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Reconstitution of cytochrome b_5 into lipid vesicles in a form which is nonsusceptible to attack by carboxypeptidase Y

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Pig liver cytochrome b_5 is reconstituted into lipid vesicles by a method whereby cytochrome b_5-lysophospholipid micelles are fused with liposomes. The reconstitution method inserts cytochrome b_5 into the lipid bilayer in a mode which renders the C-terminal part of cytochrome b_5 nonsusceptible to attack by carboxypeptidase Y. The effect of the lipid composition of the vesicles on the mode of insertion has been examined, when cytochrome b_5 is reconstituted using the presently described method and methods previously used to reconstitute this protein, namely the direct incorporation method and the cholate dialysis method.

Introduction

Cytochrome b_5, a component of the microsomal fatty acid desaturase system, is an amphiphilic protein, with a hydrophilic domain containing the catalytical activity and a hydrophobic segment anchoring the protein to the membrane [1].

Reconstitution of cytochrome b_5 into lipid vesicles has previously been performed either by the direct incorporation method or by the cholate dialysis method. Cytochrome b_5 incorporated into lipid vesicles by the direct incorporation method as described by Enoch et al. [2] results in reconstitution of cytochrome b_5 in a loosely bound or a transferable form. Cytochrome b_5 in this form is characterized by its susceptibility to attack by carboxypeptidase Y and its readily exchangeability between membranes. Cytochrome b_5 incorporated by the cholate dialysis method results in a form which is designated the tightly bound or the non-transferable form. In this form the C-terminal part of cytochrome b_5 is not accessible to carboxypeptidase nor does the protein transfer between membranes [2,3].

In the present report we describe the reconstitution of cytochrome b_5 into lipid vesicles by a method [4] which involves fusion of cytochrome b_5-lysophospholipid micelles with liposomes. This method results in insertion of cytochrome b_5 in a mode similar to the cholate dialysis method with the C-terminal part of cytochrome b_5 nonsusceptible to attack by carboxypeptidase Y.

Previously cytochrome b_5 has been reconstituted into lipid vesicles composed of a single phospholipid species, e.g. dimyristoyl-, dipalmitoylphosphatidylcholine or egg phosphatidylcholine. In most experiments described in this report the natural mixture of lipids of the endoplasmic reticulum has been used.

In the present study is also described the effect of lipid composition on the mode of insertion when cytochrome b_5 is reconstituted into lipid vesicles by the three different methods: the present method, using the natural detergents; lysophos-

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pholipids, the direct incorporation method, and the cholate dialysis method.

Materials and Methods

Chemicals

Dioleylphosphatidylcholine, dioleylphosphatidylethanolamine, egg phosphatidylcholine, egg phosphatidylethanolamine and egg lysophosphatidylcholine were obtained from Serdary Research Laboratories, London, Canada. Di-[1-^{14}C]-palmitoylphosphatidylcholine (spec. act. 156 μCi/mg) and [1-^{14}C]palmitoyllysophosphatidylcholine (spec. act. 115 μCi/mg) was obtained from Amersham International, Amersham, U.K. Phospholipase C, grade II (800 U/mg) was obtained from Boehringer Mannheim, GmbH, F.R.G. Carboxypeptidase Y was a gift from Carlbiotech, Copenhagen, Denmark. Ultrogel AcA 34 was from LKB Products (Stockholm, Sweden), Sepharose 4B from Pharmacia (Uppsala, Sweden), and Aquasol from New England Nuclear, Boston, MA, U.S.A. The organic solvents were of analytical reagent grade and was distilled prior to use. For petroleum ether the fraction with a boiling range of 60–80°C was used.

Isolation of cytochrome b_5

Amphiphilic cytochrome b_5 was isolated from pig liver essentially as described by Strittmatter et al. [5], and was obtained in sodium deoxycholate after Sephadex G-75 chromatography. Detergent-free cytochrome b_5 was prepared by removal of the sodium deoxycholate by passage through a Sephadex G-25 column as described [5]. Trypsin digestion of cytochrome b_5 was performed as described [6]. Measurements of cytochrome b_5 was performed by spectroscopy at 413 nm using an ϵ of 117 $mM^{-1} \cdot cm^{-1}$.

Detergent exchange

Sodium deoxycholate in the cytochrome b_5 preparations was exchanged by lysophosphatidylcholine by dialysis. A 45 μM solution of cytochrome b_5 (600 μl) in 20 mM Tris-acetate buffer (pH 8.1) containing 0.2 mM EDTA, 100 mM sodium chloride and 1% sodium deoxycholate was added to 13.5 μmol lysophospholipid in some cases containing a trace of [1-^{14}C]lysophosphatidylcholine. The molar ratio of lysophospholipid to cytochrome b_5 was 500, and the lysophospholipid concentration being 1.3%. The dialysis was performed for 30–48 h against several changes of the buffer, 10 mM Tris-acetate (pH 8.1) containing 0.1 mM EDTA and 100 mM sodium chloride. The cytochrome b_5-lysophosphatidylcholine complex was analyzed by gel chromatography on Ultrogel AcA 34 under the conditions as stated in Fig. 1.

Liposomes

For the preparation of liposomes either of the three different lipid mixtures was used: dioleylphosphatidylcholine and dioleylphosphatidylethanolamine (molar ratio 10 : 1), egg phosphatidylcholine and egg phosphatidylethanolamine (molar ratio 10 : 1) or a total lipid extract of Ca^{2+}-precipitated pig liver microsomes [7] prepared by chloroform-methanol extraction according to Folch et al. [8]. In order to prevent autooxidation butylated hydroxytoluene was added during extraction of the lipid from pig liver microsomes. The final concentration was approx. 1 mol butylated hydroxyltoluene per 1000 mol lipid. The lipid extract was stored under N_2 at -20°C. For the preparation of liposomes of either of the three lipid mixtures, the lipid dissolved in chloroform and containing a trace of [^{14}C]phosphatidylcholine was evaporated to dryness under N_2, leaving the lipid as a thin film in a flask. In order to ensure complete removal of the solvent, the lipid was left under vacuum for at least 1 h. The lipid was suspended by shaking on a Vortex mixer in 10 mM Tris-acetate (pH 7.0) containing 0.1 mM EDTA. The lipid concentration being 9 μmol per ml. The suspension was left at 35°C for 10 min followed by sonication for 2×5 min at 28–35°C in a 80 watt bath- type sonicator (Laboratories Supplies Co., Hicksville, NY). The solutions were centrifuged at $30\,000 \times g$ for 10 min in order to remove multilamellar vesicles.

Lipid analysis

Cholesterol was determined [9] on silica gel spots after thin-layer separation of neutral lipids and phospholipids in the solvent system petroleum ether/diethyl ether/acetic acid (70 : 30 : 1, v/v). The amount of individual phospholipids was determined as the amount of lipid phosphorus [10]

on silica gel spots after thin-layer chromatography in the solvent system chloroform/methanol/2-propanol/0.25% KCl/ethyl acetate (30 : 9 : 25 : 6 : 18, v/v) developed three times. A conversion factor of 25 was used for calculating the amount of phospholipids from the determined amount of phosphorus. The fatty acid composition was determined by gas-liquid chromatographic analysis of methyl esters on cyanosilicone (Silar 9CP) coated on acid-washed Chromosorb W. The stainless steel column was programmed between 150 and 220°C (3 deg. C per min) in a Perkin-Elmer F11 gas chromatograph equipped with a flame ionization detector.

Reconstitution

Reconstitution of cytochrome b_5 was performed by three different methods: (1) The direct incorporation method as described by Enoch et al. [2] and performed by mixing the detergent-free form of cytochorme b_5 with liposomes in the molar ratio of phospholipid to cytochrome b_5 of 200 : 1, (2) the cholate dialysis method as described by Takagaki et al. [3] with a molar ratio of phospholipid to cytochrome b_5 of 50 : 1, or (3) our method using fusion of protein-lysophospholipid micelles with liposomes [4]. Reconstitution of cytochrome b_5 by this last mentioned method was performed by mixing cytochrome b_5-lysophospholipid micelles with liposomes in a lipid to cytochrome b_5 molar ratio of 2000 and 8000 followed by incubation at 25°C for 2 h.

Carboxypeptidase Y digestion

Carboxypeptidase Y digestion was performed at 30°C for 20 h after adjusting of the pH of the samples to 6.5 with acetic acid. A cytochrome b_5 to carboxypeptidase Y molar ratio of 20 : 1 was used.

Polyacrylamide gel electrophoresis in SDS

Analysis of the carboxypeptidase sensitivity of cytochrome b_5 incorporated into the lipid vesicles was performed by polyacrylamide gel electrophoresis in 1% SDS according to Laemmli [11]. In cases of high phospholipid to cytochrome b_5 molar ratios, the phospholipid was removed before the slab gel electrophoresis by incubation with excess phospholipase C and removal of the diacylglycerol formed by extraction with petroleum ether followed by concentration of the water phase containing the proteins by lyophilization as described [12]. After electrophoresis the gel was stained for protein with Coomassie brilliant blue.

Results

Detergent exchange

The difference between the critical micellar concentration of sodium deoxycholate and lysophosphatidylcholine, 4–6 mM [13] and 0.20–0.45 mM

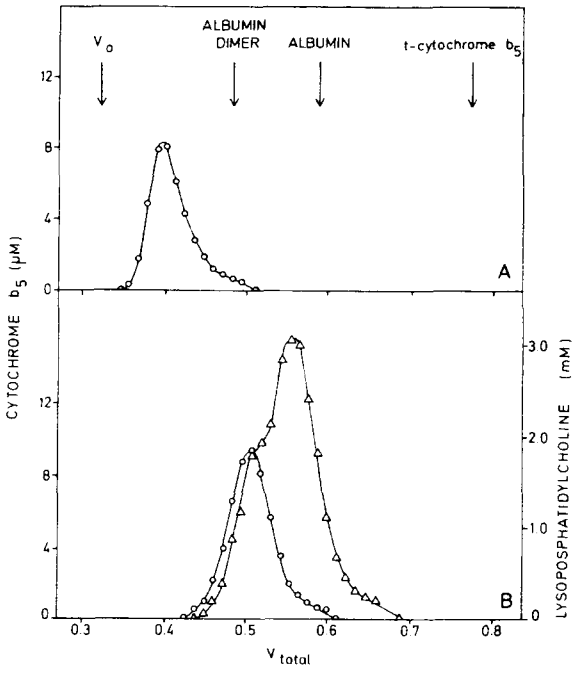

Fig. 1. Elution profile on Ultragel AcA 34 of (A), cytochrome b_5 aggregates formed by dialysis of cytochrome b_5 in 1% sodium deoxycholate against 10 mM Tris-acetate (pH 8.1) containing 0.1 mM EDTA and 100 mM NaCl for 48 h and (B), cytochrome b_5-lysophosphatidylcholine micelles formed by adding a 500-fold molar excess of lysophosphatidylcholine to cytochrome b_5 in 1% sodium deoxycholate followed by dialysis as stated above. 500 μl of either of the samples was applied to the column (0.9×58 cm, V_{total} = 37.1 ml) equilibrated and eluted at 27°C with dialysis buffer. In case of (B) the buffer contained 0.1% lysophosphatidylcholine. The flow rate was 2.0 ml/h and fractions of 0.44 ml were collected. A_{413} and phosphate, corrected for background values of buffer content of lysophosphatidylcholine, were measured. The elution volume of the marker proteins bovine serum albumin dimer (136000), monomer (68000) and the tryptic fragment of cytochrome b_5 (M_r 9400) was as indicated.

[14,15], respectively, makes it possible that cytochrome b_5-deoxycholate micelles can be converted into cytochrome b_5-lysophospholipid micelles by adding excess lysophospholipid followed by dialysis. Experiments with deoxycholate has shown that this detergent can be removed by dialysis [16] and our experiment shows that 80% of the lysophospholipid, added in 500 molar excess of the cytochrome b_5 is recovered after 48 h of dialysis at 22°C. The cytochrome b_5-lysophospholipid micelles have been analyzed by gel chromatography (Fig. 1B). The lysophospholipid micelles containing cytochrome b_5 are partly separated from the lysophospholipid micelles without cytochrome b_5 and elute in a volume corresponding to a molecular weight of about 100000. Based on cytochrome b_5 measurements and lysophospholipid determinations the cytochrome b_5 to lysophospholipid molar ratio is about 150 in the front fractions which is in agreement with the molecular weight determinations. Lysophospholipid micelles without cytochrome b_5 elute at a volume which correspond to a molecular weight of about 70000 and cytochrome b_5 alone elutes as an aggregate corresponding to a molecular weight of about 200000 (Fig. 1A).

Liposomes

The elution profile on Sepharose 4B of liposomes prepared from either of the three types of lipids was similar and in agreement with that normally obtained with liposomes prepared by sonication (17]. The liposomes appeared as two populations of vesicles, one population which elutes at about V_0 and a population with an elution volume of $0.5 \cdot V_{total}$. Electron microscopy of a negatively stained preparation of liposomes also indicates two population, one with a diameter range of 120–160 nm and one with a diameter range of 50–80 nm.

Lipid analysis of pig liver microsomal lipid showed that it contained cholesterol and phospholipid in the molar ratio of 1 : 3. The phospholipd composition is shown in Table I. The main phospholipids are phosphatidylcholine and phosphatidylethanolamine, while sphingomyelin, phosphatidylserine and -inositol each exceed 6–8% of the total phospholipids.

The fatty acid composition of egg phospholipids (phosphatidylcholine and -ethanolamine in the molar ratio of 10 : 1) and pig liver microsomal lipid was analyzed by gas-liquid chromatography and the results obtained are shown in Table II. The fatty acid composition of egg phospholipids is a mixture of 50.4% saturated fatty acid, mostly 16 : 0 and 33.6% monounsaturated and 16.0% polyunsaturated fatty acid. The polyunsaturated fatty acid being mostly 18 : 2. The pig liver microsomal lipid also contained close to 50% saturated fatty acid with 18 : 0 being the predominant saturated fatty acid. But in comparison to egg phospholipid the pig liver microsomal lipid con-

TABLE I

PHOSPHOLIPID COMPOSITION OF PIG LIVER MICROSOMAL LIPID

	% of total phospholipid
Phosphatidylethanolamine	24.5
Phosphatidylcholine	48.9
Phosphatidylinositol	8.1
Phosphatidylserine	7.0
Phosphatidic acid	1.8
Sphingomyelin	5.9
Others	3.8

TABLE II

FATTY ACID COMPOSITION OF LIPID USED IN PREPARATION OF LIPOSOMES

Fatty acid	% of total fatty acid	
	Egg (PC + PE) (molar ratio 10 : 1)	Pig liver microsomal lipid
16 : 0	35.8	16.1
16 : 1	1.8	1.1
18 : 0	14.6	30.8
18 : 1	31.8	13.6
18 : 2	12.4	12.6
18 : 3	0.3	0.0
20 : 4	3.3	19.0
22 : 5	0.0	2.2
22 : 6	0.0	4.6
Saturated fatty acid	50.4	46.9
Double-bond index [a]	0.73	1.55

[a] Double-bond index is the sum of the fraction of each fatty acid times the number of double bonds in that acid.

tained much more polyunsaturated fatty acid (38.4% of the total) and the major polyunsaturated fatty acids being 18 : 2, 20 : 4 and some 22 : 6.

The mode of insertion of cytochrome b_5 into lipid vesicles and the effect of the lipid composition

Reconstitution of cytochrome b_5 into lipid vesicles by the present method was performed by mixing of cytochrome b_5-lysophospholipid micelles with liposomes as described in Materials and Methods. The resulting lipid to lysophospholipid molar ratios were, respectively, 5 and 20.

The mode of insertion of cytochrome b_5 into lipid vesicles is examined by treatment of the reconstituate with carboxypeptidase Y. The results are analyzed by polyacrylamide gel electrophoresis in SDS. As shown in Fig. 2A using two different ratios of lipid to cytochrome b_5, cytochrome b_5 is predominantly in a form which is nonsusceptible to attack by carboxypeptidase Y, indicating that cytochrome b_5 is incorporated into the lipid bilayer with the C-terminal part shielded. The results are independent of the lipid composition of the vesicles.

In Fig. 2B are shown the results obtained with carboxypeptidase Y treatment of cytochrome b_5 reconstituted by the direct incorporation method and the cholate dialysis method using lipid vesicles of three different lipid compostions.

The direct incorporation method results in insertion of cytochrome b_5 into lipid vesicles in a form which is totally dependent of the lipid composition. Thus, when microsomal lipid is used, cytochrome b_5 is incorporated in a form which is completely susceptible to attack by carboxypeptidase Y. When egg phospholipids are used for preparing liposomes about 50% of cytochrome b_5 is in a form which is susceptible to attack by carboxypeptidase Y, while when using dioleyl-phospholipids, most of the cytochrome b_5 is nonsusceptible to attack by carboxypeptidase Y.

The cholate dialysis method results in insertion of cytochrome b_5 into lipid vesicles predominantly in a mode which is non-susceptible to attack by carboxypeptidase Y. The results are to a certain degree dependent of the lipid composition. Thus, when microsomal lipid is used, a higher amount of cytochrome b_5 is susceptible to attack by carboxypeptidase Y than when egg phospholipid is used. When dioleylphospholipid is used, cyto-

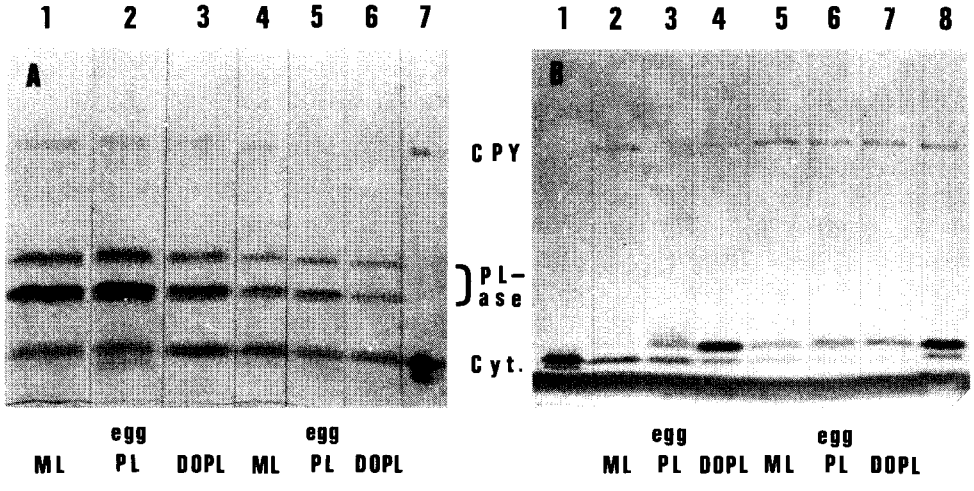

Fig. 2. SDS-polyacrylamide gel electrophoresis of carboxypeptidase Y-treated cytochrome b_5 reconstituted by three different methods (A), the present method using lipid to protein molar ratios of 8000 (lane 1–3) and 2000 (lane 4–6) and (B), the direct incorporation method (lane 2–4) and the cholate dialysis method (lane 5–7). With all three reconstitution methods three different types of lipids are used. As standards are used cytochrome b_5 only slightly converted by mild treatment at pH 8.1 with carboxypeptidase Y (Fig. A, lane 7 and Fig. B, lane 8) and cytochrome b_5 digested by trypsin (Fig. B, lane 1). ML, pig liver microsomal lipid; egg PL, egg phosphatidylcholine and -ethanolamine (molar ratio 10 : 1); DOPL, dioleylphosphatidylcholine and -ethanolamine (molar ratio 10 : 1); CPY, carboxypeptidase Y; PL-ase, phospholipase C which appears as two polypeptide bands; and Cyt, cytochrome b_5.

chrome b_5 is not sensitive to carboxypeptidase Y at all.

Discussion

Cytochrome b_5 is a small amphiphilic protein, which together with NADH-cytochrome b_5 reductase and the desaturase forms an electorn transport chain in the endoplasmic reticulum. Cytochrome b_5 is because of its size and three-dimensional structure [18] a protein, which without detergent forms stable soluble aggregates in solution; thereby producing an extreme low concentration of the monomeric species, the form which appears to insert into the lipid bilayer structure of either liposomes or the natural membrane, the endoplasmic reticulum [19].

Reconstitution of cytochrome b_5 into liposomes by the direct incorporation method using detergent-free cytochrome b_5 and preformed liposomes therefore results in incorporation of cytochrome b_5 into lipid vesicles in a form which is susceptible to attack by carboxypeptidase Y [2].

If cytochrome b_5 is incorporated into vesicles composed of dimyristylphosphatidylcholine, a lipid which forms less stable and permeable vesicles, or if cytochrome b_5 is reconstituted in the presence of detergents [2,3], the incorporation results in a form which is not accessible to attack by carboxypeptidase Y.

Experiments presented in this report show, that cytochrome b_5 can be incorporated into lipid vesicles in a mode similar to that obtained by the cholate dialysis method, namely a form which renders cytochrome b_5 nonsusceptible to attack by carboxypeptidase Y. The method, which has been described earlier, involves fusion of protein-lysophospholipid micelles with preformed liposomes [4]. In the case of cytochrome b_5 the protein-lysolipid micelles are formed by exchange of deoxycholate with lysophosphatidylcholine.

Based on the amount of cytochrome b_5 in rat liver endoplasmic reticulum [18] the molar ratio of cytochrome b_5 to phospholipid in this membrane is approximately 2000. In the present method molar ratios of 2000 and 8000 of lipid to cytochrome b_5 have been used. These two lipid to protein ratios result in lipid to lysophospholipid molar ratios in the liposomes of, respectively, 5 and 20. The present method cannot incorporate cytochrome b_5 into lipid vesicles in infinite amount, as lysophospholipid is a detergent which in high concentrations can disrupt the lipid bilayer. However, relatively high amounts of lysophospholipid are known to be able to be incorporated into natural membranes and into lipid vesicles [20], and as high as 20–35 mol% of lysophosphatidylcholine can be incorporated into liposomes without any effect on the permeability of the vesicles [21,22]. Incorporation of lysophospholipid into liposomes, especially into small lipid vesicles, is possible probably because of the 'wedge-shaped' lyso-molecule may fit into the strongly curved bilayer without disturbing their structural arrangement, and hence their permeability barrier properties [23]. Also the content of cholesterol in natural membranes and in lipid vesicles will increase the amount of lysophospholipid, which can be incorporated, as cholesterol and lysophospholipid form a 1 : 1 complex and this complex might form a bilayer similar to that formed by diacylphosphatidylcholine [24]. It is in this context noteworthy that, if cytochrome b_5 is incorporated into lipid vesicles made from pig liver endoplasmic reticulum by the present method with a lipid to protein ratio of 2000, the molar ratio of cholesterol and lysophospholipid will be approximately 1 : 1.

So far most of the experiments with reconstitution of cytochrome b_5 into lipid vesicles has been performed with a single species of lipid, namely phosphatidylcholine either as dimyristoyl-, dipalmitoyl- or egg phosphatidylcholine. In most of the experiments we have carried out with cytochrome b_5, we have used the mixture of lipids, which occur in the membrane natural for cytochrome b_5, namely the endoplasmic reticulum.

We have found a rather dramatic effect of the lipid composition of the vesicles on the difference in susceptibility of cytochrome b_5 to carboxypeptidase Y using the earlier described methods for reconstitution, e.g. the direct incorporation and the cholate dialysis method. In contrast, it is noteworthy that the lipid composition of the liposomes had no significant effect on the incorporation and thereby susceptibility of cytochrome b_5 inserted by the present described method.

The difference in behavior of cytochrome b_5 aggregates added to lipid vesicles of different com-

postion cannot be explained fully at the moment, but might be a function of the lipid bilayer. In the case of using lipid vesicles composed of dimyristoylphosphatidylcholine as used by Enoch et al. [2] or dioleylphospholipids as used in the present study, the lipid bilayer may shield the cytochrome b_5 aggregates from attack by carboxypeptidase Y or it might solubilize the aggregates and produce the monomeric species, which inserts into the lipid bilayer and become nonsusceptible to attack by carboxypeptidase Y.

In the cholate-dialysis method where cytochrome b_5 aggregates are dissolved in detergent, not all cytochrome b_5 might be in a micellar state, but some aggregates may still be present depending on the time of solubilization and removal of the detergent. The cytochrome b_5, which is still in the aggregated form, will not penetrate into the bilayer if egg phospholipids or pig liver microsomal lipids are used.

This report indicates that the lipid composition may influence the behaviour of cytochrome b_5 aggregates when added to lipid vesicles, and that only cytochrome b_5 in a monomeric state, either as itself or as cytochrome b_5-detergent micelles, will be inserted in a mode, which is not susceptible to attack by carboxypeptidase Y. This is the case with the present method, which involves formation of cytochrome b_5-lysophospholipid micelles and fusion of these micelles with preformed liposomes prepared from different lipid mixtures. As lysophosphatidylcholine is a natural lipid which occurs in small amounts in almost every biological membrane material analyzed [25], the use of this detergent is more appropriate than the use of artificial detergents.

The present method for incorporation of cytochrome b_5 into lipid vesicles can be used for studying the mode of insertion of cytochrome b_5 into the lipid bilayer. It might also be useful in studying the transport of cytochrome b_5 from its site of synthesis, the free polysomes [26,27] to the endoplasmic reticulum, where it is inserted and retained. Work is in progress for incorporation of cytochrome b_5 into the endoplasmic reticulum by the method described in this report. In this case the method is not limited by the amount of lysophospholipid added as the lysolipid in the natural membrane can be acylated into phosphatidylcholine, which have no detergent properties.

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