The Binding of Cytochrome b₅ to Phospholipid Vesicles and Biological Membranes

EFFECT OF ORIENTATION ON INTERMEMBRANE TRANSFER AND DIGESTION BY CARBOXYPEPTIDASE Y

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A method is described which allows the direct measurement of intermembrane protein transfer. We have used this method to examine the transfer of cytochrome b₅ from artificial phospholipid vesicles and biological membranes. This method involves the incubation of small, sonicated phospholipid vesicles with either biological membranes or large unilamellar phospholipid vesicles (Enoch, H. G., and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 145-149) and subsequent separation by gel filtration. We have observed cytochrome b₅ transfer between large and small single bilayer vesicles when cytochrome b₅ was bound to preformed egg phosphatidylcholine vesicles. The cytochrome b₅ of microsomes, however, did not transfer to small vesicles; neither did cytochrome b₅ reductase nor exogenous, bound cytochrome b₅. In fact, no detectable protein was transferred when high salt-washed microsomes were mixed with small, sonicated vesicles. Similar results were obtained using mitochondria and nuclear membrane fragments. We conclude that integral membrane proteins in general do not readily undergo intermembrane transfer between biological membranes. The ability of cytochrome b₅ to transfer from artificial membranes and not from biological membranes may reflect a difference in the nature of the protein binding. A nontransferable form of cytochrome b₅, which may represent the microsomal type of binding, was obtained when cytochrome b₅ was bound to preformed vesicles of dimyristyl phosphatidylcholine or when cytochrome b₅ was bound during the formation of phosphatidylcholine vesicles. A soluble, heme peptide fragment of cytochrome b₅ was released when vesicles containing cytochrome b₅ in the transferable form were incubated with carboxypeptidase Y. In contrast, the nontransferable form of cytochrome b₅ in microsomes and artificial vesicles was not released by carboxypeptidase Y treatment. We conclude that there are at least two possible orientations of cytochrome b₅ in phospholipid bilayers, and that these orientations result in either hindered or rapid intermembrane transfer.

There is considerable evidence that integral membrane proteins interact in some fashion with the hydrophobic core of the bilayer (1). Many such proteins undergo rapid rotational and translational movement in the lipid bilayer (2). Recent studies have shown that several integral membrane proteins may also undergo movement between bilayers, i.e., intermembrane protein transfer (3). It was first demonstrated with cytochrome b₅ bound to egg phosphatidylcholine vesicles (4) and with cytochrome b₅ reductase bound to dimyristyl phosphatidylcholine vesicles (3). In each case transfer was shown not to involve vesicle fusion; however, the experimental systems used in these studies precluded any direct demonstration of intermembrane transfer. Although this process could have important implications for the synthesis and function of proteins in biological membranes, it has not been reported to what extent, if any, intermembrane protein transfer occurs in vivo. Bouma et al. (5) have shown that several proteins of erythrocyte membranes may be transferred to dimyristyl phosphatidylcholine vesicles in vitro, and one of these proteins was identified as acetylcholinesterase (6).

In this report we describe a simple, rapid method for the detection and quantitation of intermembrane protein transfer. Using this method, we found that in a number of biological membranes there was no detectable transfer of any one of several membrane proteins. This suggests that these intracellular membrane proteins (integral) may have structural features which result in binding to membranes in essentially irreversible interactions. Using the binding of cytochrome b₅ to artificial phospholipid vesicles as a model system, we found two types of protein binding: one was capable of intermembrane transfer, the other was not. Based on these properties, we have proposed a model for two different orientations of the protein in membranes.

MATERIALS AND METHODS

Cytochrome b₅ (7), the cytochrome b₅ heme peptide (8), and nonpolar peptide (9) and NADH-cytochrome b₅ reductase from steer liver (10), stearoyl-CoA desaturase from rat liver (11), carboxypeptidase Y from bakers' yeast (12, 13), and egg phosphatidylcholine from hen eggs (14) were prepared as described previously. Cell fractions were prepared by differential centrifugation and density gradient centrifugation: microsomes from rat, rabbit, and steer liver (15); mitochondria (16) and nuclear membrane (17) from steer liver. Rat liver microsomal phospholipid was isolated by the method of Bligh and Dyer (18). Dioleoyl phosphatidylcholine (Supelco, Inc.) and dimyristyl phosphatidylcholine (Sigma Chemical Co.) were used without purification.

Cytochrome b₅ was determined from the oxidized spectrum (7) in vesicles and from the reduced minus oxidized difference spectrum (19) in cellular membrane fractions. NADH-cytochrome b₅ reductase (20), stearoyl-CoA desaturase (11), protein (21), and phospholipid (22) concentrations were measured as described previously. Polyacrylamide gel electrophoresis was carried out at room temperature in slab gels (15% acrylamide) using the buffer system of Laemmli (23). Unless otherwise stated all studies were carried out at 0-4°C in a buffer system consisting of 0.02 M Tris-acetate, pH 8.1, and 0.1 M NaCl.

Small, unilamellar phospholipid vesicles were prepared by sonica-
tion (11). Large, unilamellar phospholipid vesicles were prepared as described recently (24). Briefly, this method involves the treatment of a solution of small, sonicated vesicles with deoxycholate (1 mol/2 mol of lipid). The large vesicles form spontaneously and the detergent is subsequently removed by gel filtration on Sephadex G-25. The characterization of these vesicles which have an average diameter of 1000 Å has been described elsewhere (24).

RESULTS

The experimental system designed to detect and quantitate intermembrane protein transfer is based upon the ability to mix and then to separate populations of large and small phospholipid vesicles. The small vesicles used were 200 to 250 Å diameter, sonicated phospholipid vesicles, and the large vesicles were either 1000 Å, unilamellar phospholipid vesicles or purified, cellular membrane fractions (microsomes, nuclear membrane fragments, or mitochondria). Cytochrome bs was mixed with either the large or small vesicles and allowed to bind; after binding was complete, vesicles of the alternate size were added and incubated for appropriate times to allow transfer to occur. The preparations were then subjected to gel filtration (or centrifugation in the case of mitochondria) to separate the large and small vesicle populations. Cytochrome bs and phospholipid concentrations were measured in each population and the amount of intermembrane transfer was determined.

The first experiments were carried out in a completely artificial system using egg phosphatidylcholine to form both the large and small vesicles. An example of cytochrome bs transfer from large to small vesicles is illustrated in Fig. 1. As shown in Fig. 1A, the large and small vesicles were well separated by gel filtration on Sepharose 4B. We have previously reported that there was no fusion between large and small vesicles, even after 24 h of mixing (24). This was tested out using either the nonpolar peptide segment of cytochrome bs or cytochrome bs reductase, or protein appeared to undergo transfer to small vesicles (Table II, Experiment 1). By essentially reversing the order of vesicle addition, we were able to show that cytochrome bs bound to small vesicles (formed from either egg or dioleyl phosphatidylcholine) can undergo transfer to large vesicles (Table I, Experiment 2). The amount of cytochrome bs capable of undergoing transfer in this case is difficult to estimate because the affinity of the protein for the small vesicles is apparently relatively high (see above). Several transfer experiments were also carried out using either the nonpolar peptide segment of cytochrome bs or cytochrome bs reductase. Both proteins were transferred from small vesicles to large vesicles and vice versa using egg phosphatidylcholine vesicles throughout (data not shown).

We next asked the question: can cytochrome bs and other proteins of natural membranes undergo intermembrane transfer? We found that no detectable cytochrome bs, cytochrome bs reductase, or protein appeared to undergo transfer to small vesicles from high salt-washed microsomes obtained from three different species of animals (Table II, Experiment 1). The catalytic segments of both the reductase and cytochrome bs are known to be exposed to the outside face of microsomal vesicles (15) and this was verified in our preparations by showing that cytochrome bs was fully reduced by the addition of NADH. No transfer could be detected for the cytochrome bs or protein of nuclear membrane fragments or for the protein of mitochondria. This indicates that the proteins on the outer face of these membranes also do not readily undergo intermembrane transfer. When added to these membranes and allowed to bind, exogenous cytochrome bs did not transfer to small vesicles (Table II, Experiment 2) in contrast to the result obtained with cytochrome bs bound to large vesicles of egg phosphatidylcholine. In order to test whether the nontransferable nature of the cytochrome bs found in microsomes was due to the difference in the phospholipid composition of the microsomal membrane, we bound cytochrome bs to large vesicles formed from a lipid extract of microsomes. The cytochrome bs on these vesicles was bound in a form which was transferable to small vesicles (Table II, Experiment 3) suggesting that the tight binding of cytochrome bs to microsomes is not determined solely by the lipid composition.

Two types of cytochrome bs binding are thus distinguishable, one type found in microsomes (‘‘tightly bound’’ and
which would mimic the tight binding of cytochrome bs in microsomes. When cytochrome bs was included during the
another observed in some artificial membrane systems

| Experiment | Donor membranes | Cytochrome bs/ phospholipid | Time of binding (h) | Acceptor membranes | Donor lipid/ acceptor lipid | Per cent cytochrome bs transfer |
|------------|-----------------|-----------------------------|--------------------|-------------------|-----------------------------|--------------------------------|
| 1          | LUV, egg PC     | 1:100                       | 0.5                | SUV, egg PC       | 1:1                         | 76                             |
|            | LUV, egg PC     | 1:120                       | 2                  | SUV, egg PC       | 1:1                         | 72                             |
|            | LUV, egg PC     | 1:120                       | 72                 | SUV, egg PC       | 1:1                         | 70                             |
|            | LUV, egg PC     | 1:120                       | 24                 | SUV, egg PC       | 1:1                         | 86                             |
| 2          | SUV, egg PC     | 1:100                       | 2                  | SUV, egg PC       | 1:1                         | 18                             |
|            | SUV, egg PC     | 1:100                       | 2                  | SUV, egg PC       | 1:2.7                       | 23                             |
|            | SUV, egg PC     | 1:100                       | 2                  | SUV, egg PC       | 1:4                         | 16                             |
|            | SUV, dioleoyl PC| 1:100                       | 2                  | SUV, egg PC       | 1:3                         | 14                             |
|            | SUV, dioleoyl PC| 1:100                       | 2                  | SUV, egg PC       | 1:2                         | 17                             |

* LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; PC, phosphatidylcholine.

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|            | LUV, egg PC     | 1:120                       | 24                 | SUV, egg PC       | 1:1                         | 86                             |
| 2          | SUV, egg PC     | 1:100                       | 2                  | SUV, egg PC       | 1:1                         | 18                             |
|            | SUV, egg PC     | 1:100                       | 2                  | SUV, egg PC       | 1:2.7                       | 23                             |
|            | SUV, egg PC     | 1:100                       | 2                  | SUV, egg PC       | 1:4                         | 16                             |
|            | SUV, dioleoyl PC| 1:100                       | 2                  | SUV, egg PC       | 1:3                         | 14                             |
|            | SUV, dioleoyl PC| 1:100                       | 2                  | SUV, egg PC       | 1:2                         | 17                             |

* LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; PC, phosphatidylcholine.

The release of cytochrome bs from vesicles by digestion with carboxypeptidase Y

In Experiments 1, 2, 4, and 5, cytochrome bs was added to pre-formed vesicles or microsomes and incubated at 30°C under N2 for 2 h (at 1:100 to 1:200 mol of cytochrome bs/mol of phospholipid). The large vesicles used in Experiment 3 were prepared as described under "Materials and Methods" except that cytochrome bs was present during vesicle formation (initiated by the addition of deoxycholate); detergent was then removed by gel filtration as described (< 1 mol of deoxycholate remained/700 mol of phospholipid).

The protein appeared to bind in a form that was not releasable by carboxypeptidase Y (95% of the heme portion of the cytochrome bs was present during digestion for 24 h (2 h in Experiment 5) at 30°C, the preparations were subjected to gel filtration on Sephadex G-75 at 25°C to separate the vesicle-bound cytochrome bs (excluded fraction) from the released, soluble cytochrome bs (included fraction).

The release of cytochrome bs from vesicles by digestion with carboxypeptidase Y

| Experiment | Cytochrome bs-containing vesicles | Per cent cytochrome bs releasable by carboxypeptidase Y |
|------------|----------------------------------|--------------------------------------------------------|
| 1          | LUV, egg PC                       | 70-95*                                                 |
| 2          | SUV, egg PC                       | 80-90*                                                 |
| 3          | LUV, egg PC, cytochrome bs inserted during vesicle formation | <1 |
| 4          | SUV, dimyristyl-PC                | <1                                                     |
| 5          | bovine microsomes                 | <5                                                     |

* LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; PC, phosphatidylcholine.

The range of values obtained in a number of experiments.

formation of large diameter phospholipid vesicles in the presence of sodium deoxycholate as described for Experiment 3.

Table III, the protein appeared to bind in a form that was not transferable (data not shown). It was shown using NADH and the soluble catalytic fragment of cytochrome bs reductase (25, 26) that 95% of the heme portion of the cytochrome bs was exposed to the outside of this vesicle preparation.1 The vesicles were indistinguishable by electron microscopy (24) from large vesicles alone or large vesicles with cytochrome bs bound in the absence of deoxycholate. When deoxycholate was present

1 When cytochrome bs was incorporated into the bilayer during vesicle formation variable amounts of the protein (5 to 50%) were oriented with the heme peptide portion on the inside of the vesicle both by the criteria of catalytic reduction and trypptic cleavage. There is some indication that under certain conditions all of the cytochrome bs can be tightly bound to the outer surface of the bilayer. We are currently investigating the effect of a number of variables on this orientation; namely, deoxycholate/lipid ratio, time and temperature of incubation, ionic strength, and others.
during protein binding, either detergent interaction with the protein prior to binding or detergent interaction with the phospholipid during binding could lead to the insertion of cytochrome $b_5$ into the bilayer in a different orientation thereby resulting in a nontransferable form of the protein. The difference was not due to the continued presence of deoxycholate which was removed by gel filtration following binding: less than 1 mol of detergent/50 mol of cytochrome $b_5$ was present in these preparations. Tight binding of cytochrome $b_5$ to artificial vesicles can be produced by several other techniques. For example, incubation of cytochrome $b_5$ with small, sonicated vesicles of dimyristyl phosphatidylcholine alone resulted in complete “tight” binding of the protein (data not shown). Thus, in previous experiments in this laboratory using cytochrome $b_5$ bound to dimyristyl phosphatidylcholine vesicles (27) the protein was in the tightly bound form similar to microsomal cytochrome $b_5$ in terms of its ability to transfer and its susceptibility to proteolytic cleavage (see below). Incubation of cytochrome $b_5$ with large vesicles of egg phosphatidylcholine containing stearyl-CoA desaturase also led to complete tight binding of the heme protein (data not shown). The cytochrome $b_5$, thus bound was functional with cytochrome $b_5$ reductase (shown by cytochrome $b_5$ reduction) and stearyl-CoA desaturase (shown by formation of oleyl-CoA). This is a further indication that the tightly bound form of cytochrome $b_5$ in vesicles has an orientation similar to that of the protein in the microsomal membrane. It has not yet been possible to determine whether the loosely bound form of cytochrome $b_5$ is also functional with desaturase. In these same experiments no transfer of desaturase from large vesicles to small vesicles could be detected either by enzymatic activity or protein measurements.

We have also established that the transferable and nontransferable forms of cytochrome $b_5$ differ in their susceptibility to digestion by carboxypeptidase Y. When large or small, single bilayer egg phosphatidylcholine vesicles containing the loosely bound form of cytochrome $b_5$ were incubated with carboxypeptidase Y, a soluble, intermediate size fragment of cytochrome $b_5$ was initially released from the vesicles (2 to 4 h) and this fragment was then further digested (20 to 24 h) to a lower molecular weight form which may correspond approximately to the soluble, heme peptide of cytochrome $b_5$ (Fig. 2). The amount of cytochrome $b_5$ releasable by this treatment was variable with up to 95% released from large vesicles and 93% from small vesicles (Table III). We tested whether the carboxypeptidase Y-releasable fraction corresponded to the transferable fraction of cytochrome $b_5$ by measuring both fractions in a single preparation of large vesicles. Excellent correlation was obtained (72% of the cytochrome $b_5$ transferable, 71% of the cytochrome $b_5$ released by carboxypeptidase Y) suggesting that both of these functions are properties of the “loose binding” form. In contrast, no release of soluble heme peptide fragments could be detected after treatment of the tightly bound form of cytochrome $b_5$ (inserted in egg phosphatidylcholine with deoxycholate or in dimyristyl phosphatidylcholine vesicles) under the same conditions for up to 24 h (Fig. 2 and Table III). Furthermore, there was no release of the exogenously bound cytochrome $b_5$ of microsomes by carboxypeptidase Y (Table III), thus extending the correlation between proteolytic release and the ability of cytochrome $b_5$ to undergo intermembrane transfer.

A number of experiments have been carried out in order to determine whether the loose binding form of cytochrome $b_5$ can be easily and completely converted to the tight binding form. Two techniques have thus far been successful. The first (Table IV, Experiment 1) involves treating the loosely bound form of cytochrome $b_5$ in large egg phosphatidylcholine vesicles with deoxycholate (1 mol/2 mol of lipid). This level of detergent causes the vesicles to become highly permeable without disrupting the bilayer structure (24, 28). Following this treatment the cytochrome $b_5$ was no longer releasable by carboxypeptidase Y and this property was retained even after

![Fig. 2. Polyacrylamide gel electrophoresis of cytochrome $b_5$-containing vesicles after treatment with carboxypeptidase Y. The preparation of the vesicles containing cytochrome $b_5$ (1 mol/100 mol of lipid) and the digestion with carboxypeptidase Y for 0, 2, or 24 h as indicated (Lanes b to h) were performed as described in Table III. After treatment samples were rapidly denatured by pipetting into 4 volumes of 2% sodium dodecyl sulfate at 100°C. Each sample applied to the gel contained 3 to 4 µg of cytochrome $b_5$. Lanes a and i, cytochrome $b_5$ and cytochrome $b_5$ heme peptide; Lanes b to d, cytochrome $b_5$ in large egg phosphatidylcholine (PC) vesicles; b, 0 h; c, 2 h; d, 24 h; Lanes e and f, cytochrome $b_5$ in small egg PC vesicles; e, 0 h; f, 24 h; Lane g, cytochrome $b_5$ in small dimyristyl-PC vesicles, 24 h; Lane h, cytochrome $b_5$ inserted in large egg PC vesicles during vesicle formation, 24 h; Lane j, carboxypeptidase Y alone.

**Table IV**

Conversion of cytochrome $b_5$ from the loosely bound to the tightly bound form

| Experiment and treatment | Per cent cytochrome $b_5$ releasable by carboxypeptidase Y |
|--------------------------|----------------------------------------------------------|
| 1. Large egg PC vesicles | 95 |
| Add deoxycholate, 2.2 mM (10 min, 30°C) | 5 |
| Remove deoxycholate by gel filtration (< 1 mol deoxycholate/500 mol of lipid) | <1 |
| 2. Small egg PC vesicles | 81 |
| Sonicate (25–30°C under N2), 30 min | 15 |
| Sonicate (25–30°C under N2), 60 min | 12 |
| Sonicate (25–30°C under N2), 90 min | 5 |

* PC, phosphatidylcholine.
removal of the detergent by gel filtration. Lower levels of deoxycholate added to vesicles (≤0.1 mol/mol of lipid), which presumably cause considerably less perturbation of the bilayer, did not result in any change in the amount of cytochrome bs bound to small egg phosphatidylcholine vesicles was converted to the tightly bound form by sonication (Table IV, Experiment 2). Similarly, only the tightly bound form was obtained when cytochrome bs was present during initial vesicle formation by sonication. In preliminary experiments, other methods were tested for their effect on the conversion to tight binding: prolonged incubation (~24 h) of cytochrome bs-containing vesicles at elevated temperature (37°C), inclusion of other membrane-binding proteins in the vesicles (e.g. gramicidin S), and inclusion of lysophosphatidylcholine in the vesicles (up to 20% of the lipid). Thus far these methods have been only partially successful and have not led to the complete conversion of cytochrome bs from the loose binding to the tight binding form.

**DISCUSSION**

We have described an experimental system which allows one to detect and quantitate intermembrane protein transfer. The system should be directly applicable to the study of the transfer of other membrane components including other membrane proteins, phospholipids, glycolipids, steroids, proteolipids, etc. We have reported here that cytochrome bs and cytochrome bs reductase bound to artificial membranes formed from egg phosphatidylcholine are capable of undergoing intermembrane transfer, thus, confirming previous observations (3, 4), and these results were extended to include the nonpolar peptide segment of cytochrome bs.

The major and contrasting new observation is that intrinsic (or integral) membrane proteins on the endoplasmic reticulum, mitochondria, and nuclear membranes do not undergo transfer from their natural membrane loci in vitro. Since no exceptions were found, it appears that this may be the dominant situation in vivo. Although intermembrane protein transfer in vivo may not be a general phenomenon, it may occur under specific circumstances with certain proteins (5).

The orientation of endogenous and exogenously bound cytochrome bs on microsomes appears to be similar not only on the basis of the observed interactions with the cytochrome bs reductase and stearyl-CoA desaturase (15) but also in binding in a nontransferable form which is resistant to carboxypeptidase Y digestion (this report). Apparently, proper insertion of cytochrome bs in the isolated microsomes in vitro (also mitochondrial and nuclear membranes) suggesting that insertion of the protein into the membrane in vitro may occur subsequent to and independently of protein synthesis. This would be in contrast to the insertion of a class of proteins that bind to the membrane at their NH2 terminus which is then extruded through the membrane during protein synthesis (29).

Finally, we have described a model membrane system in which cytochrome bs may bind in two different orientations as recognized by differences in the ability of the protein to undergo intermembrane transfer and digestion by carboxypeptidase Y. Since only one of these forms is observed in biological membranes, it is important to know the structural basis of these two types of binding. When pure cytochrome bs is incubated with single bilayer egg phosphatidylcholine vesicles the protein binds to the membrane in such a way that (a) the COOH terminus is available for digestion by carboxypeptidase Y and (b) the protein can undergo intermembrane transfer and is referred to here as loose binding. Both of these properties are lost when cytochrome bs is bound during the formation of large, single bilayer egg phosphatidylcholine vesicles in the presence of deoxycholate (referred to as tight binding). We propose that these two operationally defined forms of cytochrome bs actually represent two different modes of orienting the nonpolar, membrane-binding segment of the protein in the bilayer. When cytochrome bs binds to pure, unperturbed bilayers, the loose binding form is predominately obtained. However, if the bilayer is in a perturbed state due to presence of deoxycholate or another integral membrane protein, i.e. desaturase, in the bilayer then cytochrome bs is inserted in the tight binding form. Only the tight binding form of cytochrome bs is observed with dimerystyl phosphatidylcholine which forms less stable, highly permeable vesicles (30). These results suggest that the insertion of cytochrome bs into the membrane in the tight binding form may require some perturbation of the phospholipid bilayer. Consistent with this, we have found that the loose binding form could be converted to the tight binding form by sonication or addition of deoxycholate, both of which may cause local disruption or perturbation within the bilayer. Barring any perturbation of the bilayer, two orientations of cytochrome bs appear to be stable and do not undergo interconversion.

We are now attempting to define the molecular basis for the difference in the properties of the two types of cytochrome bs binding and also to determine the difference in the orientation of the polypeptide chain with respect to the bilayer, or in the tertiary structure of the nonpolar peptide per se, or both. Fig. 3 diagrams some of the orientations which may account for the observed differences in cytochrome bs intermembrane transfer and in the accessibility of the COOH terminus to digestion by carboxypeptidase Y. The most direct interpretation of the data is that the loss of accessibility of the COOH terminus to carboxypeptidase Y arises from the placement of the COOH terminus at the aqueous interface in the interior of the vesicle, III, which is not accessible to the protease. Wickner (32) came to a similar conclusion concerning the orientations of the M13 virus coat protein after a study of the proteolytic digestion of the protein in artificial vesicles. Such a model for cytochrome bs binding would also explain the observed absence of intermembrane transfer. Placing the COOH terminus on the inside of the vesicle results in a thermodynamic barrier to transfer due to the energy required to move the charged residues (9) through the nonpolar interior of the bilayer as predicted by the hydrophobic effect (33, 34). We have presented several other models (Fig. 3) that are

![Fig. 3. Models for the orientation of the nonpolar, membrane-binding segment of cytochrome bs in the membrane. Heme peptide, residues 1 to 87; linkage peptide, residues 88 to 97; nonpolar peptide, residues 98 to 133 (9, 31). I, II, loose binding forms; III, IV, V, tight binding forms.](http://www.jbc.org/)
consistent with our data, but the thermodynamic basis for the stabilization of these alternate orientations is less clear. By exploiting the large vesicle preparations containing either tightly or loosely bound cytochrome $b_5$ and either carboxypeptidase Y or group-specific reagents in the interior or exterior of these vesicles, a direct and unambiguous test of the model involving transmembrane nonpolar peptide orientation in tight binding may be possible.

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