THE INTERACTION OF CYTOCHROME b_5 WITH LIPID VESICLES

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Cytochrome b_5 is a well-characterized intrinsic membrane protein found in the endoplasmic reticulum of liver cells and perhaps also in other intracellular membranes (1, 2). The protein can be isolated by detergent extraction and the amino acid sequence of cytochrome b_5 from several animal species has been published (3). The isolated detergent and lipid free protein binds rapidly and completely to preformed phosphatidylcholine (PC) vesicles and other membranes. In investigating the binding properties of the protein we observed that the protein would exchange rapidly between PC vesicles (4). Studies of the binding and exchange have been facilitated by the tryptophan fluorescence of the protein, which increases up to two-fold upon binding to PC vesicles. It should be noted that, as reported (5), the kinetics of fluorescence enhancement seen with the protein are biphasic. The slow rate ($t_{1/2} \simeq 30$ s) is due to dissociation of octomeric protein to monomer (little fluorescence change) followed by the rapid fluorescence enhancement due to binding. The enhancement kinetics can be made monophasic (rapid) by use of monomeric protein (isolated by gel filtration). This preparation is used in all our studies. A fluorescence technique has also been used to monitor continuously exchange of protein between vesicles. For these studies we have used brominated lipid vesicles. The bromolipid, made by bromination of an octadecenoic acid (either Δ9 or Δ6) followed by coupling of the dibromide to 1-palmitoylglycerophosphorylcholine, forms stable vesicles of similar size to those of 1-palmitoyl-2-oleoyl glycerophosphorylcholine (POPC).

The vesicles made from lipid containing the 9, 10 dibromide (9, 10 BrPC) show no phase transition between 5° and 50° by scanning calorimetry. Addition of 9, 10 BrPC vesicles to cytochrome b_5 (b_5) results in immediate quenching of fluorescence (to 50% of the original value). Subsequent addition of POPC vesicles results in a slow enhancement of fluorescence ($t_{1/2} \simeq 30$ min). Addition in the reverse order (POPC then 9,10 BrPC) produces an immediate enhancement, followed by slow quenching. Detailed kinetic analysis of these data and the observation that dilution had no effect on the fractional rates of b_5 exchange lead us to conclude that exchange proceeds via transfer of b_5 through the aqueous phase rather than by collision of donor and acceptor vesicles (4). Confirmation of the exchange of the protein from the donor to the acceptor vesicle is made by separating the dense 9, 10 BrPC from less dense POPC. Detailed analysis of the separated complexes show that $< 1 \pm 1\%$ lipid transfer has accompanied the protein exchange. This assay allows one to measure both the rates of exchange and the equilibrium partitioning of b_5 between different normal lipid vesicles by comparing each independently to 9, 10 BrPC. As reported previously (4), the kinetics of exchange between some lipid vesicles are multiphasic (Fig. 1). We originally suggested this was negative cooperativity, perhaps due to repulsion of the highly charged polar head groups of the protein. The data in Fig. 1 suggest that charge repulsion is not a factor, as experiments performed with different protein:vesicle stoichiometries (2–10/vesicle) give identical multiphasic curves. In addition, the kinetics are identical in 10 mM Tris acetate and in the same buffer containing 0.15 M NaCl. The multiphasic kinetics could be due to the presence of vesicle-bound b_5 in (at least) two confor-

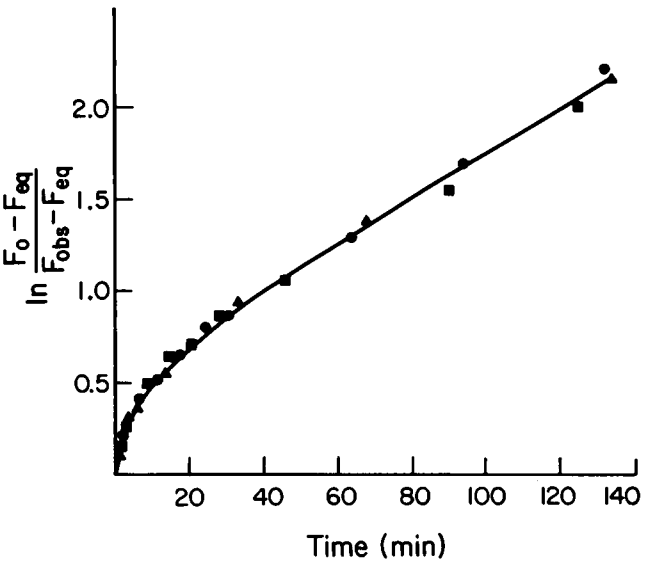

FIGURE 1 Effect of varying the cytochrome b_5:lipid ratio on the kinetics of intervesicle protein transfer. Cytochrome b_5 (1 μM) in 10 mM Tris acetate 1 mM EDTA pH 8.1 was incubated with POPC donor vesicles at 25° for 60 min. The following concentraions of POPC were used: circles, 0.25 mM; squares 0.50 mM; triangles, 1.0 mM. At zero time an equivalent amount of acceptor vesicles (6, 7 BrPc) was added. F_{obs} tryptophan fluorescence observed during the reaction time course; F_0 fluorescence at zero time, upon addition of acceptor vesicles; F_{eq} equilibrium fluorescence, fluorescence after 450 min.

TABLE I
EFFECT OF VESICLE COMPOSITION ON THE HALF TIMES OF FLUORESCENCE CHANGE AND EQUILIBRIUM PARTITIONING OF CYTOCHROME b_5*

Sample vesicle	Equilibrium partitioning‡		
	By fluorescence	By centrifugation	$t_{1/2}$ (min)
DMPC§	6.0	ND	~40
POPC‖	1.00	1.00	21
POPC:Cholestrol (9:1)¶	0.87	0.6	15
9,10 BrPc	0.83 ± 0.04 n = 6	0.83 ± 0.20 n = 4	34
6,7 BrPC	0.55 ± 0.09 n = 6	0.57 ± 0.12 n = 3	32
POPC:Cholesterol (6:4)¶	0.23	0.34	11

*Exchange of cytochrome b_5 between donor and acceptor vesicles was monitored by fluorescence changes as described in the text and reference 4.
‡The equilibrium partitioning of cytochrome b_5 was calculated from fluorescence changes and by chemical analysis of the less dense and more dense (bromolipid) fractions separated by sucrose density gradient ultracentrifugation.
§Data from a separate series of experiments.
‖All equilibrium partitioning values are expressed relative to POPC. Values for 9,10 BrPC and 6,7 BrPC are obtained directly. Other values are referenced to POPC by using the equilibrium partitioning of cytochrome b_5 between sample vesicle and BrPC and between POPC and BrPC
¶Preliminary experiments.

mations (orientations). When b_5 is initially bound to 9, 10 BrPC, exchange off is monophasic, suggesting that a slowly exchanging conformation (perhaps transbilayer [6]) is present. When b_5 is initially bound to POPC vesicles, exchange off is multiphasic, suggesting approximately equal amounts of slowly exchanging (*trans*bilayer?) and rapidly exchanging (*cis*bilayer?) are present.

In common with other intrinsic membrane proteins (7), cytochrome b_5 is made on free ribosomes (8) and released into the cytosol. The subsequent localization of these proteins may be partially determined by the relative affinity of these proteins for different intracellular membranes. This way inappropriate binding of a microsomal protein to other membranes could be reversed. To examine this hypothesis in vitro we have allowed b_5 to bind to a vesicle population (donor) for short times (5 min), mimicking intracellularly synthesized protein released adjacent to one specific membrane. We then followed the exchange to an acceptor vesicle population. As shown in Table I, both rates of exchange and final partitioning are influenced by vesicle composition. Preliminary results, prior to using lipid compositions like cell membranes, are interesting in that the presence of cholesterol in a membrane tends to reduce the protein's relative affinity to that membrane.

With this experimental procedure of short initial binding of monomeric b_5, rapid monitoring of exchange, and final monitoring of protein and lipid distribution, we feel the physiological significance of inter-membrane transport of extrinsically synthesized intrinsic membrane proteins can be evaluated.

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