Biochimica et Biophysica Acta, 1149 (1993) 1–9

BBAMEM 75968

Tight insertion of cytochrome b_5 into large unilamellar vesicles

Susan F. Greenhut [1], Kenneth M.P. Taylor and Mark A. Roseman

Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD (USA)

(Received 30 November 1992)
(Revised manuscript received 4 February 1993)

Key words: Cytochrome b_5; Liposome; Phosphatidylcholine; LUV

Cytochrome b_5 spontaneously binds to liposomes in a 'loose', or transferable form, whereas in vivo b_5 binds post-translationally to the ER in the 'tight' or nontransferable form. The mechanism of tight insertion is unknown, except that it does not require SRP or energy input. The present study shows that prolonged incubation of b_5 with large unilamellar vesicles (LUVs) of phosphatidylcholine results in slow conversion of the loose to the tight form, with a halftime of days. However, the process is complex. When the b_5-LUVs are depleted of loose b_5, by transfer of b_5 to sonicated vesicles, the tight b_5 is found to be concentrated to near saturating levels in a small fraction of the LUVs. If the LUVs devoid of tight b_5 are recovered and then reincubated with fresh b_5, the same slow transformation recurs. Apparently, a new population of vesicles, containing tight b_5, is generated during the prolonged incubation with the protein. The b_5-enriched LUVs contain about the same level of trapped sucrose as does the original vesicle preparation, indicating that vesicle integrity is maintained throughout the process. When fresh b_5 is added to these tight b_5-containing LUVs, all the freshly bound protein rapidly inserts (< 2 h) into the tight configuration. Apparently, the newly formed tight-b_5/LUV vesicle population is 'insertion-active'. A model for these complex transformations is proposed.

Introduction

It is now well recognized that many water soluble proteins spontaneously bind to and insert into biological membranes [1–3]. The thermodynamics, kinetics, and other mechanistic features of this process have been, and will continue to be, extensively studied with liposomes as a model membrane system. The present report describes some novel phenomena that occur when cytochrome b_5 is incubated with large unilamellar phosphatidylcholine vesicles for periods of time ranging from 2 to 96 h.

Cytochrome b_5, a component of the microsomal electron transport system, is an integral membrane protein with an N-terminal water-soluble catalytic domain and a C-terminal membrane-binding anchor [4]. Synthesized without a signal sequence on water-soluble ribosomes, this protein spontaneously inserts into intracellular membranes without using signal recognition particle, receptors, ATP hydrolysis, or a membrane potential [5–8].

The purified detergent-free protein is water-soluble, as an equilibrium mixture of octomers and monomers [9], yet spontaneously binds to pre-formed lipid vesicles of phosphatidylcholine [10–16]. When added to preformed vesicles, cytochrome b_5 usually inserts in a so-called 'loose' binding form, characterized by the ability of the protein to spontaneously transfer from one vesicle population to another [17,18]. In the loose configuration the hydrophobic tail forms a hairpin structure that penetrates part way into the bilayer, leaving three to eight C-terminal residues in the external aqueous phase alongside the catalytic domain [4,19]. In contrast, the endogenous cytochrome b_5 in microsomes is bound in a nontransferable or 'tight' binding form [18]. The tight binding form can be generated in liposomes by using reconstitution procedures that are commonly employed to incorporate integral membrane proteins into lipid vesicles [18,20–22]. The configuration of the hydrophobic tail in the tight binding form has not yet been resolved; photolabelling studies indicate that it spans the bilayer [23] whereas other types of studies indicate that it does not [4,24,25].

Correspondence to: M.A. Roseman, Department of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799, USA.

[1] Present address: Ogden Bioservices Corp., 685 Loftstrand Lane, Rockville, MD 20850, USA.

Abbreviations: LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; [^{14}C]POPC, 1-palmitoyl-2-[1-^{14}C]oleoyl-L-3-phosphatidylcholine; [^{3}H]sucrose, [6,6′(n)-^{3}H]sucrose; [^{3}H]triolein, [9,10(n)-^{3}H]triolein.

In a previous study of inter-vesicle protein transfer in this laboratory, it was found that incubation of cytochrome b_5 for 2 h with preformed large unilamellar vesicles (LUVs) of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dioleoylphosphatidylcholine (DOPC), or egg phosphatidylcholine leads to the spontaneous tight insertion of about 10% of the protein [26]. This result, which suggested that the barrier to tight insertion might not be as large as had previously been thought, has prompted a more detailed examination of the kinetics and mechanism of cytochrome b_5 insertion into LUVs. The present study shows that after the initial 2 h, continued prolonged incubation of cytochrome b_5 with PC LUVs leads to a slow but steady increase in the percentage of tightly bound protein. However, the mechanism of tight binding is complex. The tightly bound protein is found to be concentrated in a small fraction of the LUVs and, what is most remarkable, these vesicles, though impermeable to sucrose, are highly 'insertion-active': fresh cytochrome b_5, added exogenously, rapidly inserts with 100% efficiency into the tight configuration. The implications of these results will be discussed.

A preliminary report of these results has been presented [27].

Materials and Methods

Non-radioactive lipids were obtained from Avanti Polar Lipids and were used without further purification. 1-Palmitoyl-2-[1-^{14}C]oleoyl-L-3-phosphatidylcholine ([^{14}C]POPC), [6,6'(n)-^{3}H]sucrose ([^{3}H]sucrose), and ethyl [1-^{14}C]acetimidate hydrochloride were obtained from Amersham; [9,10(n)-^{3}H]triolein ([^{3}H]triolein) from New England Nuclear; nonlabelled ethyl acetimidate from Sigma.

Bovine cytochrome b_5 was purified to homogeneity as previously described [28]. [^{14}C]Amidinated cytochrome b_5 was prepared by modification [28] of the method used by Dailey and Strittmatter [29] to prepare amidinated catalytic fragments.

Homogeneous populations of small unilamellar vesicles (SUVs) were obtained by sonication of lipid dispersions followed by ultracentrifugation to remove larger particles [26]. The buffer in all cases was 20 mM Tris/0.1 M NaCl/0.1 mM EDTA (pH 8.1) and will be simply referred to as Tris buffer. Lipid phosphorus was determined by the Bartlett procedure [30].

Large unilamellar vesicles (LUVs) of POPC and POPC/DMPC were obtained by reverse-phase evaporation followed by extrusion through polycarbonate membranes and fractionation by gel filtration [26]. LUVs were also prepared by the detergent-removal procedure of Enoch and Strittmatter [31] with modifications [26]. An additional fractionation step had to be employed for LUVs of DOPC and DOPC/DMPC in order to obtain a population of vesicles that could be cleanly separated from SUVs by sedimentation. These LUVs were sedimented through a 0.5% glycerol step gradient (see below for composition) for 60 min at 45 000 rpm in a Ti50 rotor (Beckman ultracentrifuge). Ten fractions were collected, and the faster sedimenting vesicles (usually obtained in fractions 6 and 7) were kept for subsequent studies. These fractions were pooled and dialyzed overnight against the Tris buffer before use.

[^{14}C]POPC or [^{3}H]triolein was incorporated into the lipid bilayers by adding the labelled compounds to the chloroformic lipid solution prior to formation of the lipid dispersions. To prepare LUVs with trapped [^{3}H]sucrose, the labelled sucrose was included in the buffer during the reverse-phase evaporation procedure. External sucrose was removed by gel filtration on a Sepharose 2B-CL column (1.6 × 55 cm) equilibrated with the Tris buffer. This column also removes small vesicles that are formed during LUV preparation.

Cytochrome b_5-LUV complexes were formed by incubating the protein with preformed vesicles of POPC, DOPC, or DMPC/DOPC (1 : 1) for at least 2 h at 30°C under argon. (A 2-h incubation ensures > 95% protein binding under the conditions that we employ.) The extent of tight binding was determined by depleting the LUVs of loose cytochrome b_5 via intervesicle transfer to an excess of SUVs, then separating the vesicle populations by sedimentation and analyzing the fractions for cytochrome b_5 and lipid. The SUV-acceptor to LUV-donor phospholipid ratio was 3 : 1 or 4 : 1, which is more than sufficient to deplete the LUVs of loose cytochrome b_5 because the protein has a much higher affinity for the small vesicles [26]. Donors and acceptors were incubated for 2 h at 30°C prior to separation.

The LUVs and SUVs were separated from one another by velocity sedimentation through a glycerol step gradient consisting of 0.5 ml sample at the top, 1.4 ml of a solution in the middle that was varied from 0.5–3% glycerol (w/w) (depending on the sedimentation rates of the particles to be separated), and 0.1 ml 60% glycerol (w/w) at the bottom. Samples were centrifuged for 60 min at room temperature in a Ti50 rotor at 45 000 rpm, and then separated into ten equal fractions and analyzed for cytochrome b_5 and lipid. The concentration of non-radioactive cytochrome b_5 was determined from the 413 nm absorbance as previously described [26]. In experiments requiring levels of protein that are too low to be assayed spectrophotometrically, the labelled cytochrome b_5 was used. The concentrations of radioactive protein and lipid were determined by scintillation counting (Mark III Packard counter), using Beckman HP liquid scintillation cocktail.

A significant fraction of the radioactivity in the ^{14}C-amidinated cytochrome b_5 preparation (approx. 25%) did not bind to vesicles even at very high lipid/protein ratios. Nonbound counts, which were assumed to arise from a cytochrome b_5 dimer formed by covalent crosslinking of the monomers during the amidination reaction [28], were determined in every experiment, and subtracted from the totals to determine the fraction of cytochrome b_5 loosely and tightly bound. But after these studies were completed, the nature of this non-binding fraction was re-examined. No dimer was evident on SDS-polyacrylamide gel electrophoresis; the amidinated protein appeared to be as homogeneous as the starting protein. In a binding study, the amidinated protein was incubated with an excess of POPC-LUVs, then the mixture was subjected to gel filtration on a Sepharose 2B-CL column. The fractions were analyzed for lipid, ^{14}C, and 413 nm absorbance. The results showed that nonbound ^{14}C-containing material, which eluted in the included volume, had an insignificant absorption spectrum in the visible region. When a fresh aliquot of LUVs was added to this material, none of the counts eluted with the vesicles. From these results we conclude that the nonbound material, though still uncharacterized, does not affect the results or interpretations of our experiments.

Since there are no residues in the nonpolar tail that can be amidiniated, and since amidination is a relatively modest modification of amino groups, the lipid binding properties of amidinated cytochrome b_5 should be virtually identical to that of the native protein. This seems to be the case for several reasons: (1) Dailey and Strittmatter (1980) have shown that the amidinated protein binds to vesicles containing steoryl-CoA desaturase; (2) we have found that when excess protein is added to POPC-LUVs, the saturation levels of native and amidinated cytochrome b_5 binding are comparable, with values of 7.0 and 8–9 mol per 1000 mol lipid, respectively; (3) as will be shown in Results (Fig. 7), when amidinated cytochrome b_5 is added to freshly prepared LUVs, the extent of loose binding is the same as with native cytochrome b_5, and the rate of tight insertion is indistinguishable from that observed with native (compare Figs. 2 and 7).

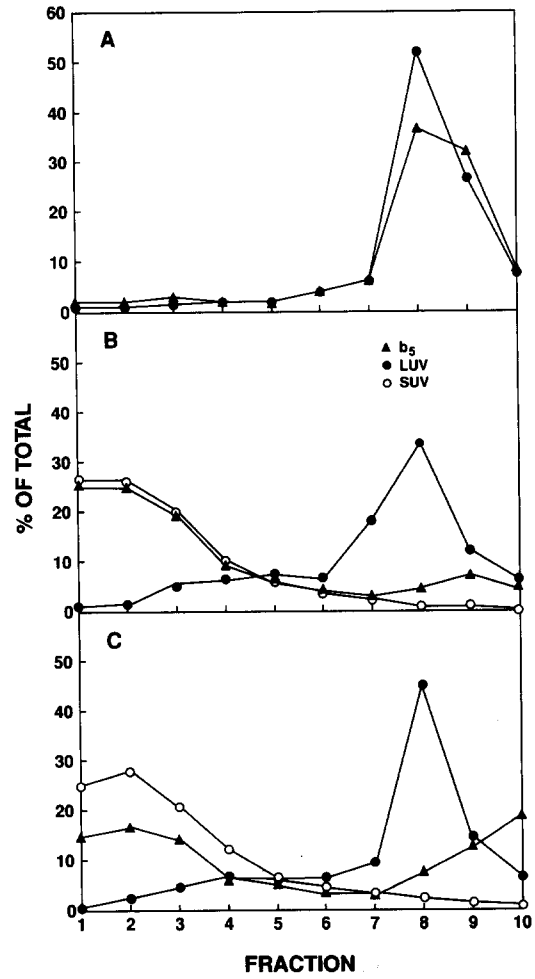


Fig. 1. Glycerol gradient analysis of cytochrome b_5-LUV incubations. (Panel A) Cytochrome b_5 (3.6 nmol) was incubated for 2 h at 30°C with POPC LUVs (3 μmol of POPC containing as a marker $4.0 \cdot 10^4$ dpm [^{3}H]triolein/μmol phospholipid) in a total volume of 0.887 ml 20 mM Tris, 0.1 M NaCl, 0.1 mM EDTA (pH 8.1). A 0.5-ml sample was then fractionated by sedimentation on a 1%-glycerol step gradient as described in Materials and Methods, and analyzed for cytochrome b_5 and ^{3}H. (Panel B) After 2 h incubation of cytochrome b_5 (3.6 nmol) with LUVs (3.0 μmol of POPC; total volume, 0.353 ml), SUV acceptors were added (12 μmol of POPC doped with $1.24 \cdot 10^4$ dpm [^{14}C]triolein/μmol PC; total volume, 0.887 ml) and the mixture was incubated for 2 h at 30°C to deplete the LUVs of loose cytochrome b_5. 0.5 ml was then applied to the glycerol gradient, fractionated, and analyzed for cytochrome b_5, ^{3}H, and ^{14}C. (Panel C) Cytochrome b_5 and LUVs were incubated for 96 h before addition of SUV acceptors. Subsequent procedures were the same as in Panel B.

Results

Appearance of nontransferable cytochrome b_5 upon prolonged incubation with LUVs

Cytochrome b_5 and LUVs of phosphatidylcholines were incubated for 2–96 h at 30°C. Aliquots were withdrawn at various times and an excess of SUV acceptors were added to deplete the LUV donors of loosely bound protein. After a 2-h incubation with acceptors, the vesicle populations were separated by sedimentation and assayed for lipid and protein to determine the fraction of cytochrome b_5 tightly bound to the LUVs.

Fig. 1 shows typical results that are obtained when cytochrome b_5 is incubated with LUVs of phosphatidylcholine for a short and a long period of time. After a 2-h incubation period (Panel A), the LUVs and protein essentially comigrate, reflecting the formation of a protein-lipid complex. The protein and lipid profiles are not exactly coincident, however: a relatively higher ratio of protein to lipid is seen towards the

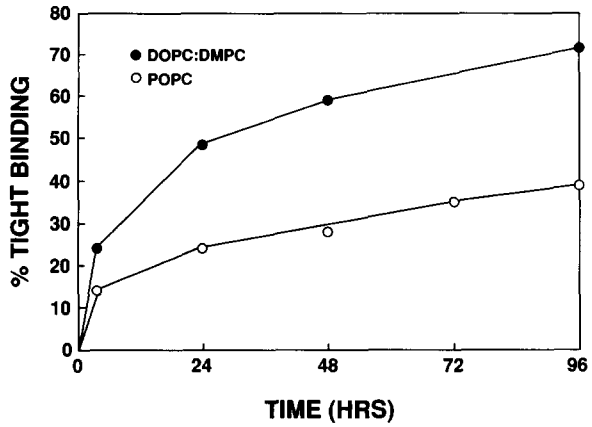


Fig. 2. Kinetics of tight binding. (Upper curve) Interaction with LUVs of equimolar DOPC:DMPC. Four identical mixtures containing cytochrome b_5 and DOPC/DMPC LUVs (2.4 nmol protein and 2 μmol phospholipid doped with $2.3 \cdot 10^4$ dpm [^{14}C]triolein/μmol lipid; total volume, 0.555 ml) were incubated at 30°C for different periods of time: 2, 24, 48, and 96 h. At the end of each incubation, SUV acceptors (8 μmol of DOPC doped with $6.25 \cdot 10^4$ dpm [^{3}H]triolein/μmol lipid in 0.445 ml buffer) were then added and the mixture was allowed to incubate for an additional 2 h. 0.5 ml was then applied to a 3% glycerol step gradient for fractionation and analysis. (Lower curve) Interaction with LUVs of POPC. Four incubation mixtures were used, as described above. The quantities, conditions and experimental protocol for measuring the amount of tight binding were the same as described in Fig. 1B.

bottom of the gradient. Addition of SUV acceptors to the protein-LUV complexes (Panel B) removes all but 10–20% of the cytochrome b_5 from the LUV population, indicating that this smaller fraction of the protein is tightly bound at the 2 h time point. Moreover, the tightly bound protein is found to be concentrated in a relatively small fraction of the LUVs.

Panel C shows the sedimentation profile after a 96 h incubation of cytochrome b_5-LUV complexes followed by a 2 h incubation with SUV acceptors. Clearly, the amount of nontransferable cytochrome b_5 is substantially increased by the long incubation as the tight form accumulates in a fraction of the LUVs. Analysis of samples taken at 2, 24, 48, 72, and 96 h shows that the changes in the sedimentation profiles evolve smoothly from the one in Panel B to the one in Panel C (data not shown).

The kinetics of tight binding (Fig. 2) indicates that the process involves an initial rapid phase followed by a much slower phase. Since we cannot determine the amount of tight binding before two hours, we can only estimate the lower limit of the rate of the fast phase: For POPC-LUVs the halftime is $\leqq 8$ h, whereas for LUVs composed of DOPC/DMPC (1:1) the halftime is $\leqq 5$ h. The halftimes for the slow phase are about 9 days for POPC-LUVs and about 1.5 days for DOPC/DMPC LUVs.

The kinetics of tight insertion were insensitive to the starting lipid concentration, which was varied from 1–10 mM phospholipid, and also insensitive to the starting mole ratio of cytochrome b_5 to phospholipid, which was varied from 1:833 to 1:208.

In a control experiment, LUVs were preincubated for 72 h in the absence of cytochrome b_5 before addition of the protein; the results were indistinguishable from those obtained with fresh LUVs. Other control experiments will be discussed later.

Characterization of the fractions having tightly bound cytochrome b_5

One cannot automatically assume that the newly formed fast-sedimenting particles are LUVs; it is conceivable that during the incubations with cytochrome b_5 the starting LUVs are converted to small vesicles, multilamellar vesicles, or even nonvesicular structures such as mixed micelles. (For small vesicles or mixed micelles to sediment so fast, supramolecular aggregates would have to form.) We therefore carried out experiments to characterize these complexes.

To determine whether the protein-enriched particles are structurally intact vesicles, the trapped volume of LUVs was determined before and after the incubation with cytochrome b_5. A protocol similar to the one described in the legend to Fig. 1 was followed, but this time with LUVs containing trapped sucrose. After addition of SUV acceptors and sedimentation through the glycerol gradient, the bottom fractions were collected, dialyzed, brought to 25% in D_2O, and centrifuged for 60 min at 45000 rpm in a Ti50 rotor. As

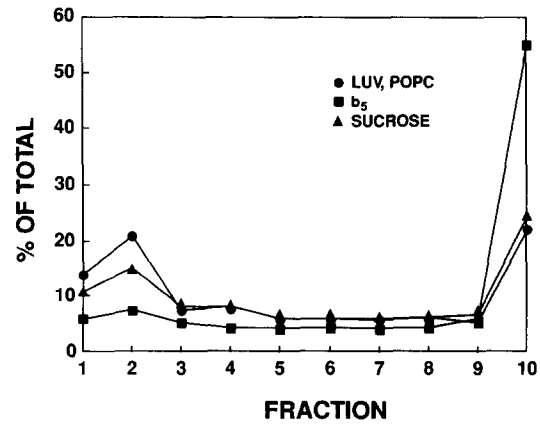


Fig. 3. Characterization of fractions with tightly bound cytochrome b_5. Cytochrome b_5 (30 nmol) was incubated with POPC LUVs (25 μmol POPC doped with $7.2 \cdot 10^4$ dpm [^{14}C]triolein/μmol phospholipid; total volume, 3.13 ml). The vesicles also contained entrapped [^{3}H]sucrose ($1.25 \cdot 10^5$ dpm/μmol phospholipid). After 90 h incubation, POPC SUVs (97 μmol) were added and the mixture was allowed to incubate for an additional 2 h. 0.5-ml aliquots were then sedimented through 3%-glycerol step gradients, and the lipoprotein complexes containing tightly bound cytochrome b_5 were recovered in the bottom fractions. The fractions were pooled, dialyzed against buffer (to remove glycerol) brought to 25% in D_2O and centrifuged for 60 min at 45000 rpm in a Ti50 rotor. The sedimentation profile from this last step is shown in the figure.

shown in Fig. 3 sedimentation through the D_2O solution cleanly separates the protein-enriched fractions from residual LUVs that have been depleted of cytochrome b_5. As shown, the trapped volume ([^{3}H]-sucrose/phospholipid) of the protein-enriched complexes is comparable to that of the protein-depleted LUVs (and to the starting LUVs). We also found that all these particles elute in the void volume of a Sepharose 2B-CL column (not shown).

These results show that the proteoliposomes are large, structurally intact vesicles. It is reasonable to conclude that they are LUVs rather than MLVs because LUVs cannot be converted to MLVs without breaking and resealing, which would result in a loss of vesicle contents. It is conceivable, however, that larger LUVs arise from non-leaky fusion of smaller ones.

The protein to lipid ratio in these fractions was found to be about 1:200. Recent studies in this laboratory have shown that this is comparable to the saturating level of binding (approx. 1:150) that occurs when excess cytochrome b_5 is added to POPC-LUVs. The significance of this observation will be disussed later.

Attempts to find evidence for a pre-existing insertion-active fraction in LUV preparations

The results presented above show that all of the tightly bound cytochrome b_5 is concentrated in a small fraction of the LUV population. One way to explain this observation is to assume that in any preparation of LUVs (prior to the addition of protein) a small fraction of the vesicles are in a strained, destabilized state that renders them 'insertion-active'. How such a population would arise is a matter of speculation: Perhaps the extrusion of multilamellar vesicles through polycarbonate membranes, which entails rupture and resealing of bilayers, occasionally leads to the formation of LUVs that seal before the lipids can organize into a stable structure.

Whatever the mechanism might be, the existence of such particles would provide the simplest explanation for the results; if such a pre-existing subpopulation were present during the prolonged incubations with protein, it would act as a sink for the cytochrome b_5 by trapping transferable protein in the tight configuration. We therefore designed several experiments to see whether these putative pre-existing insertion-active vesicles exist.

First, we pre-fractionated the LUVs obtained by the reverse-phase evaporation method by gel filtration (Fig. 4) and sedimentation velocity (Fig. 5). The subfractions were then tested for tight-insertion activity with cytochrome b_5. In all cases the results were indistinguishable from those obtained with unfractionated LUVs.

Second, we performed a series of experiments similar to those shown in Fig. 1, but with vesicles prepared by the detergent-removal procedure. Since the detergent-removal method and the extrusion method of LUV preparation are based on entirely different principles (de novo vesicle formation from mixed micelles versus mechanical breakdown of larger particles) it seems unlikely that the same kind of unstable structures could be produced by both methods. The results we obtained with the LUVs prepared by detergent removal were indistinguishable from the results obtained with vesicles prepared by reverse-phase evaporation.

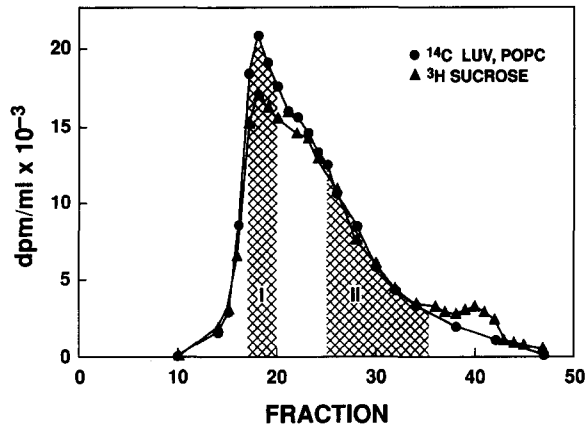


Fig. 4. Pre-fractionation of LUVs by gel filtration on Sephacryl S-1000. A 2-ml sample of POPC LUVs (13.4 μmol of phospholipid doped with $3.0 \cdot 10^4$ dpm [^{14}C]triolein/μmol lipid and loaded with $2.8 \cdot 10^4$ dpm [^{3}H]sucrose/μmol lipid) was subjected to gel filtration on a Sephacryl S-1000 column (1.6×30 cm) at 4°C and the fractions were analyzed for ^{3}H and ^{14}C. Fractions 17–20 (Roman numeral I) and 25–35 (Roman numeral II) were separately pooled.

Third, we tried to selectively remove the putative active LUVs from the LUV pool by taking advantage of the observation that the LUVs enriched in tight cytochrome b_5 sediment faster than the LUVs that have been totally stripped of cytochrome b_5 (Panel C

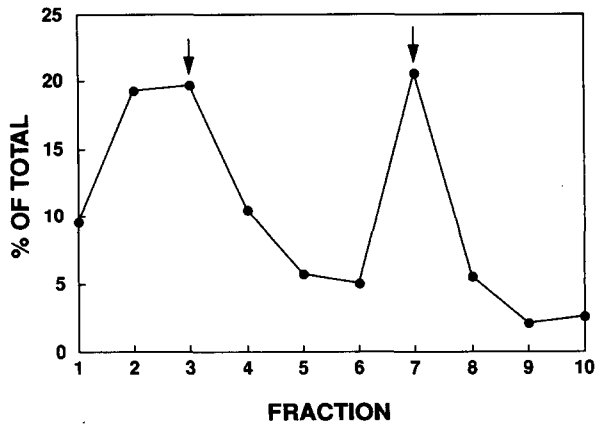


Fig. 5. Pre-fractionation of LUVs by sedimentation velocity. A 0.5-ml sample of POPC LUVs (5 μmol of POPC doped with $3.25 \cdot 10^5$ dpm [^{3}H]triolein/μmol PC) was layered onto 1.45 ml 2% glycerol and the step gradient was centrifuged for 15 min at 45000 rpm in a Ti50 rotor. Fractions were assayed for ^{3}H. Fractions 3 and 7 were used for subsequent studies.

of Fig. 1). If a clean separation were successful, and if our hypothesis is correct that LUV preparations contain pre-existing active and inactive subpopulations, we should be able to isolate a protein-free fraction of LUVs that has no tight binding activity. We should also obtain a protein-enriched fraction that could be tested for tight binding activity. The experimental protocol was as follows. After incubation of LUVs with cytochrome b_5 for 72 h, SUV acceptors were added and the mixture was sedimented through a step gradient that contained 3% glycerol in the central layer. As shown in Fig. 6, these centrifugation conditions provide a good separation of protein-enriched LUVs from protein-depleted LUVs. We refer to these as post-fractionated LUVs, since they are obtained after the incubation with cytochrome b_5. Protein-depleted LUVs (fractions 5 and 6) and protein-enriched LUVs (fractions 9 and 10) were collected and tested separately for their ability to incorporate freshly added cytochrome b_5 in the tight configuration.

The results are shown in Fig. 7. The tight insertion activity of post-fractionated protein-depleted vesicles (Fractions 5 + 6 from Fig. 6) was indistinguishable from that of unfractionated LUVs, and the sedimentation profiles changed in the familiar way with time of incubation as illustrated by the experiment in Fig. 1. When the protein-enriched fractions (9 + 10 of Fig. 6) were tested for tight insertion activity, all of the freshly added cytochrome b_5 that bound became tightly inserted within 2 h, the shortest time that can be monitored with our assay. Evidently, the protein-enriched vesicles are 'insertion-active'.

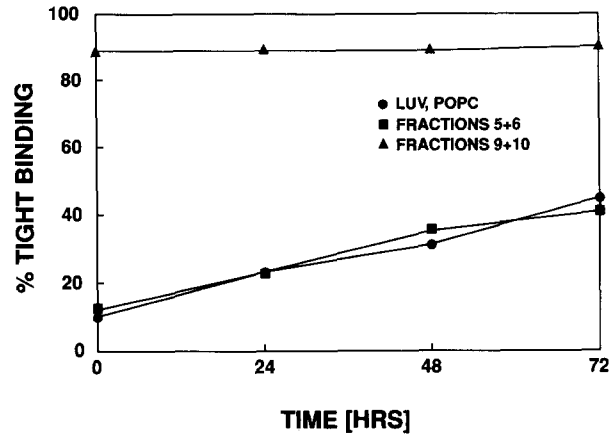


Fig. 7. Kinetics of tight binding to postfractionated LUVs. POPC LUVs obtained from the gradient shown in Fig. 6 (0.47 μmol of POPC from pooled fractions 5 + 6; 0.088 μmol of POPC from pooled fractions 9 + 10) were incubated with [^{14}C]amidinated cytochrome b_5 (1 mol protein:416 mol lipid; total volume, 0.65 ml) for 2, 24, 48 and 72 h. As a control, protein was also incubated with a sample of freshly prepared POPC LUVs (0.5 μmol phospholipid). At the end of each incubation period, a 4-fold excess of POPC SUVs was added and the mixture allowed to incubate for an additional 2 h. After separation on 3%-glycerol step gradients, the fractions were analyzed for ^{3}H and ^{14}C. Results were corrected for unbound protein (see Methods).

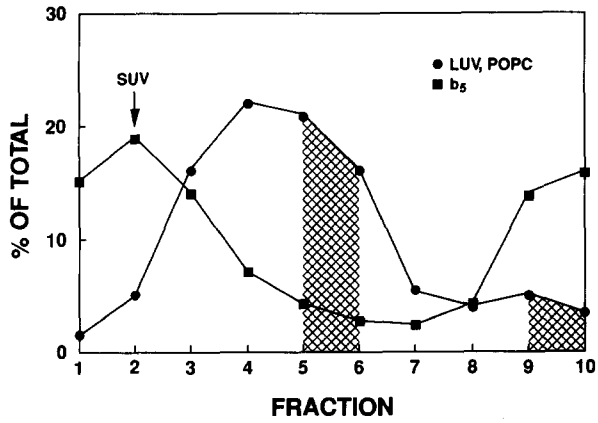


Fig. 6. Post-fractionation of LUVs after prolonged incubation with cytochrome b_5. 30 nmol of [^{14}C]amidinated cytochrome b_5 ($3.7 \cdot 10^7$ dpm/μmol) was incubated for 72 h with POPC LUVs (25 μmol POPC doped with $4.2 \cdot 10^5$ dpm [^{3}H]triolein/μmol phospholipid; total volume, 2.77 ml). POPC SUVs were then added (100 μmol POPC in 4.26 ml) and the mixture was allowed to incubate for an additional 2 h. 12 aliquots were then fractionated simultaneously by sedimentation through as many 3%-glycerol step gradients (as described in Materials and Methods), and analyzed for ^{3}H and ^{14}C. A representative gradient is shown in the figure; the shaded areas indicate the two pools (middle fractions 5 + 6, and bottom fractions 9 + 10) that were used for subsequent studies. The arrow shows where SUVs elute.

To ensure that exposure to glycerol and subsequent dialysis does not make vesicles insertion-active, control experiments were performed as follows. Fresh POPC-LUVs were incubated with either native or amidinated cytochrome b_5 for 2 h at 30°C to form complexes. Then an equal volume of 60% glycerol was added, and after a 1 h incubation, the mixture was dialyzed overnight. The fraction of protein tightly bound after this treatment was found to be, on average, 26%, which is indistinguishable from results obtained with vesicles that have not been subjected to this pre-treatment.

The results with fractions 5 + 6 strongly suggest that the results shown in Fig. 1 do not result from a pre-existing active fraction of LUVs in a LUV preparation. Rather, the protein-enriched particles appear to be generated from 'normal' LUVs during the incubation with cytochrome b_5.

The fact that post-fractionated protein-depleted LUVs show no greater insertion activity than do fresh vesicles also serves as a control to rule out the possibility that tight insertion is caused by lipid degradation during the long incubations. If lipid degradation were responsible for tight insertion, all the vesicles should be equally insertion-active since there is no obvious reason why a subpopulation of the LUVs should selectively accumulate such degradation products. It should be noted that all the vesicles contain bound cyto-

chrome b_5, either loose or tight, during the incubation period. Therefore, one cannot invoke a mechanism wherein only the protein-enriched particles become degraded.

Additional controls showed that tight insertion into the protein enriched insertion-active vesicles (fractions 9 + 10 of Fig. 6) is not due to lipid degradation. The insertion-active proteoliposomes were subjected to a Folch extraction, and the extracted lipids were used to form a second batch of LUVs. These LUVs, when tested for tight insertion activity, were found to be indistinguishable from fresh LUVs. Experiments performed with sterile solutions (Millipore filtered with sodium azide included) showed the same results: this rules out the possibility that bacterial contamination, which could cause lipid breakdown, might be responsible for the insertion activity. Finally, no lipid breakdown products were evident on thin-layer chromatograms.

Additional studies on the insertion-active vesicles

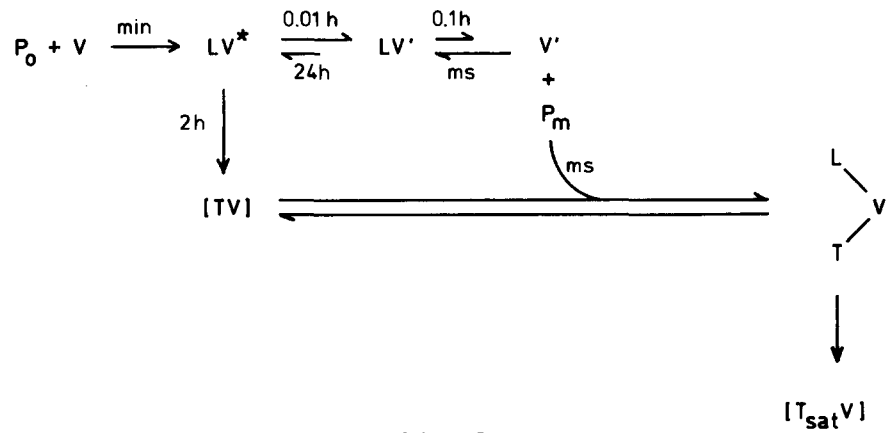
A simple explanation for the insertion activity of fractions 9 + 10 is that cytochrome b_5 in the tight configuration facilitates the tight insertion of additional cytochrome b_5. To test this possiblility, we first prepared proteoliposomes of cytochrome b_5/POPC by a detergent-reconstitution procedure that incorporates cytochrome b_5 into the tight configuration [18]. Exogenous cytochrome b_5 was then added to the proteoliposomes, and after a 2 h incubation period the fraction of tightly bound protein was determined by transfer to the SUVs. The results showed that 79% of the bound protein was transferable. This demonstrates that the proteoliposomes prepared by detergent-removal are not insertion-active, which seems to rule out the possibility that tightly bound cytochrome b_5, per se, catalyzes tight insertion of more protein.

Discussion

We have shown that when cytochrome b_5 is incubated with LUVs, (1) initially, a small fraction of the protein is rapidly inserted in the tight configuration, (2) subsequently, the major fraction of the protein becomes tightly inserted on a time scale of days, (3) all of the tightly inserted protein is found concentrated (at approximately saturating levels) in a small fraction of the vesicles, (4) all exogenously added cytochrome b_5 that binds to the isolated protein-enriched vesicles becomes tightly inserted on a time scale too fast to measure with our assay (< 2 h).

A reaction scheme to account for these results is shown (Scheme I). The numbers are postulated approximate halftimes for the individual steps. P_o represents the initial form of cytochrome b_5, i.e., mainly octomers in equilibrium with a small amount of monomers, which is added to the vesicles, V. The initial protein–lipid interaction forms a complex, LV*, comprised of a destablized vesicle, V*, with loosely bound protein, L. (Cytochrome b_5 has been shown to transiently destabilize vesicles [28]). LV* has two fates: conversion to a relatively relaxed vesicle with loosely bound protein, LV′, and conversion to insertion-active vesicles with tightly bound protein, [TV]. If, for the sake of illustration, the rate of LV′ formation is 20-times faster than the rate of [TV] formation, the ratio of LV′ to all vesicles containing tightly bound protein will be about 20 after the first two hours of incubation, when all the P_o is bound.

LV′ is the predominant lipoprotein particle present throughout the long incubation periods. It exists in equilibrium with monomeric cytochrome b_5, P_m, and can be converted on a time scale of days to [TV] via LV*. The initial interaction of P_m with [TV] is postulated to produce a short-lived loose binding protein,



Scheme I.

which is rapidly converted to the tight binding form. Continued binding of P_m to insertion active vesicles eventually produces $T_{sat}V$, a subpopulation of vesicles saturated with tight binding protein.

The halftimes for the initial binding of cytochrome b_5 to vesicles and the halftimes for the transfer of cytochrome b_5 between vesicles has been experimentally determined [18,34]. We have also determined that cytochrome b_5 added to insertion-active vesicles forms tightly bound protein within 2 h (Fig. 7). What remain as hypothetical halftimes in this scheme are the ones for interconverting the hypothetical intermediates LV*, LV', and [TV].

This scheme is rather elaborate because it is necessary to account for origin of the initial subpopulation of activated vesicles (i.e., those that have perhaps one or two tightly inserted proteins per vesicle) in a population of proteoliposomes that is expected to behave uniformly. While heterogeneity in vesicle activity might be understandable if the protein to vesicle ratio was very low, compositional heterogenity is unlikely in our system where the cytochrome b_5 to LUV ratio is at least 60:1. Though difficult to explain, functional heterogeneity has been observed in systems that appear to be nearly homogeneous in composition; in this laboratory, we have recently shown that addition of magainin 2a to a population of phosphatidylserine LUVs causes a burst of solute release, via bilayer destabilization, from only a fraction of the vesicles even though the ratio of bound peptide per vesicle is at least 1000:1 [35]. And detergents, in relatively high ratios of bound detergent per phospholipid, have also been reported to induce all-or-none release of solutes from cells [32,33].

An apparent inconsistency in this model is the following: If the protein-enriched vesicles were saturated with protein, it should not have been possible to incorporate additional protein into these isolated proteoliposomes by adding exogenous cytochrome b_5 (Fig. 7). This inconsistency can be explained by assuming that a true saturation of the protein-enriched vesicles is not attained in the original incubation mixture because as saturation is approached, charge repulsion of the acidic catalytic domain of the protein, and perhaps steric effects, substantially reduce the affinity constant for remaining sites on the vesicle. In such a case, the partitioning of loosely bound protein between vesicle species could so heavily favor the vesicles with low protein/lipid ratio that further accumulation in the protein-enriched fractions would cease short of true saturation. However, when protein is added exogenously to the isolated proteoliposomes (Fig. 7) some binding occurs because in this case the protein is partitioning between the aqueous phase and a site on the vesicle rather than between donor and acceptor vesicles; even with a greatly reduced affinity constant, the bound state is preferred to the unbound state.

We must also account for the observation that when tight cytochrome b_5 is produced by traditional methods such as reconstitution with detergents, the reconstituted vesicles are not insertion-active as are the protein-enriched vesicles obtained after long incubation. One possiblity is that the lipid packing is different in vesicles generated in different ways. But there is also the possibility that there may be more than one tight binding form of the protein. Since tight binding is defined operationally (as nontransferable protein) rather than structurally, it is possible that the nontransferable form generated by detergent-reconstitution differs structurally from the nontransferable form generated by prolonged incubation. If so, these forms could have different effects on the insertion of exogenous protein.

There is at present no way to know which reconstitution method produces the native stucture of nontransferable cytochrome b_5 and/or the physical properties of the native membrane. It has been shown that when cytochrome b_5 is incubated with liver microsomes, all the protein inserts rapidly into the nontransferable form [18]. This suggests the possibility that the insertion-active vesicles we have obtained represent a more native-like membrane than those obtained by traditional reconstitution methods.

From a practical standpoint, these studies show that complex transformations leading to compositional and structural heterogeneity occur in a system that was chosen for its simplicity.

Acknowledgement

This work was supported by NIH grant DK30432.

References

1 Wickner, W.T. and Lodish, H.F. (1985) Science 230, 400–407.
2 Blumenthal, R. and Klausner, R.D. (1982) in Membrane Reconstitution (Poste, G. and Nicolson, G.L., eds.), pp. 43–82, Elsevier Biomedical Press, Amsterdam.
3 Jain, M.K. and Zakim, D. (1987) Biochim. Biophys. Acta 906, 33–68.
4 Strittmatter, P. and Dailey, H.A. (1982) in Membranes and Transport (Martinosi, A.N., ed.), Vol. 1, p. 71, Plenum Press, New York.
5 Okada, Y., Sabatini, D.D. and Kreibich, G. (1979) J. Cell. Biol. 83, 437a.
6 Rachubinski, R.A., Verma, D.P.S. and Bergeron, J.J.M. (1978) J. Cell. Biol. 362a.
7 Bendzko, P., Prehn, S., Pfeil, W. and Rapoport, T. (1982) Eur. J. Biochem. 123, 121–126.
8 Anderson, D.J., Mostov, K.E. and Blobel, G. (1983) Proc. Natl. Acad. Sci. USA 80, 7249–7253.
9 Calabro, M.A., Katz, J.T. and Holloway, P.W. (1976) J. Biol. Chem. 251, 2113–2118.
10 Remacle, J. (1978) J. Cell. Biol. 79, 291–313.
11 Dufourcq, J., Bernon, R. and Lussan, C.C.R. (1974) Acad. Sci. Ser D, Paris 278, 2565–2568.

12 Strittmatter, P., Rogers, J.J. and Spatz, L. (1972) J. Biol. Chem. 247, 7188–7194.
13 Enomoto, K. and Sato, R., (1973) Biochem. Biophys. Res. Commun. 51, 1–7.
14 Sullivan, M.R. and Holloway, P.W. (1974) Biochem. Biophys. Res. Commun. 54, 808–815.
15 Holloway, P.W. and Katz, J.T. (1975) J. Biol. Chem. 250, 9002–9007.
16 Rogers, M.J. and Strittmatter, P. (1975) J. Biol. Chem. 250, 5713–5718.
17 Roseman, M.A., Holloway, P.W., Calabro, M.A., Thompson, T.E. (1977) J. Biol. Chem. 252, 4842–4849.
18 Enoch, H.G., Fleming, P.J. and Strittmatter, P. (1979) J. Biol. Chem. 254, 6483–6488.
19 Takagaki, Y. Radhakrishman, R., Gupta, C.M. and Khorana, G.H. (1983) J. Biol. Chem. 258, 9128–9135.
20 Poensgen, J. and Ullrich, V. (1980) Biochim. Biophys. Acta 596, 248–263.
21 Christiansen, K. and Carlsen, J. (1985) Biochim. Biophys. Acta 815, 215–222.
22 Christiansen, K. and Carlsen, J. (1983) Biochim. Biophys. Acta 735, 225–233.
23 Takagaki, Y., Radhakrishnan, R., Writz, W.A. and Khorana, G.H. (1983) J. Biol. Chem. 258, 9136–9142.
24 Ozols, J. (1989) Biochim. Biophys. Acta 997, 121–130.
25 Arinc, E., Rzepecki, L.M. and Strittmatter, P. (1987) J. Biol. Chem. 262, 15563–15567.
26 Greenhut, S.F., Bourgeois, V.R. and Roseman, M.A. (1986) J. Biol. Chem. 261, 3670–3675.
27 Greenhut, S.F. and Roseman, M.A. (1989) Biophys. J. 55, 323a.
28 Greenhut, S.F. and Roseman, M.A. (1985) Biochemistry 24, 1252–1260.
29 Dailey, H.A. and Strittmatter, P. (1979) J. Biol. Chem. 254, 5388–5396.
30 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
31 Enoch, H.G. and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. USA 76, 145–149.
32 Scharff, T.G. (1960) Biochem. Pharmacol. 5, 79–86.
33 Silhankova, L. (1959) Folia Microbiol. 4, 29–40.
34 Leto, T. and Holloway, P.W. (1979) J. Biol. Chem. 254, 5015–5019.
35 Grant, E., Beeler, T.J., Taylor, K.M.P., Gable, K. and Roseman, M.A. (1992) Biochemistry 31, 9912–9918.