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ASSOCIATION OF LIPIDS AND PROTEINS IN CHLOROPLAST LAMELLAR MEMBRANE

TAE H. JI AND A. A. BENSON

Scripps Institution of Oceanography, University of California, San Diego, La Jolla, Calif. (U.S.A.)

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SUMMARY

1. To study the nature of lipid association with protein in biological membranes, lipids with different hydrophilic groups were reassociated with chloroplast membrane proteins.

2. For stoichiometric reassociation of lipids with protein, a convenient method, a double dialysis procedure, was developed and its validity was examined.

3. Saturation of lipid association occurred when 36 moles of palmitic acid, 30 moles of chlorophyll, 16 moles of monogalactosyl diglyceride, 15 moles of phosphatidyl glycerol or digalactosyl diglyceride, 6 moles of dihydrophytyl ether phosphatidyl glycerophosphate or 1 mole of β-carotene was bound per mole of lamellar protein.

4. In the presence of competitors, reassociation of monogalactosyl diglyceride or chlorophyll with protein was shown to be dependent upon the nature of the hydrophobic moiety of competing amphipathic lipids and independent of their hydrophilic groups.

5. These data provide evidence for hydrophobic association of lipids with chloroplast lamellar proteins.

INTRODUCTION

Restoration of physiological functions by reassociation of lipids with the extracted membrane has been demonstrated with mitochondrial membrane[1] and chloroplast systems (I. SHIBUYA AND B. MARUO, personal communication). Detergent-solubilized membranes of Mycoplasma[2] and of mitochondria[3] have been reconstituted with recovery of morphological integrity. A reconstituted membrane system should exhibit a structural or functional relationship to the original membrane.

In a previous paper we reported evidence of specific hydrophobic association of β-carotene with chloroplast lamellar protein[4]. A red complex formed upon 80% aqueous acetone extraction exhibited an absorption maximum at 538 nm compared to 493 nm *in vivo* or 487 nm in phytol. The red shift was interpreted as a result of enhancement of the π–π^* transition by the polarization effect of a specific hydrophobic binding site for β-carotene within the protein. The red protein could be reconstituted

Abbreviation: PGP, dihydrophytyl ether phosphatidyl glycerophosphate.

from β-carotene and extracted protein by a double dialysis procedure. Its characteristic absorption spectrum indicated specific association of lipid and protein.

The structure of biological membranes has been visualized as a lipoprotein monolayer[5–7] or as a lipid bilayer[8,9]. The former involves hydrophobic while the latter requires ionic interaction between lipid and protein. In this communication, we report the specific affinity of lamellar protein for lipids of varying hydrophilicity. Several lipids have been introduced into chloroplast lamellar protein by the double dialysis procedure. The results are discussed with respect to the nature of molecular interactions between lipids and proteins in native biological membranes.

MATERIALS AND METHODS

Preparation of lamellar protein

Chloroplasts were prepared from spinach obtained from a local market. The de-ribbed leaf tissue was macerated in a Waring Blendor in a volume of buffer, 0.8 M sucrose, 0.01 M NaCl, and 0.02 M Tricine (Tris(hydroxymethyl)methyl glycine) at pH 7.9, equal to the weight of tissue. After 15 sec at maximum grinding speed, the homogenate was filtered twice through 4 layers of cheese cloth. The chloroplasts which sedimented between 250 × *g* and 650 × *g* for 10 min each were resuspended in 0.01 M NaCl and 0.02 M Tricine at pH 7.9, and sonicated at 20 kHz for 60 sec at 0–5°. After removal of whole chloroplasts, the lamellae were centrifuged for 40 min at 39000 × *g*. The chloroplast fragments were then sequentially extracted with 60, 70, 80% aqueous acetone and 100% acetone, as described previously[4].

Preparation of lipids

Spinach leaves were homogenized in a Waring Blendor in methanol–acetone (1:1, v/v) solvent. Total extract was filtered through cheese cloth and filter paper and concentrated in a rotatory evaporator. The concentrated extract was taken up in the same solvent and chromatographed on silica gel G thin-layer plates using chloroform–methanol–water (75:25:2, v/v/v) solvent. To purify chlorophyll and β-carotene, the extract was chromatographed in 30% acetone in hexane. The positions of galactolipids, monogalactosyl and digalactosyl diglyceride, on the thin-layer plates were determined with diphenylamine spray[10]. Phospholipid, phosphatidyl glycerol, was detected with the molybdate spray[11]. Spray reagents were applied either on the edge of thin-layer plates or on a separate control plate. Each lipid spot was collected in a glass column and eluted with methanol. Quantity of the lipids was assayed colorimetrically as described previously[4]. Palmitic acid and linolenic acid were purchased from Applied Science Laboratories Inc., State College, Pa. [1-^{14}C]Palmitic acid was obtained from Volk Radiochemical Co., Burbank, Calif. Phytol and methyl palmitate were obtained from Calbiochem, Los Angeles, Calif. Phytylsulfate was synthesized by Dr. P. P. M. BONSEN and dihydrophytyl ether phosphatidyl glycerophosphate (PGP) and [^{32}P]PGP from Halobacterium were kindly provided by Miss JOELLEN SARNER.

Preparation of [^{14}C]lipids

The buffer medium for the ^{14}C-labeling experiment contained 5 mM K_2HPO_4, 1 mM $MgCl_2$ and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Spinach leaves were cut into 1-cm^2 pieces and 10 pieces (250 mg wet wt.) were

placed in a gas-tight vessel containing 4 ml of the buffer medium and 1 mC of sodium [^{14}C]bicarbonate. The vessel was held in a water bath at 20° and was illuminated through the transparent bottom of the water bath with an incident light intensity of 4000 ft-candles. After 2 h of photosynthesis, the leaf pieces were dropped into boiling ethanol with 1 mg of butylhydroquinone antioxidant. Lipids were extracted until the leaf became colorless. The concentrated total extract was chromatographed as described above.

Lipoprotein reconstitution

Lamellar protein was suspended in methanol by homogenization and ultrasonic oscillation at 0° for 15 sec. 3 ml of lamellar protein suspension (0.1 mg/ml) was placed in a dialysis tube (diameter, 0.52 inch, purchased from Arthur H. Thomas Co., Philadelphia, Pa.) and dialyzed against 20 ml of methanol containing 20–500 μg (200 counts/min per μg) of lipid per ml (first dialysis) at 5°. In the case of unsaturated lipids, dialysis was carried out under nitrogen. After the lipid was equilibrated between the two solutions, which took 6–9 h, the whole system was transferred into a large dialysis tube (diameter, 1.1 inch) and dialyzed against aqueous methanol (second dialysis). Methanol concentration was controlled from 60 to 20% by adding water to the aqueous methanol. This prevents sudden dilution of methanol concentration which could favor lipid micelle formation rather than lipoprotein formation. When the methanol concentration approached 20% in water after 2–3 h of the second dialysis, the lipoprotein solution (in the small dialysis bag) and lipid solution (in the large dialysis bag) were recovered for analysis of lipid content. Protein was measured according to the method of LOWRY *et al.*[12] as well as gravimetrically. Lipid content was measured by radioactivity. An aliquot of the solutions was evaporated in a scintillation vial, redissolved in scintillation fluid[13] and counted with a Beckman DPM-100 liquid-scintillation counter at ambient temperature. The difference of external standard ratio between samples was about 5%. Chlorophyll was re-extracted in 80% acetone and assayed spectrophotometrically using the absorption coefficients of MACKINNEY[14]. The amount of bound lipid was determined by subtracting the concentration of lipid in the lipid solution (in the large dialysis bag) from the concentration of lipid in the lipoprotein solution (in the small dialysis bag). The amount of lipid left on the dialysis membrane was corrected by comparing with control dialyses in which protein was absent[15]. Concentration of bound lipid was divided by the concentration of protein to determine weight ratio of bound lipid to lamellar protein. Molar ratios of bound lipid to lamellar protein were derived from weights; lamellar protein, 23000 (ref. 16); chlorophyll, 900; monogalactosyl diglyceride, 770; digalactosyl diglyceride, 930; phosphatidyl glycerol, 780 (ref. 5); PGP, 960 (ref. 17); β-carotene, 540; and palmitic acid, 260.

Competitive reassociation

A suspension of 300 μg of lamellar protein in 3 ml methanol was dialyzed against 20 ml methanol containing 250 μg of monogalactosyl diglyceride or 400 μg of chlorophyll per ml in the presence of varying amounts of competitor. After the second dialysis, the content of monogalactosyl diglyceride, or chlorophyll was measured to determine bound lipid.

Reassociation of heat-treated lamellar protein

A suspension of lamellar protein in 1 mM phosphate buffer at pH 6.5 was placed in a glass tube. The tubes were sealed and maintained at 75° for 0.25–24 h. The recovered protein after heat treatment was dialyzed against distilled water and suspended in methanol to make the concentration 100 μg/ml. Monogalactosyl diglyceride in methanol (200 μg/ml, 200 counts/min per μg) was used for reassociation studied as described above.

RESULTS AND DISCUSSION

Reassociation of lipids with lamellar proteins

To determine the possible influence of ionic (glycerophosphate, glycerol 1,3-diphosphate and carboxylic group) or hydrophilic groups (galactose and digalactose) of lipids upon interaction with lamellar protein, several lipids with different hydrophilicities were tested for reassociation. Reassociation data presented in Fig. 1 deviate from KLOTZ's[15] equation, $r/(A) = kn - kr$, to give a curve rather than a straight line. Since the equation is based on the assumption that all binding sites are identical and there is no interaction between them, the deviation from the equation would indicate that there are differences between binding sites and/or association of lipid with one binding site influences another binding site. Probably lamellar protein associates with an individual lipid as well as groups of lipids[4]. The reassociation data revealed that lamellar protein has the faculty to accommodate a certain number of lipids. Hence the saturation of bound lipids was determined by extrapolating the plateau of each curve. As shown in Fig. 1, 30 moles of chlorophyll or 36 moles of palmitic acid were bound per mole of lamellar protein. A mole of lamellar protein associated with 16

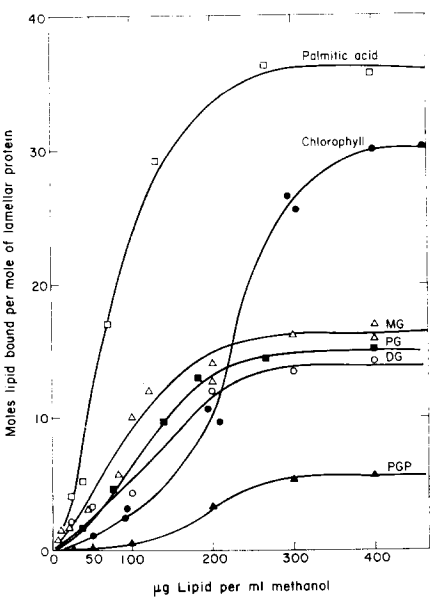


Fig. 1. Reassociation of lipids with lamellar protein. Reassociation of lipids with lamellar protein as function of the initial concentration of lipid in methanol. Lipid was introduced to protein by the double dialysis procedure described in the text. MG, monogalactosyl diglyceride; DG, digalactosyl diglyceride; PG, phosphatidyl glycerol.

TABLE I

REASSOCIATION OF CHLOROPLAST LAMELLAR LIPOPROTEIN

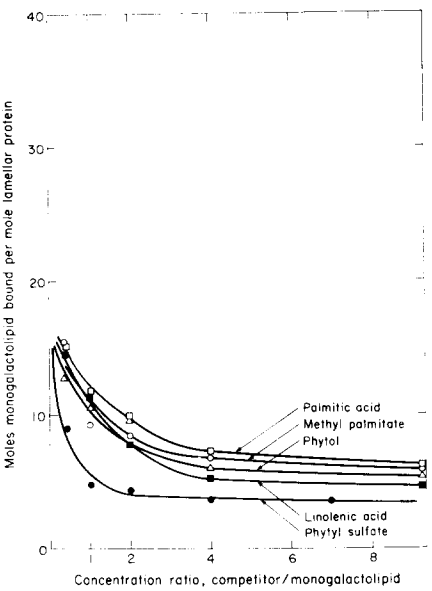
Saturation number of lipid bounds to lamellar protein is presented. Low binding affinity of PGP is identical to the association ratio to Halobacterium cell membrane *in vivo*, as discussed in the text. The rest of lipids occur in chloroplast lamellae. The data indicated the hydrophobic specificity of reassociation of chloroplast lamellar lipids with protein.

Lipid	*Moles of lipid bound per mole of lamellar protein*	*Moles of hydrocarbon chains per mole of lamellar protein*	*Hydrophilic group*	*Hydrophobic group*
Palmitic acid	36	36	carboxylic acid	palmitic acid
Chlorophyll	30	30	carbonyl	phytol, porphyrin ring
Monogalactosyl diglyceride	16	32	galactose	linolenic acid
Phosphatidyl diglyceride	15	30	glycerophosphate	linolenic acid
Digalactosyl diglyceride	14.5	29	digalactose	linolenic acid
PGP	5.7	11.4	glycerol 1,3-diphosphate	

moles of monogalactosyl diglyceride, 15 moles of phosphatidyl glycerol and 14.5 moles of digalactosyl diglyceride, which contains two fatty chains in each molecule. Therefore, the combining ratio of lipid with lamellar protein is 29–36 hydrocarbon chains per molecule of lamellar protein, regardless of significant differences in the ionic or hydrophilic character of the lipids. These data, appearing in Table I, might be interpreted as evidence that lamellar proteins may contain independent hydrophilic or ionic sites for each lipid. This possibility was eliminated by the competitive reassociation study. Reassociation of monogalactosyl diglyceride or chlorophyll with lamellar protein could be inhibited by a variety of long chain competitors, as shown in Figs. 2 and 3. No remarkable difference in competitiveness of the inhibitors was noticeable, although the competitors possess a wide range of hydrophilicity: from the relatively hydrophobic methyl palmitate and weakly hydrophilic phytol to ionic palmitate and strongly ionic phytyl sulfate. The results indicate that the long chain competitors compete with monogalactosyl diglyceride or chlorophyll for the same binding sites by other than hydrophilic groups. Therefore, it is reasonable to conclude that the hydrophobic moieties of lipids associate with the hydrophobic interior of the membrane. The diphytyl ether phosphatide, PGP, exhibited a remarkably different binding affinity. PGP occurs only in Halobacteria where it is the major membrane lipid[17]. The unique feature of PGP is its two dihydrophytyl ether chains instead of the fatty ester found in other biological membranes[5]. This may account for the fact that Halobacterium cell membranes accommodate only 17–23% lipid[18], compared with 40–50% in erythrocyte membranes[19] and chloroplast membranes[20]. Thus the weight ratio of lipid to protein in Halobacterium membranes is approx. 1:4, which is consistent with the weight ratio of bound PGP to lamellar protein.

As shown in Table II, lamellar protein reassociated with lipids after heating at 78° for 4 h. After 24 h, however, it lost its faculty for reassociation with monogalactosyl diglyceride. A protein may be heat stable by virtue of either its rigidity or its potential flexibility. Present results do not distinguish between these possibilities.

The probable flexibility of membrane protein in carrying out its physiological functions, active transport as an example[6], is consistent with the latter interpretation of the heat stability of lamellar protein.



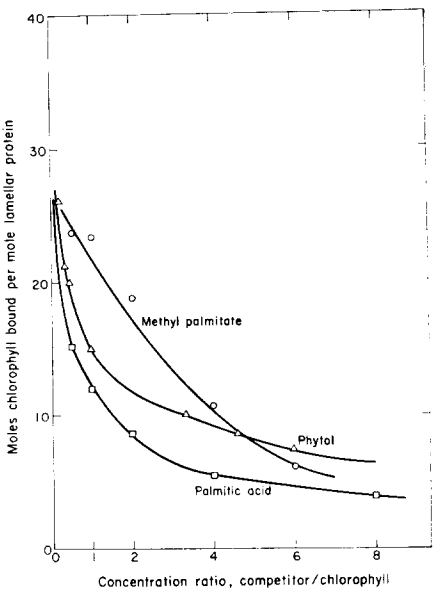


Fig. 2. Reassociation of monogalactosyl diglyceride with lamellar protein in presence of competitor. Reassociation of monogalactosyl diglyceride with lamellar protein in the presence of competitor lipid was plotted against the initial concentration ratio of the competitor to monogalactolipid.

Fig. 3. Reassociation of chlorophyll with lamellar protein in presence of competitor. Reassociation of chlorophyll with lamellar protein in the presence of competitor as described in Fig. 2.

TABLE II

REASSOCIATION OF DENATURED LAMELLAR PROTEIN

Lamellar protein was incubated in 1 mM phosphate buffer, pH 7.3, in sealed tubes at 75°. After heat treatment, the protein was dialyzed against distilled water. Reassociation with monogalactosyl diglyceride was carried out as described in the text.

Denaturation time (h at 75°)	*0*	*0.25*	*0.75*	*1.0*	*2*	*4*	*24*
Moles of bound monogalactosyl diglyceride per mole of lamellar protein	16	16.3	14.4	15.0	14.5	15.2	0

Solvent effect of lipoprotein formation

Various methods have been applied in forming lipid–protein complexes of biological significance. Extreme insolubility of membrane protein and the difference in solubility of lipid and protein have limited stoichiometric studies of their association. An ingenious method for associating lipids with proteins in aqueous–organic solvent systems was developed[21,22]. HENNINGER AND CRANE[23] demonstrated that quinone in heptane could be added back to the extracted chloroplast by evaporating heptane, followed by homogenization of the complex in an aqueous solvent. The reconstituted chloroplast quinone complex recovered its original electron transport activity.

Principles of this method include changing conformation of protein and solubility of lipids which effects micelle formation of lipid. KAUZMANN[24] presented the thermodynamic basis for hydrophobic association of hydrophobic molecules and non-polar segments of protein. The orientation of hydrophobic amino acid side chains in the interior of a protein while hydrophilic groups predominate at the aqueous interface at the exterior was attributed to the great entropy increase upon hydrophobic association of hydrocarbon groups within a protein or within a lipid micelle. SINGER[25] followed similar thermodynamic principles in interpreting protein conformational changes in non-aqueous media. In non-aqueous solvent systems both hydrogen and ionic bonding may increase as a result of decreased free energy of hydrophobic association.

In the double dialysis procedure, lipids and lamellar proteins are suspended in methanol where hydrophobic groups of protein and lipid are free to associate. As the methanol concentration decreases upon addition of water, the hydrophobic groups of protein are restored to their internal location, as are the associated lipid hydrocarbon chains. Since association of protein and lipids is expected to result from random motion, lipids may bind at every available site in lamellar protein, as revealed in Table I, unless there is an exclusively specific interaction between some lipid and lamellar protein. As an example, β-carotene associated with lamellar protein with saturation at a 1:1 molar ratio.

Reconstitution by the double dialysis procedure

Although recovery of biological activity after reassociation of lipids with lamellar protein by the double dialysis procedure was not established, we present evidence that the reassociation data represent neither simple coprecipitation of lipids with lamellar protein nor random association with no specificity for native membrane organization. (1) Stability of membrane protein toward organic solvent treatment has been demonstrated (refs. 1, 23; I. SHIBUYA AND B. MARUO, personal communication) (2) Heat-denatured lamellar protein lost its ability to associate with lipids, as revealed in Table II. (3) β-Carotene, coprecipitated with lamellar protein in an aqueous solvent, could be extracted by 80% aqueous acetone while 80% acetone could not remove β-carotene from reconstituted red protein (T.H. JI AND A. A. BENSON, unpublished observations). (4) Reconstituted red protein exhibited the typical absorption spectrum of the original red protein[4]. (5) Specific stoichiometric reassociation of β-carotene with lamellar protein[4]. (6) The saturated reassociation ratio of lipid hydrocarbon chains bound per molecule of lamellar protein, 29–36, is comparable to the ratio of lipids to protein *in vivo* (recalculated from ALLEN *et al.*[29] and PARK AND BIGGINS[26]). (7) The observed affinity of lamellar protein to PGP is consistent with the binding affinity of PGP in Halobacterium cell membrane protein.

These data provide evidence for hydrophobic association of lipids with lamellar proteins. One may envision the chloroplast lamellar membrane as an aggregate of hydrophobically associated lipoprotein.

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