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Phospholipid-Metal Complexes

INTERACTION OF TRIPHOSPHOINOSITIDE- AND PHOSPHATIDYLSERINE-METAL COMPLEXES WITH ETHYLENEDIAMINE, POLYAMINOACIDS, AND PROTEIN

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SUMMARY

When ovalbumin, poly-L-lysine, poly-L-aspartic acid, or ethylenediamine is added to a biphasic chloroform-methanol-water system containing the Mg(II), Ni(II), or Ca(II) complex of triphosphoinositide or phosphatidylserine, mixed complexes are formed as evidenced by the formation of an interfacial precipitate similar to that observed by Dawson containing phospholipid, metal, and protein or polyamino acid, or by the presence of ethylenediamine in the chloroform-rich phase where it is ordinarily insoluble. Stable mixed complex formation was shown by gel filtration studies. Analysis of complexes before and after gel filtration indicates stable binding of one ethylenediamine per phospholipid-metal unit and weak binding of an additional molecule of the amine. Polylysine is bound to the triphosphoinositide-Ni(II) complex in a 0.64:1 ratio on a weight basis.

Binding of metal ions by phospholipids may be quite important in such biochemical processes as ion transport and lipoprotein formation. The possibility of mixed chelation as a type of interaction in lipoproteins suggested determination of the stability constants for Ca(II), Mg(II), and Ni(II) complexes of phosphatidylserine and triphosphoinositide, which has been reported previously from this laboratory (1). Further study has supported the hypothesis of lipid-protein binding through metal ion chelation in natural lipoprotein systems. Papahadjopoulos and Hanahan (2) have reported the requirement for Ca(II) in the formation of a prothrombin activating lipoprotein. A model system for lipid-protein interaction has been shown by Dawson (3) with the use of triphosphoinositide-Ca(II) complex with serum albumin. The results of Dawson's study of the albumin binding are confirmed by identical observations in this laboratory. In addition, mixed complexes were formed in model systems containing phospholipid, metal ions, and coligands such as poly-L-lysine, poly-L-aspartic acid, and ethylenediamine. Liquid-liquid partition and gel filtration studies have shown the formation of stable phospholipid-metal-coligand complexes.

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EXPERIMENTAL PROCEDURE

Materials—Phosphatidylserine and triphosphoinositide were obtained from fresh beef brain by fractionation according to Folch (4, 5) and were further purified by DEAE-cellulose chromatography (6). An antioxidant, 4-methyl-2,6-di-*tert*-butyl phenol, was used at 0.005% concentration in chromatographic solvents (7). Purity of the lipids was determined by chromatography on thin layer silicic acid, paper chromatography of deacylated lipids (6), and by ester (8) to phosphorus (9) ratios. Lipids were lyophilized from water emulsion following dialysis against distilled water, and then lyophilized from benzene solution and stored at 2°. For binding studies phosphatidylserine was dispersed in 0.05 M *N*-ethylmorpholine buffer (pH 8.0) by ultrasonic irradiation at 20 kc (10, 11). Triphosphoinositide was water-soluble; greater clarity of solution was produced by buffering to pH 8.0.

Ethylenediamine (Eastman Kodak, 98%)[1] was diluted and titrated to give a pH 8.0, 0.5 M stock solution. Used as received were poly-L-lysine·HCl (mol wt 185,000) and poly-L-aspartic acid sodium salt (mol wt 24,000) from Mann, poly-L-lysine·HBr (mol wt 3,000 to 5,000) from New England Nuclear Corporation, and ovalbumin from Worthington. *N*-Ethylmorpholine from Matheson, Coleman, and Bell was diluted and titrated to pH 8.0 to give a 0.05 M stock solution. Stock 0.5 M solutions of Ni(II), Ca(II), and Mg(II) were prepared from analytical grade nitrates or chloride in the case of Mg(II). Analytical reagent grade chloroform and methanol were mixed in a volume ratio of 2:1 with 1 volume of deionized, distilled water giving a stock biphasic mixture with phases of approximately equal volume for use in partition studies.

Partition Studies—For partition studies, lipids, metal salts, and coligands were shaken with equal volumes of both aqueous and chloroform-rich phases of the stock solvent mixture. Partition of phospholipid material was determined by phosphorus analysis of the solvent phases, and partition of coligands was determined by reaction with ninhydrin (12). Because the lipids were isolated as ammonium salts, interference from ammonium ion reaction with ninhydrin was avoided by washing the chloroform-rich phase with the stock aqueous phase after formation of

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

the phospholipid-metal complex before adding coligand. Quantities used in 10 ml of biphasic solvent were 10 μmoles of phospholipid, 20 μmoles of metal ion (before washing), and 10 mg of polyamino acid or protein, or 10 μmoles of ethylenediamine.

Gel Filtration—For quantitative gel filtration studies, ethylenediamine complexes were prepared in the biphasic solvent mixture as described above. The chloroform-rich phase was evaporated to a small volume and dispersed in 2 ml of eluting buffer by ultrasonic irradiation, after which the remaining organic solvent was evaporated giving a stable aqueous dispersion of the complex.

For qualitative gel filtration studies, ethylenediamine, polyamino acid, and protein complexes were prepared by dispersing 10 mg of phospholipid and dissolving 10 mg of coligand together in 2 ml of pH 8.0 *N*-ethylmorpholine buffer. Metal ion, when used, was added to the phospholipid-coligand solution until the clarity of solution was affected (approximately 5 μmoles of metal ion). Coligand-metal controls contained amounts of coligand and metal ion identical with those in the coligand-metal-phospholipid mixture. Gel filtration was carried out in a 100-ml, 1-cm diameter column of Sephadex G-25, Sephadex G-200, or Bio-Gel P-300 in studies with ethylenediamine, and Bio-Gel P-300 in studies with ovalbumin and polylysine. Elution was effected by pH 8.0 *N*-ethylmorpholine buffer at a flow rate of 1 to 2 ml per hour. Phospholipids in the eluate were detected by phosphorus analysis, ovalbumin or polylysine by reaction with ninhydrin, and ethylenediamine by reaction with ninhydrin and by the carbon disulfide-silver nitrate spot test described by Feigl (13), which gave a brownish precipitate with ethylenediamine visibly distinguishable from the gray precipitate produced by phosphatidylserine in experiments with that lipid. Nickel to phosphorus ratios were determined by x-ray fluorescence spectroscopy in comparison with a carefully prepared 1:1 nickel to phosphorus preparation. Insoluble complexes were solubilized ultrasonically in 0.001 N hydrochloric acid for analysis.

RESULTS AND DISCUSSION

Although triphosphoinositide was soluble in the aqueous phase of the chloroform-methanol-water mixture, and phosphatidylserine was partially soluble in that phase, addition of Ni(II), Ca(II), or Mg(II) in molar amounts equivalent to phospholipid caused partition of the lipid-metal complex formed entirely into the chloroform-rich phase, results also observed by Dawson (3) with triphosphoinositide and Ca(II) or Mg(II). Ethylenediamine, polylysine, polyaspartic acid, and ovalbumin each were found to be partitioned into only the aqueous methanol phase of the solvent system when tested alone or in the presence of either metal ion or phospholipid. However, when shaken in the presence of both phosphatidylserine or triphosphoinositide and Ni(II), Ca(II), or Mg(II), ethylenediamine was found in the chloroform-rich phase. Polyamino acids or protein shaken in the presence of both phospholipid and metal ion formed an interfacial precipitate, very insoluble except in acid solution. Polyamino acid or protein complex formation with phospholipid and metal appears rather nonspecific in the cases studied. All combinations of the two polylysines, polyaspartic acid, or ovalbumin with Ni(II), Ca(II), or Mg(II) and triphosphoinositide or phosphatidylserine gave precipitates similar in appearance and solubility characteristics whether prepared from the two-phase solvent system or from coprecipitation of the ligands from aqueous solution by addition of metal ions.

TABLE I

Elution volumes from gel columns

All runs used 0.05 M (pH 8.0) *N*-ethylmorpholine buffer for elution from a 100-ml, 1-cm diameter column with a 1 to 2 ml per hour flow rate. Coligands except as marked[d] were detected by ninhydrin; phospholipids were detected by phosphorus analysis.

Phospholipid	Coligand	Metal	Eluting buffer to phospholipid peak	Eluting buffer to coligand peak
			ml	*ml*
	Ethylenediamine	Ni(II)		50[a]
Triphosphoinositide	Ethylenediamine	Ni(II)	22	22[a]
Triphosphoinositide	Ethylenediamine		27	54[b]
Triphosphoinositide	Ethylenediamine	Ni(II)	27	27[b]
Triphosphoinositide	Ethylenediamine	Ca(II)	27	27[b]
Triphosphoinositide	Ethylenediamine	Mg(II)	27	27[b]
	Poly-L-lysine			27[c]
Triphosphoinositide	Poly-L-lysine	Ni(II)	18	18[c]
	Ovalbumin			36[c]
	Ovalbumin	Ni(II)		36[c]
Triphosphoinositide	Ovalbumin	Ni(II)	18	18[c]
Phosphatidylserine	Ethylenediamine[d]		27	54[b]
Phosphatidylserine	Ethylenediamine[d]	Ni(II)	27	27[b]

[a] Sephadex G-200.
[b] Sephadex G-25.
[c] Bio-Gel P-300.
[d] Analyzed qualitatively by CS_2-$AgNO_3$ spot test.

TABLE II

Analysis of triphosphoinositide-coligand ratios

Phospholipid was determined on a phosphorus basis; ethylenediamine was determined by ninhydrin reaction.

Complex	Molar ethylenediamine to triphosphoinositide ratio	
	Before gel filtration	After gel filtration
Triphosphoinositide-Ni-ethylenediamine	1.99 to 1	0.97 to 1
Triphosphoinositide-Mg-ethylenediamine	1.58 to 1	1.03 to 1
Triphosphoinositide-Ca-ethylenediamine	0.88 to 1	0.44 to 1

Table I shows the elution volumes from Sephadex G-25 and G-200 and from Bio-Gel P-300 of phospholipid and ethylenediamine, and from Bio-Gel P-300 of phospholipid and ovalbumin or polylysine in the presence and absence of metal ion. It should be noted that coligands are not retarded on the column in the presence of both phospholipid and metal ion, but are eluted exactly with the lipid, clearly showing complex formation.

Results of analyses for triphosphoinositide-coligand ratios of complexes before and after gel filtration are shown in Table II. One molecule of ethylenediamine per phospholipid-metal unit is apparently bound stably in Ni(II)- or Mg(II)-containing complexes with an additional molecule of the amine bound weakly enough to be dissociated upon passage through the gel. The Ca(II)-containing complex appears to be less stable, the 1 molecule of ethylenediamine bound being subject to dissociation by passage through the gel. The polyamino acid-triphosphoinositide ratio of a triphosphoinositide-Ni-polylysine complex formed in the biphasic solvent mixture with the use of polylysine of 185,000 molecular weight was determined by ϵ-amino and phosphorus content to be 0.64:1 on a weight basis after washing with water. Nickel to triphosphoinositide ratios in all nickel-containing complexes were very nearly 1:1.

These studies show that as many as 2 molecules of a bidentate ligand such as ethylenediamine can combine with a phospholipid-metal complex such as triphosphoinositide-Ni(II) or triphosphoinositide-Mg(II) to form mixed complexes. Proteins such as polylysine, polyaspartic acid, and ovalbumin can similarly combine with phospholipid-metal complexes to form lipo-protein complexes. There seems to be little or no specificity with these proteins. However, more recent study in this laboratory, to be reported later, indicates some specificity for protein binding by phospholipid-metal complexes may exist with certain naturally occurring protein mixtures. Knowledge of the nature of this specificity would certainly be helpful in understanding lipoprotein structure and formation.

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