed against distilled water and lyophilized before being dissolved in electrophoresis buffers.

Protein Modification

Carboxyl groups were methylated and amino groups acetylated by standard methods (21). The products were lyophilized after dialysis.

Electrophoresis

Starch gel electrophoresis in aluminum lactate buffer, pH 3.1, was carried out as described by Cole and Mecham (22). Gels were photographed while still wet after the destaining process. Free boundary electrophoresis was conducted in a Perkin-Elmer model 38A electrophoresis apparatus using solutions of 0.5% protein and(or) 0.5% phospholipid in 0.05 M *N*-ethylmorpholine buffer, pH 8.0. Solutions were made 0.1 M in NaCl or KCl to give proper conductivity. Runs were made with the sample in an ice-water bath near 1°C at 100 v; current varied somewhat from run to run between 13 and 15 ma. Photographic records were made with a Land camera attachment to the instrument.

Carbohydrate was determined with phenol–sulfuric acid (23) and a xylose standard.

Amino acid compositions of bound and unbound protein fractions were determined with a Beckman Spinco model 120 amino acid analyzer. Proteins were hydrolyzed in 6 N HCl at 110°C for 22 hr.

Protein in solution was determined by the biuret reaction (24).

RESULTS

Fig. 1 shows the starch gel electrophoresis patterns of protein fractions produced by interaction of wheat flour water-soluble protein with phosphatidyl serine in the presence of Ca(II). The total protein extract is shown, which gave no visible interaction with the phospholipid until calcium ions were added. The addition of Ca(II) produced a precipitate, leaving in solution those protein components labeled as "unbound." These species, 68% of the total protein, will not bind to additional phosphatidyl serine added with Ca(II). 39% of the precipitated protein was solubilized by acidic chloroform–methanol–water; 33% of the bound lipid was simultaneously freed into the chloroform-rich phase. The solubilized protein is shown in the next lane, and last is that portion of the precipitate which was not solubilized by acidic chloroform–methanol–water. Streaking resulted in this pattern from incomplete solubility of the fraction in the electrophoresis buffer–urea solvent which efficiently dispersed the other precipitated complexes. The specificity demonstrated seems particularly noteworthy. The electrophoresis pattern of the total extract was unaltered by addition of metal ions alone, phospholipid alone, or by shaking with chloroform–methanol. No effect on the pattern of bound components was seen by changing the lipid or metal concentration; only the amount of the same components bound was changed. In order to determine

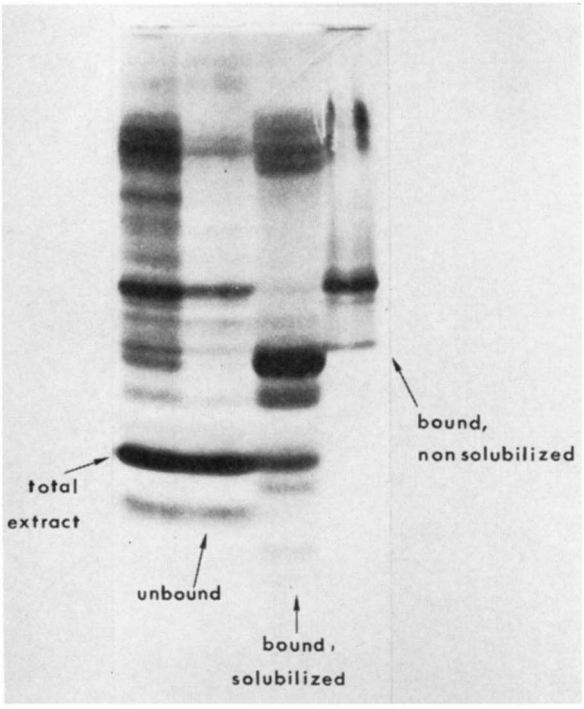

FIG. 1. Starch gel electrophoresis patterns of wheat flour protein fractions precipitated with phosphatidyl serine + Ca(II). Bound protein was partially solubilized by acidic chloroform–methanol–water.

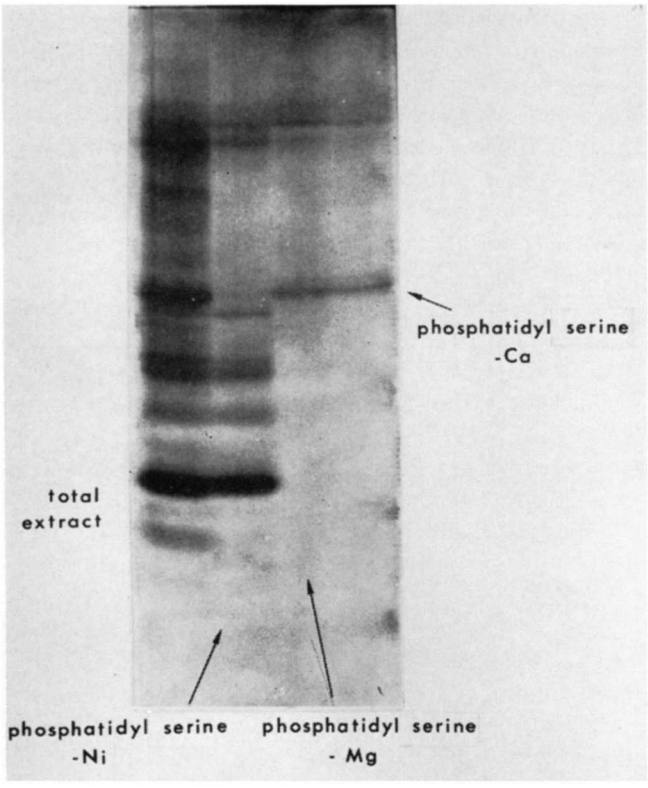

FIG. 2. Starch gel electrophoresis patterns of wheat flour protein components bound by phosphatidyl serine in the presence of Ni(II), Mg(II), and Ca(II).

TABLE 1 CARBOHYDRATE CONTENT OF FRACTIONS OF WHEAT FLOUR EXTRACT

Fraction	Carbohydrate
	% of total dry wt
Initial (total) extract	67
Unbound	71
Bound by phosphatidyl serine + Ca(II)	
(*a*) solubilized by acidic chloroform–methanol–water	30
(*b*) not solubilized	1

the partitioning of the carbohydrate known to exist in the mixture, we analyzed the fractions and obtained the results in Table 1.

Similar precipitation reactions were carried out with Ni(II) and Mg(II). In Fig. 2 are shown the total sets of protein components bound and precipitated by phosphatidyl serine in the presence of the various metal ions. Although the samples on the gel are not comparable in concentration, the qualitative differences are evident for Ni; slight differences between the complexes with Mg and Ca are not clear in the photograph. Recent experiments have shown that phosphatidyl serine with Co(II) binds a still different pattern of protein species from the mixture. Fig. 3 likewise shows qualitative differences in protein components bound in the presence of the three metal ion species, but in this case with triphosphoinositide. Differences are again evident in the bound components, and also between components bound in the presence of the same metal ion but different lipid.

In order to determine if particular amino acid residues of the protein might be involved in binding to phospholipid–metal complexes, we analyzed the amino acids in bound and unbound fractions resulting from the reaction of triphosphoinositide–Ca(II) with wheat flour water-soluble protein. No attempt was made to correct for hydrolysis losses since the results were for comparison only and both samples underwent identical hydrolysis. Electrophoretograms of the fractions analyzed are shown in Fig. 4, and results of the analyses are collected in Table 2.

Fig. 5 shows the starch gel electrophoresis pattern produced by methylated and acetylated water-soluble protein of flour and the products of interaction with triphosphoinositide alone and with triphosphoinositide + Ca(II). Acetylation of the protein reduced binding to triphosphoinositide + Ca(II) by 34% as determined by the biuret reaction. The methylated mixture was bound to phospholipid micelles apparently in its entirety in the absence of metal; a major component disappeared completely from the electrophoresis pattern.

In Fig. 6 are schlieren patterns produced in three separate free boundary electrophoresis experiments. The uppermost pattern is that of triphosphoinositide, which

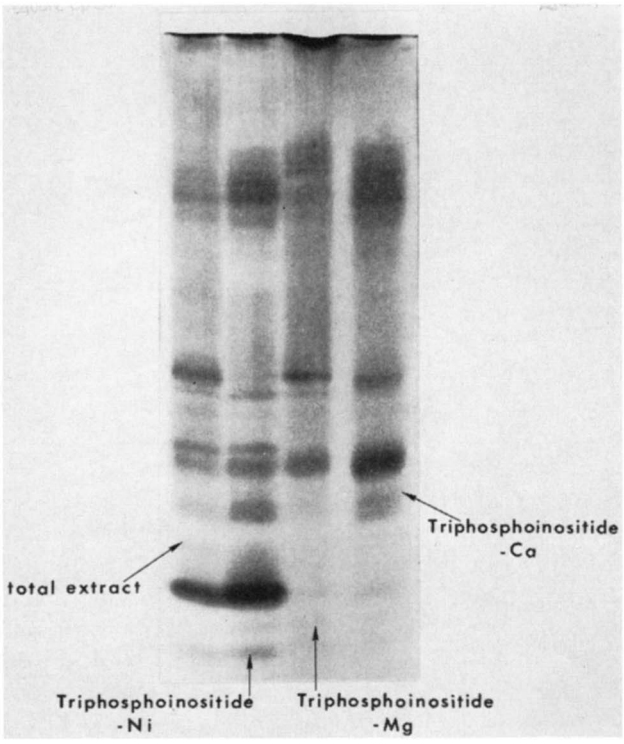

FIG. 3. Starch gel electrophoresis patterns of wheat flour protein components bound by triphosphoinositide in the presence of Ni(II), Mg(II), and Ca(II).

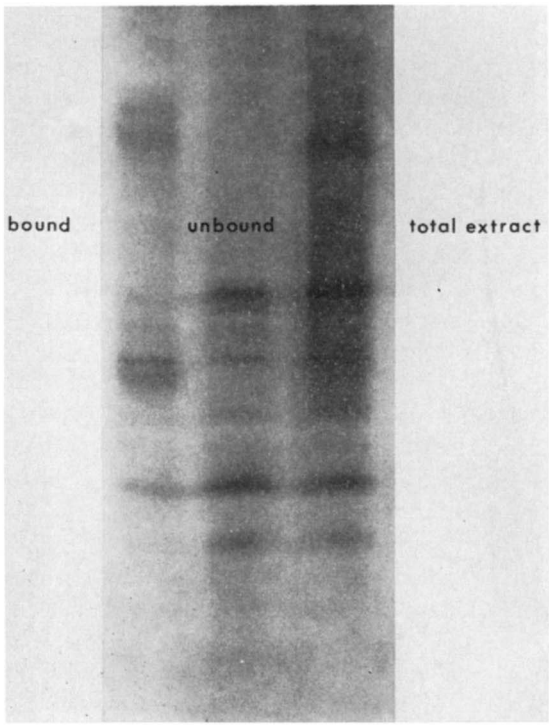

FIG. 4. Starch gel electrophoresis patterns of the wheat flour protein fractions that were analyzed for amino acid content. The bound and unbound fractions were analyzed.

TABLE 2 AMINO ACID CONTENT OF BOUND AND UNBOUND FRACTIONS

Amino Acid	Moles in Bound Protein	Moles in Unbound Protein
	%	
Lysine	3.8	4.0
Histidine	2.0	2.1
Arginine	3.4	4.8
Aspartic acid	5.5	8.5
Glutamic acid	21.3	14.9
Threonine	4.1	1.6
Serine	6.0	3.6
Tyrosine	2.2	tr.
Cysteine	3.2	0
Methionine	2.3	0
Cysteic acid	0	2.7
Proline	9.8	9.2
Glycine	7.5	10.2
Alanine	6.3	11.6
Valine	6.6	7.8
Leucine	7.5	9.5
Isoleucine	4.0	4.2
Phenylalanine	3.5	2.5
Unknown	1.0	2.8

after 40 min migrated through half the field. The unmodified protein components of the flour extract are nearly immobile in this buffer system as shown in the middle pattern by their position after 75 min. In the bottom pattern the lipid from a run using a mixture of both the inositide and protein did not migrate as far in 60 min as it did alone in 40 min, and the shape of the peak indicates inhomogeneity; the protein peak was reduced also. Although a large fraction of the protein is not involved in the interaction, recovery of the lipid-containing peak from the electrophoresis cell and subsequent starch gel electrophoresis showed that the bound protein gives the same pattern as the total extract rather than demonstrating specific removal of some proteins as with metal ions.

The metal ion-dependent interaction between phosphatidyl serine and wheat flour water-soluble protein exhibited only a quantitative dependence on pH between pH 6.0 and pH 9.0. Although less protein was bound as the pH was raised, the pattern of components bound remained unchanged. In solutions more acidic than pH 6.0 a decrease in binding also occurs.

DISCUSSION

Interactions of phospholipid and protein in the presence of metal ions most probably arise from mixed chelation of protein and phospholipid upon the metal ions added, as postulated in a previous paper (5). Hakomori, Ishimoda, Kawauchi, and Nakamura have also suggested this type of lipid–metal–protein interaction in an ox brain fraction (4). Alternative explanations for the interaction might

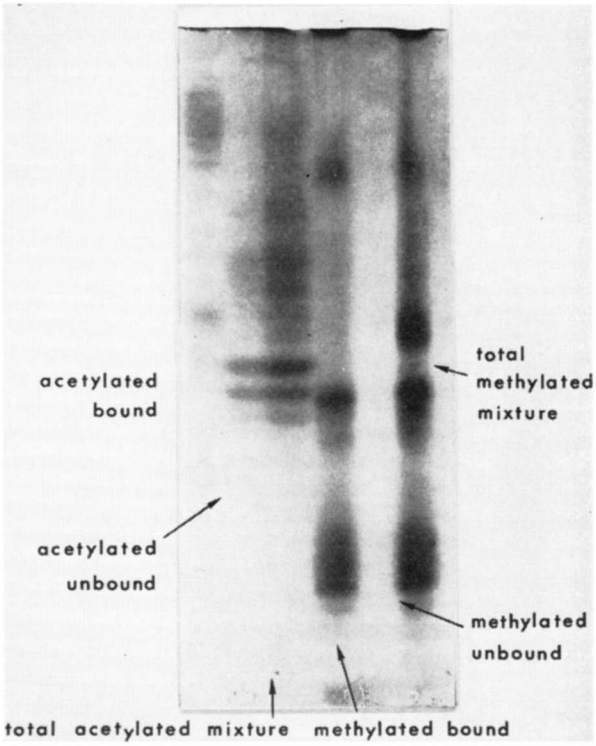

FIG. 5. Starch gel electrophoresis patterns of mixtures with modified proteins. Portions of acetylated protein bound and unbound by triphosphoinositide + Ca(II) and of methylated protein bound and unbound by triphosphoinositide alone.

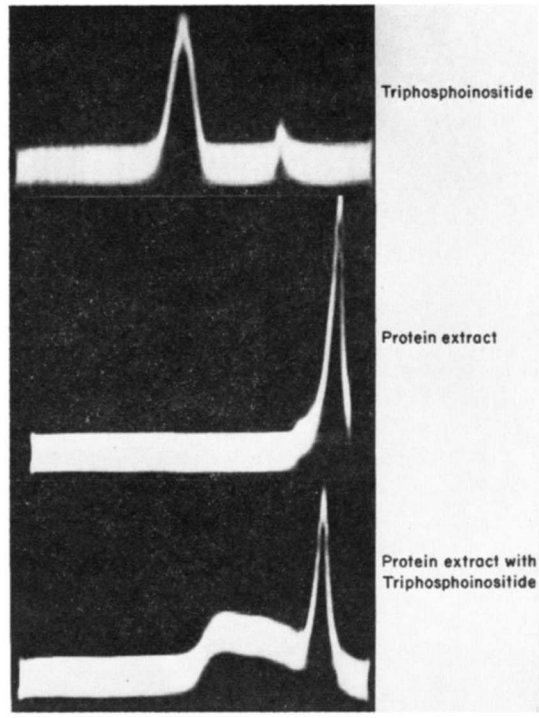

FIG. 6. Schlieren patterns from free-boundary electrophoresis. Top, triphosphoinositide, 40 min, 13.6 ma, 100 v; center, protein extract, 75 min, 13.3 ma, 100 v; bottom, protein extract with triphosphoinositide, 60 min, 14.3 ma, 100 v. All runs were made at 1 °C in 0.05 M *N*-ethylmorpholine pH 8.0 buffer which was 0.1 M in KCl. Migration as shown is right to left.

include binding of lipid to protein to form soluble structures which are aggregated by metal ions, but the soluble complexes that were observed by free boundary electrophoresis differed in protein composition from the metal-dependent complexes. Since the surface of the phospholipid micelles is polar, the interaction with protein in the absence of metal is probably ionic. The experiments with modified (methylated and acetylated) protein support this concept because the changes are all explicable on the basis of alteration of the charged groups rather than of the hydrophobic side chains of the protein. Binding occurs rapidly and extensively between triphosphoinositide micelles having a high potential negative surface charge and methylated protein having potential negative groups blocked, whereas decrease in interaction is observed between triphosphoinositide and acetylated protein, which has potential positive groups blocked. Changes in the conformation of the protein brought about by the chemical treatment would not be expected to cause such opposite binding effects in the two cases studied if exposure of hydrophobic groups were the determining factor in this kind of binding. The observations of binding in the absence of metal ions almost certainly result from charge effects in this system.

The amino acid analyses (Table 2) of unmodified protein bound and unbound in the presence of metal ions show some increase of acidic groups in the protein bound to triphosphoinositide + Ca(II). The opposite would be expected if metal ions were merely precipitating previously bound complexes. The positively charged metal ion could act as an intermediate in binding by virtue of its charge neutralization effects between two negative bodies. This charge consideration would account for the fact that the species bound through metal ions to the lipid differ from those bound in the absence of metal. However, the difference in specificity noted upon changing the metal ion or phospholipid, and the lack of change in specificity with change in pH suggest a more specific requirement for binding than total charge on the protein.

In the light of present observations the most likely criterion for binding to phospholipid–metal complexes by protein appears to be the proper arrangement of appropriate groups on the protein molecule so that chelation onto the remaining coordination positions of phospholipid-bound metal ions can take place. Differences in specificity with different metal ions would then be related to the electronic structure of the metal ion, which determines the nature of groups coordinated. The difference of solubility in acidic chloroform–methanol–water between carbohydrate-containing protein complexes and those containing little carbohydrate raises the possibility of

binding through the carbohydrate portion of the molecule rather than directly to the protein. The amino acid analyses have furnished guidelines for further study but little direct evidence of the protein groups involved in binding. Further analysis of bound protein must be carried out with the various metal ions known to coordinate preferentially with certain groups.

In addition to providing information that is possibly related to the structure of membranes and natural lipoproteins, the observed interactions may find application to the precipitation of specific components from protein mixtures for purposes of separation and identification.

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