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β-Galactosidase fused to the hydrophobic domain of cytochrome b_5 spontaneously associates with liposomes

Sathish K. George, Lydia Najera, Ramona Sandoval and Garret M. Ihler

Department of Medical Biochemistry and Genetics, Texas A&M College of Medicine, College Station, TX (U.S.A.)

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Since liver microsomal cytochrome b_5 spontaneously associates with liposomes and membranes by means of its C-terminal hydrophobic domain (HP), chimeric proteins containing HP prepared by genetic fusion might also spontaneously associate with liposomes or cellular membranes. Synthetic DNA corresponding to the hydrophobic domain of cytochrome b_5 was enzymatically fused in-frame to cloned DNA corresponding to the C-terminus of the *Escherichia coli* enzyme, β-galactosidase. This protein, LacZ:HP, synthesized in *E. coli* and purified from a crude *E. coli* membrane extract, was shown to spontaneously associate with liposomes, as does cytochrome b_5. Association is rapid and stable in the presence of salt and high pH and the fusion protein behaves as an integral membrane protein. LacZ:HP can be readily and extensively purified from crude extracts by association with liposomes and this procedure may provide a convenient purification scheme for proteins not otherwise readily purified, for example polypeptides from cloned gene fragments to be used for antibody production. These hybrid proteins may represent a new potentially useful class of polypeptides capable of hydrophobic interactions with membranes.

Introduction

Polypeptides capable of direct association or insertion into membranes, or interaction with hydrophobic surfaces, might have various scientific or pharmaceutical uses. Integral membrane proteins however do not in general spontaneously insert into the membranes of preformed liposomes or into cellular membranes. Membrane proteins can be reconstituted directly into liposomal membranes in a functional form, for example by sonication. Alternatively, reconstitution may occur if the membrane bilayer is formed in the presence of the protein, as occurs for example when detergent is removed by dialysis from a mixture of membrane proteins and lipid. Such liposomes have proven to be useful in the study of the properties of integral membrane proteins, particularly transport proteins [1]. Peripheral membrane proteins, which are held to the membrane predominantly by electrostatic interactions with integral membrane proteins or with lipids, can bind to membranes containing appropriate integral membrane proteins, but are released by exposure to high or low ionic strengths or pH values.

A few proteins are known to spontaneously associate in vitro with preformed, protein-free phosphatidylcholine liposomes and among these is cytochrome b_5, which also binds to liver microsomal membranes [2]. Cytochrome b_5 is regarded as an integral membrane protein, released by disruption of the lipid bilayer with detergents or organic solvents, although it is probably not a transmembrane protein. Cytochrome b_5 probably spontaneously associates with membranes in vivo, since the gene lacks an N-terminal signal peptide sequence and the protein is synthesized on cytoplasmic ribosomes [3]. A short C-terminal hydrophobic amino acid sequence, which is coded for by a single exon in the DNA [4], anchors the protein in the membrane and this sequence is required for association with liposomes in vitro [5]. A model for the interaction of this hydrophobic sequence with the membrane, involving a hairpin which does not completely cross the membrane, has been proposed [5,6].

That this hydrophobic amino acid sequence is sufficient for association with membranes in vivo was demonstrated by construction of a hybrid protein, LacZ:HP, consisting of E. coli β-galactosidase linked

Correspondence: G.M. Ihler, Department of Medical Biochemistry and Genetics, Texas A&M College of Medicine, College Station, TX 77843, U.S.A.

at its carboxyl terminus to the hydrophobic peptide of cytochrome b_5 (HP). β-Galactosidase is a cytoplasmic enzyme whose DNA does not encode an N-terminal signal sequence. When expressed in *E. coli*, most of the LacZ : HP was associated with the *E. coli* inner membrane, as was demonstrated by membrane fractionation and immunocytochemical studies on intact cells and membranes [7].

If hybrid proteins containing HP are capable of spontaneous association with hydrophobic surfaces such as liposomes, or cell membranes, several biochemical or pharmaceutical applications could be imagined. We show here that, like cytochrome b_5 itself, LacZ : HP spontaneously associates with preformed phosphatidylcholine liposomes in vitro resulting in liposomes bearing β-galactosidase activity on their external face. The protein is not dissociated by high ionic strengths or high pH values. This property could be useful biochemically since recombinant proteins bearing the HP domain could be readily purified by co-flotation with liposomes. If proteins containing HP remained associated with liposomes in vivo after infusion, these hybrid proteins might display altered pharmacodynamics including increased life span or confinement to the vascular system, and perhaps could serve to direct specific interaction of the liposomes with target cells, as has been demonstrated for liposomes bearing monoclonal antibodies, antigens, mannose-bearing glycoproteins, or lectins [8,9]. If proteins containing HP do not remain associated with liposomes in vivo, transfer of the hybrid protein to endothelial cell membranes or other hydrophobic acceptors might be possible.

Experimental procedures

Construction of the β-galactosidase : HP gene fusion

The construction of the β-galactosidase : HP gene fusion has been described previously [7]. Briefly, a DNA sequence corresponding to the hydrophobic domain of rabbit liver microsomal cytochrome b_5 [10] was synthesized and cloned between the *Bam*H1 and *Sal*1 sites of the expression vector pUC19. Plasmid vector pUR278 [11] which contains a multiple cloning site cloned in-frame at the C-terminus of the *lacZ* gene was then used to locate the hydrophobic domain (Fig. 1A) at the C-terminus of the *E. coli* enzyme β-galactosidase. The resulting plasmid, pUR278 : HP (Fig. 1B), codes for a fusion protein consisting of 1070 amino acids. The proximal 1024 amino acids correspond to the entire *lacZ* gene, and the extension beyond the normal C-terminus of β-galactosidase contains an additional 46 amino acids, the last 43 of which correspond to HP.

Isolation of the LacZ : HP fusion protein

An *E. coli* K-12 strain MB392 [7] transformed with pUR278 : HP was grown in a liter of broth to an A_{600} of 0.3, induced with IPTG at a final concentration of 1 mM, and growth was allowed to continue to an A of

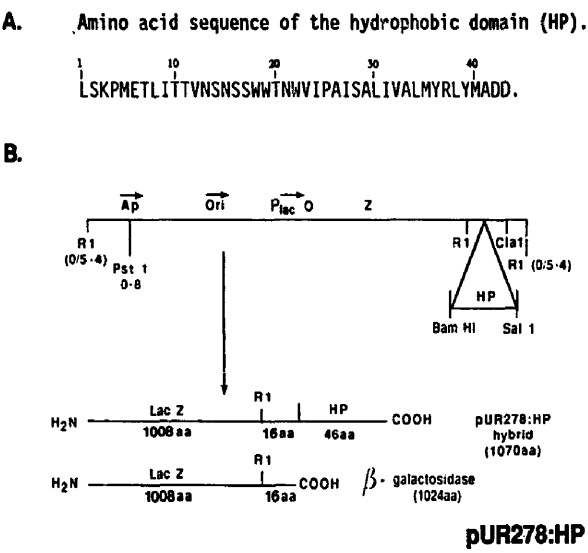

Fig. 1. Peptide sequence and plasmid construct. (A) Amino acid sequence of the hydrophobic domain (HP) of rabbit cytochrome b_5 (10). (B) Plasmid pUR78 : HP containing the DNA sequence corresponding to HP cloned between BamH1 and Sal1 sites at the C-terminus of the *lacZ* gene. The resulting LacZ : HP fusion protein is expected to consist of an additional 46 amino acids fused to the C-terminus of β-galactosidase, 43 of which correspond to HP.

1.0. The cells were collected by centrifugation (2000 × *g* for 10 min at 4°C), resuspended in 40 ml of Tris-acetate (TA) buffer (0.01 M Tris, 0.1 M NaCl, 0.1 mM EDTA, 0.02% sodium azide, pH 8.1), made 1 mM with respect to phenylmethylsulfonyl fluoride and lysed by passage through a French pressure cell (16000 psi). Unlysed cells were removed by centrifugation (2000 × *g* for 10 min at 4°C), and the lysate separated into supernatant and membrane fractions by ultracentrifugation (250000 × *g* for 1 h at 4°C in a Ti70 rotor).

The resulting membrane pellet was solubilized in 10 ml of a 2% solution of sodium deoxycholate in Tris-acetate buffer (pH 8.1). Aliquots of the supernatant and membrane pellet fractions were analyzed by western blotting using a β-galactosidase antibody (Promega Biotec) to determine the size and distribution of the fusion protein. The distribution of the LacZ : HP fusion protein activity between the membrane and supernatant fractions was also assayed enzymatically using the colorimetrie ONPG method [12].

Processing of membrane extracts

Membrane extracts containing the fusion protein were either directly dialyzed against Tris-acetate buffer (pH 8.1) or delipidated by chromatographing on an anion exchange column (Mono-Q, Pharmacia). Bound proteins were eluted with 0.6% (w/v) sodium cholate, 1 M sodium chloride in Tris-acetate buffer (pH 8.1) and dialyzed prior to use in binding studies. Purification of the LacZ : HP fusion protein employed two chromatographic steps. Crude membrane extracts were subjected to gel filtration on a Sepharose-4B column (1.5 × 90 cm) eluted with 0.6% (w/v) sodium cholate in Tris-acetate buffer. Fractions containing β-galactosidase activity were pooled and loaded onto an anion exchange column (Mono-Q). Bound protein was eluted with 50 ml of a sodium thiocyanate gradient (0–0.5 M) in TA buffer at a flow rate of 1 ml/min, and the fractions analyzed by SDS-polyacrylamide gel electrophoresis to determine the purity of the recovered fusion protein.

Preparation of liposomes

Liposomes were prepared as described by Barenholz, et al. [13]. Briefly, egg yolk lecithin (phosphatidylcholine 20 mg/ml in ethanol/chloroform, Avanti lipids) was evaporated to dryness under nitrogen leaving a film of lipid on the walls of the tube. In some instances, rhodamine-labeled phosphatidylethanolamine was included as a marker at a molar ratio of 1 : 10 with respect to phosphatidylcholine. Following lyophilization overnight, the lipid film was resuspended in TA buffer at a concentration of 22 mg/ml. Small unilamellar liposomes (SUVS) were prepared by ultrasonication (Cell Disruptor, Model W185F, Heat Systems-Ultrasonics Inc,) at 30% of full cycle power for 2 h on ice under an inert atmosphere of nitrogen. Undispersed phospholipid was removed by ultra-centrifugation (3 h at 150000 × *g* in TLA 100.3 rotor at 15°C) and the supernatant containing the liposomes was decanted and stored under nitrogen at 4°C.

Incubation of liposomes with the LacZ : HP fusion protein

Phosphatidylcholine (PC) liposomes were incubated with the LacZ : HP fusion protein, in a total volume of 0.3 ml, under conditions of varying concentration, time, temperature and pH. Incubation times from 0–2 h were examined. Likewise, the efficiency of binding in the pH range from 5 to 10 and temperatures between 0 and 42°C was assessed. The liposome-associated protein was separated from the free protein by flotation through a sucrose density gradient. The incubation mixtures were made 60% (w/v) with respect to sucrose and loaded beneath a 50–20% (w/v) linear sucrose gradient (3.0 ml). After centrifugation for 4 h at 240000 × *g* and 15°C in a SW 50.1 rotor, the gradient was fractionated into 0.5 ml aliquots and assayed for β-galactosidase enzymatic activity. Aliquots of each fraction were also analysed by SDS-polyacrylamide gel electrophoresis. Liposome flotations under identical centrifugation conditions were also carried out using cesium chloride gradients, where the incubation mixture was made 42% (w/v) with respect to CsCl and loaded beneath 3 ml of a 14% (w/v) CsCl cushion. The location of the liposomes in the gradient was determined both by light scattering and by measurement of lipid phosphate in the fractions [14].

The influence of liposome concentration on binding was studied by incubating a constant amount of the fusion protein (25 μg) with varying amounts of phosphatidylcholine liposomes (0.22–2.2 mg) in a final volume of 0.3 ml. The amount of protein in the incubation corresponding to the fusion protein was estimated from a Coomasie blue-stained SDS-polyacrylamide gel calibrated with BSA standards. After determining the protein : lipid ratio at which most, but not all of the protein was bound to the liposome, the concentration of the protein and lipid was varied while holding this ratio constant. The stability of the association was studied by re-incubating (37°C for 1 h) sucrose gradient purified fusion protein bound liposomes and re-isolating these liposomes by flotation.

Results

After transformation of lac⁻ MB392 bacteria with plasmid pUR278 : HP and induction with IPTG, the bacteria produce β-galactosidase activity. Enzymatic assay of the membrane and supernatant fractions previously demonstrated that over 80% of the total β-galactosidase activity was membrane bound [7]. Analysis of the membrane fraction by SDS polyacrylamide gel electrophoresis and Western blotting identified a

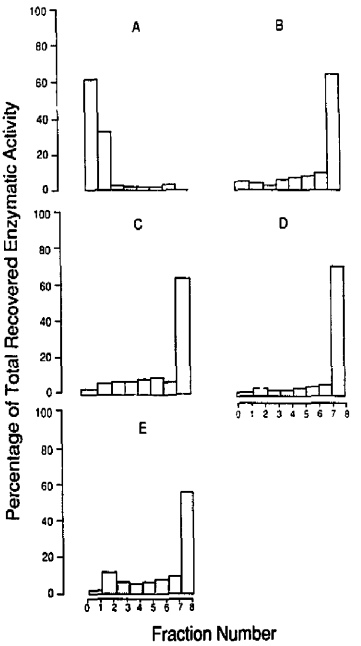

Fig. 2. Effect of temperature. Identical 1 h incubations of liposomes with LacZ:HP fusion protein of containing membrane extracts were carried out at 0°C (B), 15°C (C), 37°C (D) and 42°C (E). (A) The membrane extract incubated in the absence of liposomes at 37°C. Liposome-associated protein was separated from free protein by sucrose density gradient centrifugation. Fraction 8 corresponds to the banding position of the liposomes in the gradient while the free protein remains at the bottom (fraction 1 and 2).

β-galactosidase immunoreactive polypeptide consistent in size with the expected fusion protein (121 kDa). This protein could be extracted from the membrane by solubilization with sodium deoxycholate and exhibited approx. 50% of the specific activity of native β-galactosidase.

Both purified LacZ:HP and LacZ:HP present in delipidated crude extracts spontaneously associate with phosphatidylcholine liposomes (Figs. 2 and 3). The liposomes were separated from unbound protein by flotation in sucrose or cesium chloride gradients. Under the conditions used, the liposomes band at the top of the gradient, as demonstrated either by light scattering, following the fluorescence associated with the rhodamine labeled phosphatidylethanolamine or lipid phosphate analysis of the gradient fractions. Most of the added LacZ:HP fusion protein co-migrated with the liposomes, as demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 4), immunoblotting and enzymatic assay (Figs. 2 and 3). No enzymatic activity co-migrated with the liposomes when identical incubations were carried out with *E. coli* β-galactosidase derived from strains carrying the parent plasmid, pUR278. LacZ:HP, either purified or present in extracts, did not move out of the bottom (60%) step of the gradient when incubated in the absence of liposomes (Figs. 2A and 3A).

A substantial purification of the fusion protein could be achieved from delipidated crude extracts. In Fig. 4, the only protein associated with the liposomes (fraction 7) is the LacZ:HP fusion protein. Although LacZ:HP is a major protein in the crude membrane extract (Fig. 4 lane E), at least 20 other polypeptides detected in the unbound fraction (Fig. 4 lane 1) could have been detected in the liposome fraction, but they were not present. This represents a substantial purification of LacZ:HP. In the absence of delipidation, a small fraction of a few other proteins in the extract were found to associate with the liposomes. Even when the extract was not delipidated, the major proportion of these proteins

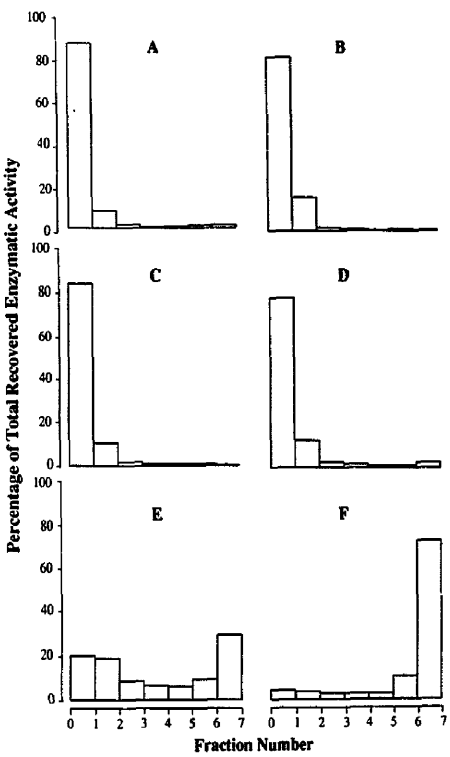

Fig. 3. Effect of liposome concentration. An anion exchange chromatographed membrane protein extract containing approx. 25 μg of the LacZ:HP fusion protein was incubated with either A. 0.0 μl B. 0.01 μl C. 0.01 μl D. 0.1 μl E. 1.0 μl or F. 10.0 μl of 22.0 mg/ml PC liposomes. All incubations totalled 300 μl in volume and were carried out at 37°C for 1 h. Separation of liposome associated protein from free protein was achieved by centrifugation in sucrose density gradients. Fraction 7 corresponds to the banding position of the liposomes in the gradient and fraction 1 corresponds to the position of the free protein.

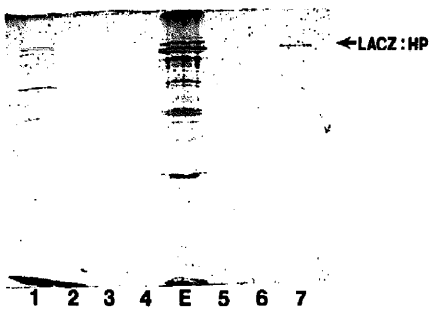

Fig. 4. Analysis of liposome associated proteins. Coomassie blue-stained SDS polyacrylamide gel of sucrose density gradient fractions. The fractions result from the floatation of liposomes incubated for 1 h at 37°C with a LacZ:HP fusion protein containing membrane extract. The extract was delipidated and concentrated by chromatography on an anion exchange column prior to use in the incubations. The position of the liposomes in the gradient corresponds to fraction 7, while the free protein remains at the most dense part of the gradient (fraction 1). Lane E consists of an aliquot of the membrane extract used in these liposome incubations.

were still found in the unbound fraction, in contrast to LacZ:HP. Approx. 90% of the recovered LacZ:HP enzymatic activity binds to the liposomes and is found at the top of the gradient (Fig. 2). The presence of only relatively small amounts of trailing activity in the gradient suggests that the binding affinity of the fusion protein to liposomes is high or that the fusion protein is stably inserted into the membrane.

To characterize the binding of the fusion protein to PC liposomes, the effect of parameters such as time, temperature, pH and concentration were examined. Incubation of the fusion protein with liposomes for 1 h at either 0, 15, 37 or 42°C, followed by flotation at 15°C, resulted in no significant differences in the percentage of recovered fusion protein bound to the liposomes (Fig. 2). Similar results were obtained when the floatations were carried out at 0°C. Subsequent incubation studies were carried out at 37°C. The binding kinetics of the fusion protein to PC liposomes were also studied. Maximal binding of the fusion protein to the liposomes was achieved with no incubation beyond the time required for mixing and flotation irrespective of whether the flotations were carried out at 0° or 15°C.

The influence of pH on the liposome binding ability of the fusion protein was investigated at pH 5, 6, 7, 8.1, 9 and 10 by adding a predetermined volume of either acid or base to the liposomes prior to adding the fusion protein and incubating for 1 h at 37°C. Bound from free protein was separated on sucrose gradients adjusted to the respective pH. Similar results were obtained at all pH values. To determine the level of maximal binding, the concentration of the fusion protein was held constant (85 μg/ml) and that of the liposomes varied between 0.73–73 mg/ml. Maximal binding (Fig. 3E), corresponded to approximately one molecule of LacZ:HP bound per 350 molecules of phosphatidylcholine. The number of LacZ:HP monomers bound per liposome was estimated to be 12, from the surface area of the liposomes (calculated from an average diameter of 30 nm as determined in electron microscopic studies) and the area occupied by the phospholipid head group [15].

The effect of concentration on binding was studied by varying the concentrations of the reactants while maintaining the fusion protein to liposome ratio constant at approximately that point at which saturation was reached. No concentration dependance was observed. Re-incubation and flotation of previously purified LacZ:HP bound liposomes did not result in dissociation of the fusion protein already bound to the liposome. Furthermore, isolation of the fusion protein bound liposomes by flotation through a cesium chloride gradient gave results similar to those obtained by flotation through sucrose, indicating that the interaction of the fusion protein with the liposome was unlikely to be ionic in nature.

Resistance to extraction at pH 11 is thought to reflect integration of the protein into the lipid bilayer [16]. For this reason, both the liposome incubation and separation of bound from free protein was also carried out at pH 11. In support of the idea that the fusion protein is inserted into the liposomal bilayer, no difference in the binding of the fusion protein to the liposomes was observed when the incubation and flotation was carried out at pH 11. The binding of rabbit liver cytochrome b_5 to PC liposomes was also unaffected at this pH.

Discussion

The enzymatically active fusion protein, LacZ:HP, contains the entire amino acid sequence of *E. coli* β-galactosidase coupled to the hydrophobic domain of rabbit liver cytochrome b_5. The hydrophobic domain constitutes the C-terminus of cytochrome b_5 and for this reason was located at the C-terminus of LacZ:HP. This small domain, consisting of 43 amino acids, is responsible for the association of cytochrome b_5 with membranes [2,5]. When expressed in E. coli, LacZ:HP is associated with the bacterial membrane in vivo and remains associated with membranes after disruption of the cells [7]. After solubilization with sodium deoxycholate, LacZ:HP could be readily purified by several standard procedures previously developed for β-galactosidase.

Purified LacZ:HP was found to spontaneously associate with preformed phosphatidylcholine liposomes, as does cytochrome b_5. The fusion protein was enzymati-

cally active while associated with liposomes and its presence could be monitored by enzymatic activity, immunoblotting or SDS-PAGE. *E. coli* β-galactosidase, like other soluble cytoplasmic proteins, did not associate with liposomes. Also, *E. coli* membrane proteins, like other membrane proteins, did not spontaneously associate with the liposomes.

Cytochrome b_5 is regarded as an integral membrane protein and several models for its interaction with membranes have been proposed [6,17,18]. Like cytochrome b_5, LacZ:HP was found to copurify with liposomes in density gradients of sucrose or cesium chloride. Detergent was required to release LacZ:HP from *E. coli* membranes and both the membrane and liposome associated protein was resistant to extraction with high concentrations of cesium or sodium chloride and elevated pH. These observations argue that spontaneous binding involves primarily hydrophobic, rather than ionic, interactions. It seems clear that the hydrophobic peptide of cytochrome b_5 endows LacZ:HP with the same membrane-associative properties of cytochrome b_5, and probably would do so for nearly any other HP-containing fusion protein.

Association of either cytochrome b_5 or LacZ:HP with liposomes is rapid. Essentially, all of the LacZ:HP bound to the liposomes without any additional incubation beyond the time required for mixing and flotation. Furthermore, temperatures between 0 and 42°C and pH values between 5 and 10 did not significantly influence the rate or extent of binding. As will be discussed elsewhere, association of LacZ:HP with red cells does occur readily, but the rate is much slower and association exhibits both temperature and pH dependance.

Variable quantities of liposomes were added to a constant amount of fusion protein to determine the stoichiometry of binding. It was found that when 25 μg of protein was incubated with 22 μg of phosphatidylcholine, only about 40% of the protein co-migrated with the liposomes, while nearly all the protein bound when larger amounts of lipid were added. Limited binding was not a consequence of the decreased concentration of lipid per se, since the same maximal ratio of bound fusion protein to lipid was obtained at several different concentrations of lipid. Moreover, liposomes bearing the maximal amount of fusion protein could be diluted and reincubated before flotation without loss of the fusion protein from the liposomes.

Maximal binding corresponds to about 350 phosphatidylcholine molecules per hydrophobic sequence. It seems unlikely that the 43 amino acids of the hydrophobic peptide would require 350 lipid molecules in the membrane with which to interact. The β-galactosidase portion of the polypeptide is considerably larger than the hydrophobic domain, since it contains about 25 times as many amino acids as the hydrophobic sequence. At maximal saturation of 1 LacZ:HP monomer per 350 phosphatidylcholine molecules the interhead distance between LacZ:HP monomers is approximately 15 nm, based on a phospholipid head group area of 0.7 nm^2 per molecule [15]. This indicates that steric hinderance between the β-galactosidase domains could be significant, and suggests that maximal binding corresponds to close packing of the β-galactosidase domain.

Using the liposome flotation procedure, highly purified LacZ:HP was obtained directly from the delipidated membrane fraction of the crude lysate. Purification by liposome flotation is not especially useful for LacZ:HP, since the subsequent delipidation step is likely to be as time-consuming as either affinity chromatography or anion exchange and gel filtration chromatography, both of which procedures yield highly purified fusion protein. This purification procedure might, however, be of some use for identifying and isolating fusion proteins expressed in bacterial or eucaryotic expression systems. Use of a linker of appropriate amino acid sequence between the protein and HP could permit the subsequent proteolytic or chemical cleavage of the hydrophobic domain, if its presence were not desired.

The use of the cytochrome b_5 hydrophobic sequence to associate arbitrary proteins with membranes or hydrophobic surfaces might have pharmacological implications. LacZ:HP is not removed *in vitro* from liposomes by red cells or from red cells by plasma and since liposomes with certain compositions have useful circulatory half lives [9], the protein molecules might remain associated with the liposomes for a useful period of time before they are either taken up by phagocytosis with the liposome or transferred to other hydrophobic acceptors. *In vitro*, cytochrome b_5 on the surface of either DMPC liposomes or phosphatidylcholine liposomes which had been exposed to detergent (deoxycholate at 1 mol/2 mol lipid) is not transferred to other liposomes [19]. This suggests that conditions might be found which would permit the hybrid proteins either to remain associated with the liposomes or to be transferred to other hydrophobic acceptors.

The molecular mechanism by which HP directs the association of proteins to membranes is not well understood. By introducing mutations into the DNA, we hope to determine the effect of amino acid substitutions at various positions in the hydrophobic domain on association with liposomes, to understand at a molecular level the nature of the association.

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