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BINDING OF BOVINE CYTOCHROME b_5 TO PHOSPHATIDYLCHOLINE LIPOSOMES

CHARACTERIZATION OF THE RECONSTITUTED LIPID-PROTEIN VESICLES

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SUMMARY

Cytochrome b_5 was extracted and purified from beef liver by a detergent method (cytochrome *d*-b_5). The hydrophilic moiety which carries the heme group (cytochrome *t*-b_5) was prepared by trypsin action upon pure cytochrome *d*-b_5.

Single-shelled lecithin liposomes form complexes with cytochrome *d*-b_5 up to a molar ratio of one protein for 35 phospholipids. The lipid-protein complexes were isolated by gel filtration on Sepharose 4B. They are hollow vesicles in which [^{3}H]-glucose can be trapped. Their diameter is greater than that of the initial liposomes.

Cytochrome *t*-b_5 does not interact with the vesicles. These results show that the hydrophobic tail is necessary for the binding and that the hydrophilic part of the protein is located on the outer face of the vesicles. This asymmetry is also proved by the action of reducing agents.

Experiments with saturated phosphatidylcholines show that the protein interacts with the lipids both below the transition temperature T_M, i.e. when the aliphatic chains are in a crystalline state, and above T_M, when the aliphatic chain are in a fluid state.

^{1}H NMR spectra show that even at the maximum cytochrome *d*-b_5 concentration the presence of the proteins does not markedly change the dynamics of the phospholipid molecules. An asymmetric single-shelled vesicle structure is proposed for the complex.

INTRODUCTION

Lipid-protein interactions are a key to our understanding of the structure-function relationship in membranes. One way to approach this problem involves reconstitution experiments using the dissociated components, proteins and lipids [1].

Two methods have been used: (a) Reconstitution of complete functional system, for example the cytochrome oxidase system (Racker [2]), chemically excitable membrane from *Torpedo marmorata* (Hazelbauer and Changeux [3]) and the redox chain from liver microsomes (Archakov et al. [4] and Lu and Levin [5]). (b) Re-

constitution of incomplete systems to look at specific interactions between intrinsic proteins and lipids (cf. Vanderkoi [6] on the cytochrome oxidase and rhodopsin; Redwood and Patel [7] on ATPase binding to phospholipids; Segrest et al. [8, 9] on the glycoprotein from erythrocytes; Warren et al. [10] on the reconstitution of the calcium pump from the sarcoplasmic reticulum and Grant and McConnell [11] on interaction of glycophorin with selected phospholipids).

Strittmatter et al. [12] and Enemoto and Sato [13] used cytochrome b_5 for reconstitution experiments, but these authors bound the protein to the intact microsomes or to total lipid extracts. More recently Sullivan and Holloway [14] showed by ultracentrifuge experiments that a complex between rabbit cytochrome b_5 and egg lecithin may exist. We have already shown [15] that it is possible to isolate egg lecithin/bovine cytochrome *d*-b_5 complexes of defined lipid/protein ratio using gel chromatography. Dehlinger et al. [16] used EPR probes to look at the lipid dynamics of cytochrome b_5/microsomal lipid complexes. While we were writting this paper Robinson and Tanford [17] reported some peculiarities of detergent binding to pig cytochrome b_5 and also that this protein binds to lecithin vesicles.

Cytochrome b_5 was first extracted from rat and rabbit endoplasmic reticulum membranes by Ito and Sato [18] and by Spatz and Strittmatter [19] using detergents. They showed that this protein (cytochrome *d*-b_5) is composed of two moieties. One is hydrophilic and contains the heme; it can be obtained by trypsin action on microsomes, and so is termed cytochrome *t*-b_5. The other containing 54 amino acid residues in the case of bovine liver [20], is responsible for the binding of the whole protein to the microsomal phospholipid matrix. Strittmatter et al. [12] and Enemoto and Sato [13] showed that it is possible to bind large amounts of purified cytochrome *d*-b_5 to microsomes compared to the amount of endogenous cytochrome b_5 present, but that cytochrome *t*-b_5 cannot be bound.

In this paper we report on the interaction between detergent-purified bovine liver cytochrome b_5 and well-defined phosphatidylcholine vesicles.

MATERIALS AND METHODS

Extraction and purification of cytochrome d-b_5. The protein is extracted from bovine liver microsomes following a method close to that first described by Spatz and Strittmatter [19] for rat and rabbit liver microsomes. Ozols [20] has published an extraction and purification procedure for bovine cytochrome *d*-b_5 similar to ours.

In our slightly modified method, fresh liver was homogenized in a Waring blendor. About 40 g of washed microsomes were obtained from 1 kg of liver, after selective sedimentation of subcellular fractions at +4 °C in 0.1 M Tris acetate buffer, pH 8.1, 0.25 M sucrose, 1 mM EDTA. Microsomes are sonicated in 1 M NaCl and then washed twice with 20 mM Tris/acetate, pH 8.1. Then the lipids were extracted with cold acetone and glycerol at −20 °C. Membrane proteins were extracted from the washed acetone precipitate by overnight incubation in buffer (Tris/20 mM acetate, 1 mM EDTA, pH 8.1) on which 1.5 % of Triton X-100 was added.

Purification of cytochrome b_5 was carried out according to the following steps: (i) The Triton solution containing detergent solubilized proteins was added to a DEAE-Sephadex column (2.5 cm diameter, 30 cm high). After washing with

buffer (Tris/20 mM acetate, 0.25 % deoxycholate) the cytochrome was eluted at pH 8.1 by a linear ionic strength gradient of SCN^- (0–0.4 M). The fractions were pooled and concentrated on a PM 10 membrane using the Amicon system. (ii) The protein solution was added to a Sephadex G-100 column (5 cm diameter, 100 cm high). Several high molecular weight proteins are removed and cytochrome *d*-b_5 is eluted as a dimer. (iii) Deoxycholate was eliminated by dialysis and passing through a Sephadex G-50 column (2.5 cm diameter, 100 cm high). The final amount of detergent evaluated with labelled [^{3}H]deoxycholate was about one deoxycholate molecule percent of cytochrome. The final yield was 30 mg of protein/1 kg of liver.

The purity of cytochrome b_5 was estimated by measuring the ratio $A_{413\,nm}/A_{280\,nm}$ which increases from a very low value on starting the purification, to about 1.0 after the DEAE-cellulose chromatography and 2.7 after the final step. The ultraviolet/visible spectrum of the protein is in good agreement with those published by Spatz and Strittmatter [19] and by Ozols [20]. Furthermore, the absorbance ratio of the reduced to oxidized form $A_{423\,nm}/A_{413\,nm}$ is equal to 1.46 as pointed out by Ozols and Strittmatter [21]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done according to Weber and Osborn [22]. The protein migrates essentially as a single band of 16 000 molecular weight.

In absence of any detergent, cytochrome *d*-b_5 exists in solution in a highly aggregated form. By gel chromatography on Sepharose 4B the molecular weight of the oligomer is estimated to 390 000 compared to standard globular proteins. This is different from the value obtained by Spatz and Strittmatter [19] of molecular weight 120 000 for the rabbit protein, but in good agreement with the size of the oligomer $R = 60$ Å obtained by Robinson and Tanford [17] for pig cytochrome b_5.

Preparation of cytochrome t-b_5. For the isolation of the trypsin core of cytochrome b_5, previous authors used the proteolytic enzyme directly on microsomes [21]. We proceeded in a different way by tryptic cleavage of the purified cytochrome *d*-b_5 itself. The pure protein (about 3 mg/ml) was incubated with trypsin at a molar ratio 1/400 for 18 h at room temperature. Then the mixture was fractionated on a Sephadex G-50 column. A protein fraction eluted in the void volume has a very weak heme absorption spectrum and was mainly composed of highly aggregated hydrophobic species. Another component which moved as a low molecular weight protein had the same characteristic ultraviolet/visible spectrum as that obtained for cytochrome *t*-b_5 by others [21]. The absorbance ratio $A_{413\,nm}/A_{280\,nm}$ is equal to 4.5 and the oxidized $A_{413\,nm}$ to reduced $A_{423\,nm}$ were in good agreement with that of cytochrome *t*-b_5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band corresponding to a molecular weight of 11000. So, according to the above criteria, the tryptic core obtained is identical with cytochrome *t*-b_5.

Phospholipid extraction and purification. Natural lecithin was extracted from hen egg yolks according to the method of Singleton et al. [23], and the purity of the final product was checked by thin-layer chromatography. In order to follow and to determine the exact molar amount of lipid, a small quantity of labelled [^{14}C]phosphatidycholine was added. The two lipids were mixed in benzene, lyophilized and stored under vacuum at −30 °C. The labelled phospholipid was obtained by extraction and purification [24] from soybean after germination on a water [^{14}C]-glycerol medium.

Reagents. Gel filtration products were from Pharmacia, sodium deoxycholate

from Sigma, Triton X-100 from Eastman Kodak, trypsin from Boehringer GmbH, and liquid scintillation products from Intertechnique. In some experiments we used labelled phosphatidylcholine from New England Nuclear, which has a higher specific activity than that which we prepared. [^{3}H]glucose is produced by the Commissariat à l'Energie Atomique.

Apparatus. All centrifuge experiments have been carried out with a MSE 65 high speed centrifuge. The ultraviolet and visible spectra were obtained with a Cary 16 spectrophotometer. Radioactivity measurements were done on an SL 30 Intertechnique multichannel liquid scintillation spectrometer. The sonication was done with an Annemasse 150 T model sonicator. Electron micrographs were obtained on a Siemens Elmiskop 101 microscope.

Quantitative determination of concentrations. Cytochrome content was estimated from the absorbance at 413 nm for the oxidized form of the cytochrome (extinction coefficient $\varepsilon = 117\ 000\ M^{-1} \cdot cm^{-1}$) [21].

Phospholipid content was calculated for each solution by measuring the specific activity of the benzene solution of labelled and unlabelled lipids and by titration of the phosphorus content. Stock quantities of labelled phospholipids were prepared in this way (^{14}C activity of 6000 dpm/mg).

Preparation of reconstituted systems. The cytochrome *d*-b_5 solution in 20 mM Tris/acetate, pH 8.1, 0.2 mM EDTA had a concentration of 3 mg/ml. The phospholipids (40 mg/ml) were suspended in the same buffer and sonicated under nitrogen at 0 °C for 15 min. When synthetic saturated lipids were used, sonication was done a few degrees above the aliphatic chain transition temperature. Large undispersed particles and titanium from the sonicator were removed by centrifugation at 100 000 $\times g$ for 1 h.

Our standard reconstitution conditions were the following: protein and sonicated phospholipid solutions in variable amounts were mixed to a final total volume of 0.1 ml. The mixture was incubated at 37 °C for 20 min; afterwards the samples were stored and studied at +4 °C.

RESULTS

Egg lecithin-cytochrome d-b_5 complexes. Fig. 1a shows the elution pattern of a sonicated egg lecithin solution with large particles in the void volume (A) and fractionated vesicular single-shelled liposomes retained (B). This is consistent with the well-known result of Huang [25]. In Fig. 1b, it can be seen that cytochrome *d*-b_5 eluted under the same conditions moves as a sharp single band corresponding to a high molecular weight oligomer (see Materials and Methods). After the vesicles and cytochrome *d*-b_5 were mixed and incubated (as described under Materials and Methods), a quite different pattern is obtained (Fig. 1c). The protein and the phospholipid vesicles are now eluted at exactly the same position. This indicates that after incubation a stable complex, undissociable by gel filtration, is formed between the two components. Furthermore, its elution position indicates that the complex is larger than either of the components alone.

When increasing the protein to lipid ratio in the incubation mixture, there is a parallel increase of bound cytochrome. But finally a new protein band appears on

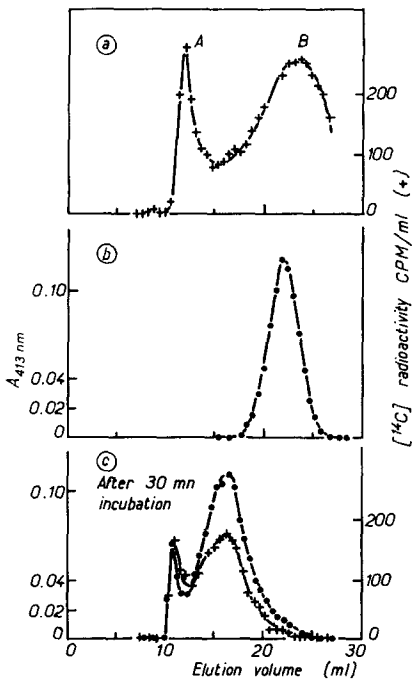

Fig. 1. Interaction of cytochrome *d*-b_5 with egg phosphatidylcholine: analytical gel filtration on a Sepharose 4B column (diameter 1.3 cm, length 50 cm) at 4 °C. 0.1 ml mixture in Tris 20 mM acetate buffer, pH 8.1, were introduced. 0.7-ml fractions were collected and analyzed for lipid (— + —) and protein contents (— ● —). (a) Sonicated egg phosphatidylcholine alone. (b) Cytochrome *d*-b_5 oligomers. (c) Reconstituted system after incubation as described under Materials and Methods, incubation ratio 1 mol of protein for 200 mol of phospholipids.

the elution pattern attributable to free cytochrome, since its position is identical with that of the protein alone.

The phospholipid to protein molar ratio in the incubation mixture R_i was varied from 1000 to 33. The same ratio was measured in each fraction along the elution pattern R_r. For the systems with a large lipid content (R_i between 1000 and 400), R_r decreased with increasing elution volume so that it was not possible to isolate complexes of constant composition. But as seen in Fig. 2, with R_i levels from 172 to 39, the variation of R_r with elution volume differed markedly. In the first region, starting at the void volume and corresponding to large non-fractionated particles, R_r decreases sharply. Furthermore, the protein content in each of these fractions is smaller than that in the incubated solution. This is due to the occurrence of particles, composed of many concentric spheres with low accessibility to externally added protein. In the second elution region, there is a plateau, indicating that homogeneous particles of well-defined composition were being isolated. But finally, the system saturates and any further addition of protein has no effect. At low R_i values a limit is obtained for R_r corresponding to 35 phospholipid molecules for one protein. If we compare the position of the maximum concentration of the complex, we observe a regular decrease in the elution volume as the protein content increases. This can be correlated with changes in the size of the complex.

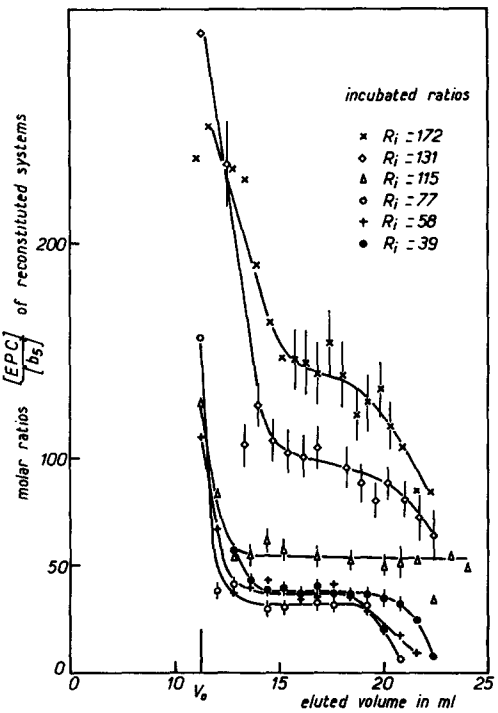

Fig. 2. Evolution of the egg phosphatidylcholine (EPC) to cytochrome bound molar ratio (R_r) for different values of the incubated ratio (R_i). Incubation time, 20 min; incubation temperature, 37 °C. It was, however, verified that the amount of bound cytochrome b_5 remains constant with incubation times up to several days.

Proof of the vesicular nature of the isolated complexes

The classical way to show that liposomes are hollow vesicles is to trap ions or small molecules inside them [25, 26]. We used [^{3}H]glucose added to initial lipid dispersion; the system is sonicated as usual. A protein solution containing the same concentration of labelled glucose is added, the usual reconstitution is then carried out as described under Materials and Methods. After chromatography, as shown in Fig. 3, glucose is mainly eluted as free glucose in the solution. However, a small fraction migrates with the lipid-protein complex, which means that it is trapped inside; so the isolated complexes are hollow vesicles. There is a 3-fold increase in the amount of trapped [^{3}H]glucose for the same phospholipid concentration on going from pure phospholipid liposomes of 250 Å diameter, to the protein-lipid vesicles, with $R_r = 40$.

As the maximum activity of the trapped glucose and that of ^{14}C correspond to the same eluted volume and since there is no "tail" in ^{3}H activity after elution of the complex, glucose diffusion through the vesicle wall is not noticeable during the time of the experiment. However, if the temperature at which the gel chromatography is done is increased to 28 °C, both a decrease of the ^{3}H activity at the maximum and a "tail" due to leaking are observed.

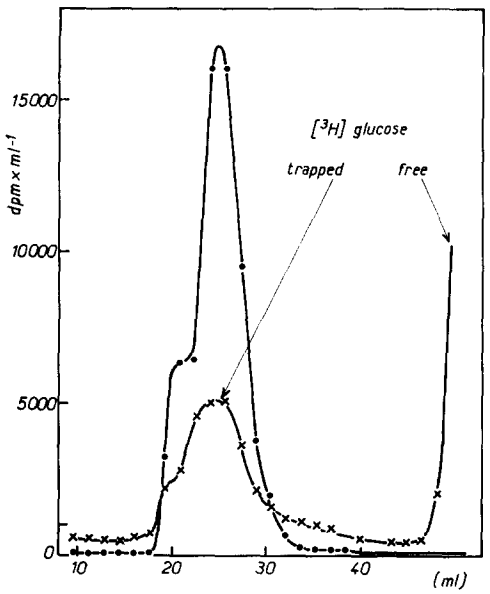

Fig. 3. Evidence for trapping [^{3}H]glucose inside reconstituted vesicles, R_r = 104. Lecithin liposomes preincubated in [^{3}H]glucose were incubated with cytochrome *d*-b_5 containing [^{3}H]glucose (see text). The mixture is then eluted through a Sepharose 4B column (diameter 1.3 cm, length 50 cm) following by Sephadex G-25 column (diameter 1.3 cm, length 10 cm).

Interaction of cytochrome t-b_5 with egg lecithin liposomes

Cytochrome *t*-b_5 does not form oligomers in solution, and it is therefore eluted from Sepharose 4B only by very large volumes. Fig. 4 shows the elution pattern obtained when cytochrome *t*-b_5 is incubated with egg lecithin liposomes. It is clear that there is no complex between the two components. This result is consistent with that of Strittmatter et al. [12] who showed that there is no interaction between cytochrome *t*-b_5 and microsomes, and that of Robinson and Tanford [17] involving pig cytochrome *t*-b_5 and lecithins.

We have previously reported [15] the action of trypsin on reconstituted systems formed between cytochrome *d*-b_5 and egg lecithin and showed that following

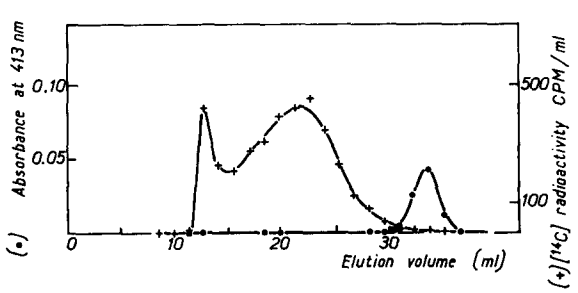

Fig. 4. Elution on Sepharose 4B of sonicated egg phosphatidylcholine (— + —) incubated as usually with cytochrome *t*-b_5 (— ● —).

such trypsin treatment, phospholipid vesicles containing the hydrophobic tails and a small protein identified as cytochrome t-b_5 are separated. Moreover, independent experiments by tryptophan fluorescence show that after similar trypsin treatment hydrophobic peptide is still bound to the phospholipid vesicles [27].

Asymmetry of the lipid-protein vesicles

The previous experiment also suggests that almost all the hydrophilic parts of the bound proteins are exposed to the solution, on the outer face of the vesicles.

It is also possible to demonstrate this asymmetry chemically. On reduction, the Soret band of cytochrome b_5 in solution moves from 413 to 423 nm and the observed ratio $A_{423\,nm}/A_{413\,nm}$ is 1.46. This value is in agreement with previous results [21] since $\varepsilon_{413} = 117\,000$ and $\varepsilon_{423} = 171000$. 1 ml of reconstituted oxidized cytochrome d-b_5 lecithin suspension obtained after elution from Sepharose 4B ($R_f = 45$) is reduced by 30 μl of 1 M dithionite solution. The 423 nm absorbance is measured immediately after mixing. The observed ratio of $A_{423\,nm}$ reduced/$A_{413\,nm}$ oxidized = 1.42; this corresponds to the reduction of 95 % of the total cytochrome content. Since the reduction time is very short it is unlikely that dithionite has entered the vesicles by passive diffusion, and its concentration (30 mM) is too low to induce breakdown of the vesicles. The reconstituted systems are therefore probably asymmetric vesicles with almost all the heme-containing moities of the proteins facing the outside.

Morphology and size of the complexes determined by electron microscopy

Reconstituted systems were isolated as described above and negatively stained. The vesicles obtained after incubation with cytochrome t-b_5 have almost the same size as protein free liposomes, with a diameter of 250 Å [25, 26]. In contrast with the reconstituted cytochrome d-b_5/egg lecithin complexes one obtains very large vesicles. Their diameters range from 500 Å to more than 1000 Å depending on the protein to lipid ratio.

Interaction of cytochrome d-b_5 with saturated lecithins

It was also of interest to see using lipids showing a transition temperature whether there is a different affinity of the protein for lipids below the transition temperature when all the aliphatic chains are in a crystalline state. The transition temperature of dimyristoyl and dipalmitoylphosphatidylcholine are 23 and 41 °C, respectively.

Fig. 5 shows the elution patterns obtained with dipalmitoyllecithin after incubating lipid/protein mixtures ($R_i = 90$) at temperatures ranging from +7 to +44 °C. At +7 °C a considerable fraction of the protein is still free, but part of the cytochrome forms a complex with lipids. As the incubation temperature is raised to 30 °C the amount of bound protein increases until no more free cytochrome remains. This occurs more than 10 °C below the melting point of the aliphatic chains. Above 41 °C the elution profile shows that all the reconstituted species are eluted in the void volume.

Reconstitution experiments with dimyristoylphosphatidylcholine gave identical results. High protein content complexes are isolated with about the same maximum

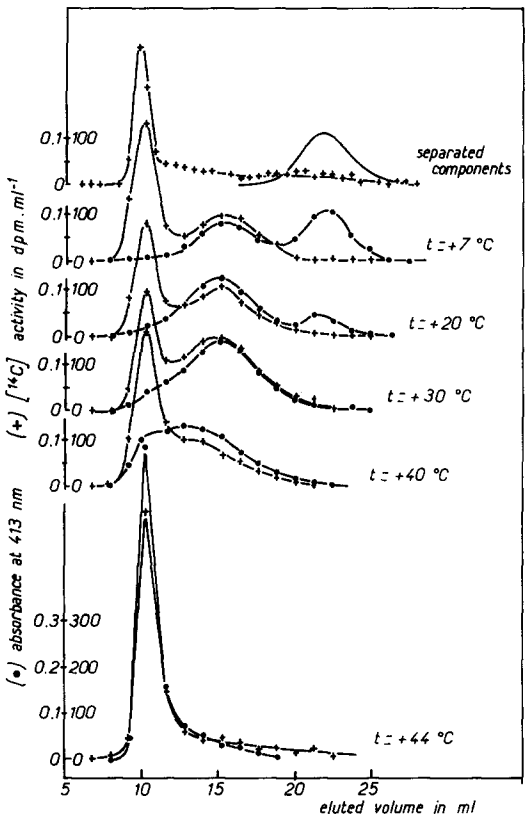

Fig. 5. Interaction between cytochrome *d*-b_5 and synthetic dipalmitoylphosphatidylcholine. —|—, phospholipids detected by ^{14}C labelling; —●—, cytochrome b_5 detected at 413 nm. On the top: separated components run in two successive experiments on Sepharose 4B column. Below: lipid-protein systems eluted on the same column after incubation during 1 h at the indicated temperatures from 4 to 44 °C. Incubated ratio $R_i = 90$, temperature of gel filtration, +4 °C.

ratio as for egg lecithin, one cytochrome b_5 molecule for 40 dimyristoylphosphatidylcholine molecules. It is also possible to trap [^{3}H]glucose inside these vesicles.

Proton NMR spectra of the reconstituted systems

Fig. 6a shows the 270 MHz NMR spectrum of pure egg lecithin liposomes, and Fig. 6b that obtained for the lipid-protein vesicles containing one protein for 40 lecithin molecules. The two spectra are almost identical.

The characteristic sharp band at 3.23 ppm is due to the protons of the choline polar group. The strong methylene band at 1.2 ppm and a methyl band at 0.9 ppm are from the aliphatic chains. Weak bands at 5.1 ppm from glycerol and double bond protons, and two bands from methylene groups of the polar head of the phospholipids are also well resolved.

The NMR spectrum of cytochrome *t*-b_5 from rabbit, published by Keller and Wüthrich [28], does not show any sharp line in the region of the NMe_3^+ line. The spectrum of cytochrome *d*-b_5 oligomers from bovine liver has the same general

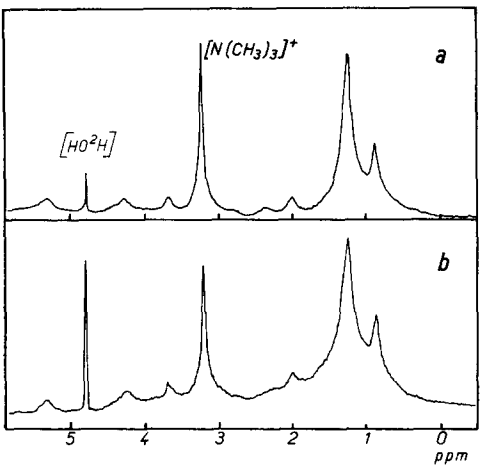

Fig. 6. ^{1}H NMR spectra obtained on a 270 MHz Brüker spectrometer by Fourier transform at 20 °C. (a) Egg phosphatidylcholine liposomes in ^2H_2O. (b) Reconstituted vesicles with cytochrome *d*-b_5, isolated by gel filtration, $R_r = 40$, exchanged with ^2H_2O. 200 scans are accumulated with a repetition time of 4 s, residual water protons are mainly eliminated by gated decoupling.

shape (Dufourcq, J., Bernon, R. and Lussan, C., unpublished results). So the observed band at 3.23 ppm is due only to the lecithin choline protons. Integration of all the resolved lines indicates that they derive from the phospholipid and not from the protein. There is no shift of any line in the lipid-protein vesicles, the only difference between the two spectra being a weak broadening of the foot of the peaks. In natural membranes the observed spectra have been interpreted as mainly due to the lipids [29, 30]. Another interesting point is that even when the lecithin vesicles are saturated with proteins the major part of the phospholipid is still in a dynamic state very close to that of the phospholipid alone in small liposomes.

DISCUSSION

We have shown that cytochrome *d*-b_5 from bovine liver microsomes is similar to that from rat, rabbit [12–14] and pig [17]. The monomeric hydrophilic cytochrome *t*-b_5 does not interact with lecithin but the total cytochrome *d*-b_5 forms a complex. So cytochrome *d*-b_5 probably binds to lecithin vesicles by its hydrophobic tail, like in natural microsomes.

The high affinity of cytochrome *d*-b_5 for egg lecithin allows isolation of complexes with a maximum molecular ratio of one protein for 35 ± 5 phosphatidylcholine molecules; this corresponds to a weight ratio of protein to lipid of 0.5. For saturated dimyristoylphosphatidylcholine almost the same maximal composition is observed. By binding extra cytochrome b_5 to natural microsomes, Strittmatter et al. [12] obtained a similar limiting composition of one cytochrome for 40 phospholipids. But we should notice that even though phosphatidylcholine is the major lipid component (about 65 % [31]), the natural system contains both proteins and lipids.

Protein binding is followed by a size change in the phospholipid vesicles. Due to the lack of high molecular weight standards we cannot obtain quantitative measure-

ments of the diameter of large vesicles by gel filtration. However, the experiments involving trapped glucose in presence of protein can only be interpreted: (i) by a decrease in membrane permeability, or (ii) by a change in trapped volume. The first hypothesis can be ruled out since similar results are obtained with egg lecithin in which the chains are melted and with dipalmitoylphosphatidylcholine at room temperature. In the latter case, the liposomes alone are not leaky at all for glucose [32]. So from the increase of trapped species we calculate an increase of the inner volume of the vesicles. By making the assumptions that the vesicles are single shelled and that the proteins do not contribute significantly to the vesicular surface area we calculate a mean diameter of about 800 Å. This value is in the same range as that obtained independently from electron microscopy.

Size increases have also been mentioned in reconstitution experiments with pig cytochrome b_5 [17] and after interaction of alamethicin with lecithin liposomes [33], the diameter obtained in this case being similar to the one we have calculated. The number of phospholipid molecules necessary to yield such bigger vesicles is more than ten times the amount in the initial liposomes. The mechanism of aggregation or fusion by which small liposomes of 250 Å diameter, grow to 800 Å vesicles following protein binding is now being studied. Perhaps these results may be related to the biogenesis of membranes.

Until now no intrinsic protein has been shown to form complexes with saturated phospholipids when the lipids are in the crystalline state, i.e. when all the chains are in the *trans* conformation. Incubation at temperatures up to 30 °C below the melting point of dipalmitoylphosphatidylcholine with cytochrome *d*-b_5 results in the formation of a complex, but binding is slower than that to phospholipids with melted chains at the same temperature. This is in agreement with the results obtained by Pownall et al. [34] for the binding of apolipoproteins to dimyristoylphosphatidylcholine. For a constant composition, at the melting temperature there is an increase in the size of the vesicles. This shows that at a saturating protein lipid ratio, melting of phospholipid chains still occurs at the same temperature. This is in agreement with differential scanning calorimetry results on natural membrane and lipid extracts [35].

NMR experiments showed that at the maximal protein concentration the major part, if not all, of the phospholipids give rise to a high resolution spectrum. This indicates firstly that the increase of correlation time due to the increase in vesicles diameter has no broadening effect, and secondly that most of the lipids have a dynamic behaviour on the NMR time scale close to that of pure lipids in liposomes. This does not contradict the results of Dehlinger et al. [16] who showed that protein binding decreases the fluidity of the membrane as detected by an EPR probe, or of fluorescence polarization which we interpreted in the same way [36]. These NMR results, among the first obtained on interaction of lipids with intrinsic proteins, are totally different from those obtained with other proteins, especially with alamethicin [33] where the high resolution spectra disappear totally at a lipid to protein ratio close to 30. Nevertheless the result we obtained agrees with the observation that in some cases whole functional membranes gave high resolution spectra [29, 30].

In summary we can propose a structural model for the complex. It is probably a single bilayer-closed vesicle with the protein heme moiety on the outer side. Two possibilities remain for the location and the structure of the hydrophobic tail, that

it goes through the bimolecular leaflet, or that it interacts only with the outer phospholipid monolayer.

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