Thermodynamics of the Membrane Insertion Process of the M13 Procoat Protein, a Lipid Bilayer Traversing Protein Containing a Leader Sequence†

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ABSTRACT: For the first time, the standard free energy change, $\Delta G°$, of a membrane-inserting protein with a leader sequence has been determined experimentally, using M13 procoat protein as an example. The partition coefficient for the distribution of the procoat protein between the aqueous phase and the membrane phase of preformed lipid vesicles yielded a value of $\Gamma = 6.5 \times 10^5\ M^{-1}$, corresponding to a $\Delta G°$ of −10.4 kcal/mol, based on measurements of the fluorescence energy transfer between the intrinsic tryptophan of the protein and a suitably labeled lipid membrane of POPC. For comparison, the partition coefficient of the M13 coat protein between the aqueous and the POPC lipid bilayer phase was determined to be distinctly lower: $\Gamma = 1 \times 10^5\ M^{-1}$ ($\Delta G° = -9.3$ kcal/mol). Proteinase K digestion experiments have been performed, showing that 20% of the procoat protein bound to lipid vesicles spontaneously integrate in a transbilayer form, whereas 80% remain inserted in the interfacial membrane region. By taking together these results, an upper limit for the free energy change of the transmembrane insertion of procoat protein was estimated to be −14.8 kcal/mol. In order to distinguish further the contribution arising from insertion of the procoat protein into the membrane interfacial region from that due to transmembrane insertion, the partition coefficient of the mutant procoat protein OM30R [which contains a positively charged amino acid in its mature hydrophobic segment (exchange of a Val to an Arg residue at position 30)] was determined, yielding $\Gamma = 0.3 \times 10^5\ M^{-1}$ ($\Delta G° = -8.6$ kcal/mol). Previously reported *in vivo* experiments have shown that the OM30R mutant protein is not translocated across *Escherichia coli* membranes but only binds to the inner surface. The results presented here indicate that although the insertion of the procoat protein into the interfacial region of the lipid bilayer contributes the major part to $\Delta G°$, it is the final energy gain of the interaction of the hydrophobic portions of the folded pre-protein with the lipid chains which drives the transmembrane insertion of the M13 procoat protein. Neither the leader sequence nor the mature coat protein alone yields this free energy gain. For the different proteins investigated here, spontaneous membrane insertion occurs only for fluid lipid bilayers, but not for membranes in the crystalline lipid phase. Furthermore, by using lipid bilayers with negative membrane surface charges, it was shown that both procoat and coat proteins are electrostatically attracted to the surface of the lipid membrane, though only to a small extent, with apparent partition coefficients of the same order of magnitude as for the phosphatidylcholine lipid membrane.

The insertion of newly synthesized membrane proteins into lipid bilayers is in many cases directed by N-terminal signal (leader) sequences, that are removed from the mature part of the protein after insertion [for reviews, see von Heijne (1994) and Wickner (1988)]. Complicated protein insertion and translocation machineries have been characterized genetically and biochemically in prokaryotic and eukaryotic cells. However, there are several examples of relatively small proteins which insert into membranes independently of the translocation machinery. One classical example is the coat protein of the filamentous phage M13 (Kuhn, 1995; Broomesmith et al., 1994).

The M13 coat protein is synthesized with a 23 amino acid long signal sequence in the cytoplasm of *Escherichia coli* and is then inserted into the plasma membrane. The membrane insertion process of this so-called M13 procoat protein has been extensively studied genetically and biochemically (Kuhn & Troschel, 1992). There are experimental indications that the binding of the protein to the negatively charged membrane surface occurs via the positively charged amino acid residues at the amino and carboxy termini of the procoat protein (Gallusser & Kuhn, 1990). The subsequent partitioning of the two hydrophobic regions into the membrane results in an U-like configuration. This *in vivo* insertion process requires a transmembrane potential for the wild-type procoat protein (Kuhn & Wickner, 1985). Biophysical investigations have shown that at least part of the predicted transmembrane regions adopt an α-helical conformation (Thiaudière et al., 1993). The topology of this intermediate has been investigated using procoat proteins with either N-terminal or C-terminal extensions (Kuhn et al., 1986; Kuhn, 1987). It was also found that the N- and C-termini do not leave the cytoplasm during the insertion

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process and only the negatively charged central region traverses the membrane in accordance with the "positive-inside" rule (von Heijne, 1992). After membrane insertion, the signal sequence is cleaved off by the *E. coli* leader peptidase which has its enzymatic activity at the periplasmic face of the cytoplasmic membrane (Dalbey, 1991; Dalbey & von Heijne, 1992).

The membrane insertion process of the procoat protein is apparently independent of any "helper" protein complex, such as *E. coli* translocase and other factors like the ffh protein (Kuhn, unpublished results). In this context, it is interesting to note that newly synthesized M13 procoat protein is capable of insertion into liposomes from the aqueous phase (Geller & Wickner, 1985), suggesting that the insertion process is mainly driven by hydrophobic interactions. This makes quantitative investigation of the membrane insertion of this protein attractive. In the present paper, we determined experimentally the free energy change $\Delta G°$ occurring during the spontaneous transfer of the procoat protein from the aqueous to the lipid bilayer phase. These data will serve as a basis for a thermodynamic model describing the membrane insertion of procoat protein. In order to distinguish the energetic contributions which arise from the protein binding to the interfacial membrane region and from the membrane-spanning insertion, parallel experiments were also performed with the coat protein as well as with the procoat mutant protein OM30R. The OM30R mutant differs from the wild-type procoat protein in having a Val → Arg mutation at position +30, inserting a positive charge into the hydrophobic stretch of the mature part of the protein. This mutant protein is known from *in vivo* experiments to interact with the membrane, but not to be translocated across the *E. coli* plasma membrane. The interest in the present context is to learn whether or not this changed biological property is correlated with a change of the partitioning into lipid bilayer membranes. Furthermore, electrostatic contributions of the procoat protein interaction with membranes were taken into consideration by investigating the protein insertion into membranes with different membrane surface charge densities.

The experiments were performed in a reconstituted system using purified proteins which, after mixing, spontaneously bind to lipid bilayers either during or after vesicle formation. The process of protein incorporation into the lipid membranes was then observed by measuring the fluorescence energy transfer (FET)[1] between the (pro-) coat protein's tryptophan and tyrosine residues, which act as fluorescence donors, and a diphenylhexatriene (DPH) moiety coupled to phosphatidylcholine molecules in the bilayer membrane which acts as a fluorescence acceptor. Instead of the wild-type M13 procoat protein, we used in the course of this work the noncleavable procoat mutant protein H5 (with a Phe at position −3; see Figure 1) which inserts into the lipid bilayer as the wild-type protein but can be isolated by a simple procedure in chemical quantities as was shown elsewhere (Thiaudière et al., 1993).

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PC, 2-[3-(diphenylhexatrienyl)propanoyl]-1-hexadecanoyl-*sn*-3-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-3-phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; FET, fluorescence energy transfer; L/P, lipid to protein molar ratio; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol; SUV, small unilamellar vesicle(s).

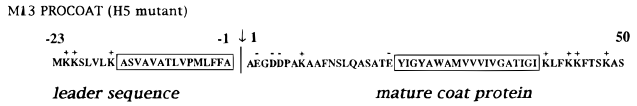

FIGURE 1: Amino acid sequences of M13 H5 procoat and coat proteins. The arrow indicates the cleavage site of M13 procoat protein by leader peptidase. This cleavage site separates the leader sequence (amino acids −23 to −1) from the mature coat protein sequence (amino acids 1−50). The charged amino acids are indicated; the rectangles symbolize the hydrophobic, putative transmembrane domains. The H5 procoat mutant protein used in the present work is derived from the wild-type procoat protein by a single amino acid mutation at sequence position −3 which changes Ser to Phe. This mutation does not affect the membrane translocation pathway but prevents procoat cleavage by leader peptidase (Kuhn & Wickner, 1985). The OM30R procoat mutant protein has a Val → Arg mutation at position 30.

EXPERIMENTAL PROCEDURES

Materials

The lipids 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine (POPC), 1,2-dipalmityol-*sn*-3-phosphatidylcholine (DPPC), and 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol (POPG) were from Avanti Polar Lipids (AL); 2-[3-(diphenylhexatrienyl)propanoyl]-1-hexadecanoyl-*sn*-3-phosphatidylcholine (DPH-PC) was from Molecular Probes (Eugene, OR); all other chemicals were from Fluka (Buchs, Switzerland) and of the best quality available.

M13 H5 procoat and M13 coat proteins were isolated and purified on HPLC as previously described (Thiaudière et al., 1993). The procoat mutant protein OM30R was constructed by oligonucleotide-directed mutagenesis and expressed in *E. coli* as described (Kuhn et al., 1987); the isolation was performed as for the H5 procoat protein. The buffer used in the experiments was 1 mM Tris-HCl, pH 7.4, containing 0.1 mM EDTA, degassed with He.

Methods

Two different reconstitution protocols were used in the present studies.

Protein Binding to Preformed Lipid Vesicles. Appropriate volumes of solutions of unlabeled lipids and DPH-PC [both 2 mM in chloroform/methanol = 1/1 (v/v)] were mixed. The solvent was evaporated first under a continuous flow of N_2, and then under vacuum. The resulting films were rehydrated with degassed buffer, yielding a dispersion of multilamellar liposomes. For the production of small unilamellar vesicles (SUV), the liposomes were sonicated until the preparation became transparent. By this procedure, vesicles with an average diameter of about 30 nm were produced as determined by electron microscopy. Alternatively, large unilamellar vesicles with an average diameter of 100 nm were formed by extruding the liposome preparation through a Nucleopore polycarbonate filter from Castor Corp. (Cambridge, MA) using an extruder from Lipex Biomembranes, Inc. (Vancouver, Canada). The lipid concentration of the corresponding vesicle preparations was typically 2 mM with 2 mol % DPH-PC. Phospholipid concentration was determined according to Rouser et al. (1970).

A small volume of a protein solution in organic solvent was injected under stirring into either buffer or a suspension containing the appropriate quantity of lipid vesicles. The final amount of organic solvent in the samples was less than 0.5 vol %. The concentrations of the coat and procoat

proteins were determined by the UV absorbance at 280 nm using $\epsilon = 8000\ M^{-1}\ cm^{-1}$ (Thiaudière et al., 1993). For each vesicle preparation, the 90° light scattering signal was measured at 280 nm before and after addition of the particular protein; because no signal change was observed, we concluded that the vesicles remained intact during interaction with the proteins.

Protein Reconstitution by Dilution of a Mixed Lipid/Protein Solution (Mixed Injection Method). Appropriate volumes of solutions of lipids [2 mM in chloroform/methanol, 1/1 (v/v)] containing 2 mol % DPH-PC and coat or procoat protein (in 2-propanol) were mixed. An aliquot of this mixture was injected under stirring into degassed buffer. Under these conditions, large lipid vesicles are formed and the protein spontaneously integrates into the bilayers during formation.

Determination of Protein Topology in Lipid Membranes. Either coat or procoat protein was bound to preformed vesicles (L/P = 200) as described above. After addition of proteinase K (Sigma) to a final concentration of 100 μg/mL, the samples were incubated for 30 min at 0 °C. Proteinase was inactivated by addition of phenylmethanesulfonyl fluoride (Sigma), and the protein was precipitated by 20% trichloroacetic acid. The pellets (typically 100 μg of protein) were dissolved by electrophoresis buffer, applied to SDS−PAGE (Ito et al., 1980), and analyzed by Western blots using polyclonal antibodies which preferentially recognize the mature N-terminal coat sequence, both in the coat and in the procoat proteins. The bands were visualized by the Amersham ECL kit (Beaconsfield, U.K.). Similar samples were prepared for lipid vesicles with proteins reconstituted by the mixed injection method. Parallel controls were performed for each sample by adding detergent (octyl glucoside, final concentration in sample buffer of 1% w/v) prior to the incubation with proteinase K. In the controls, it is expected that the mature N-terminal coat sequences both in coat and in procoat proteins are exposed to the enzymatic digestion.

Fluorescence Measurements. At 2.5 min either after protein binding to preformed lipid vesicles or after protein reconstitution by the mixed injection method, fluorescence spectra were recorded using a Spex Model 1681 fluorometer (Spex, NJ). Longer incubation times were tested as well (up to 1 h), but did not result in detectable changes of the deduced partition coefficients; conditions: excitation at 280 nm, slit width 1 mm; emission 300−450 nm, slit width 5 mm; quartz cuvettes of 0.4 cm × 1 cm; 23 °C. Under the applied experimental conditions, the intrinsic protein fluorescence arises from Tyr and Trp residues between 300 and 400 nm. In the presence of diphenylhexatrienylphosphatidylcholine, DPH-PC, the fluorescence of the DPH chromophore appears at 380−450 nm. In order to obtain the change of the fluorescence spectra due to fluorescence energy transfer (FET) of membrane-bound protein molecules, a blank spectrum of a particular vesicle suspension was recorded (i) either directly prior to injection of protein into the samples or (ii), in the case of the "mixed injection" method, from an additional pure vesicle suspension, and subtracted for base line correction from each raw spectrum acquired with the samples containing the protein.

Analysis of the Fluorescence Data. For the titration curves of (pro-)coat proteins, both the Trp/Tyr and the DPH fluorescence signals were monitored as a function of the lipid to protein molar ratio, L/P. The Trp/Tyr and DPH fluorescence of individual samples was measured as the integral intensity of the emission from 300 to 356 nm and from 390 to 450 nm, respectively.

FET from the natural tryptophan and tyrosines of the proteins to a lipid-attached chromophore, DPH-PC, was used to evaluate the membrane affinity of M13 procoat and coat proteins. FET was characterized as the increase of the DPH fluorescence due to the close proximity of Tyr and Trp residues to the DPH-PC molecules in the membrane. Upon irradiation at 280 nm, DPH is directly excited to some extent; therefore, the background fluorescence of DPH is observed in the absence of FET. Spectra were corrected for the intrinsic DPH fluorescence by subtracting the spectrum of a sample which contained the appropriate amount of lipids, but no protein. The DPH fluorescence intensity, F, is taken to be the integral of the corresponding difference spectra from 390 to 450 nm. Measuring the FET-induced decrease of the Tyr/Trp fluorescence yielded identical information on the membrane partitioning. However, the experimental scatter of the data was higher than for DPH fluorescence for reasons we do not yet understand. We assume that at the moment of sample injection the protein equilibrates between the aqueous and the lipid bilayer. All protein molecules which are not lipid-associated and do not contribute to the DPH fluorescence will be included in a term called "free" protein concentration, c_f.

The association of polypeptides to membranes has been quantitatively evaluated in the literature in terms of a partition equilibrium between the aqueous and the lipid bilayer phase for several amphipathic polypeptides [see Pawlak et al., (1994b) and references cited therein; Roise, 1993]. FET measurements have already been used by others to detect protein association to lipid membranes (Vaz et al., 1977; Tamm & Bartoldus, 1988). Here we apply this method to M13 procoat protein and M13 coat protein using the formalism of Schwarz et al. (1986). An apparent partition coefficient Γ_{app} is defined as

$$rf(r) = \Gamma_{app} c_f \tag{1}$$

with

$$r = c_{as}/c_l \tag{2}$$

being the molar ratio of lipid-associated protein molecules (concentration c_{as}) per lipid molecules (c_l). $f(r)$, the activity coefficient as a function of r, may describe deviations from ideal partitioning ($f = 1$) due to protein−protein interaction at the water−membrane interface or in the lipid bilayer. The molar fraction of lipid-associated protein, c_{as}/c_p, was measured by applying

$$c_{as}/c_p = F/F_\infty \tag{3}$$

where

$$c_p = c_{as} + c_f \tag{4}$$

is the total concentration of protein, F represents the FET-induced increase in DPH fluorescence, and F_∞ is the corresponding limiting fluorescence intensity at high L/P ratios for the case where all protein molecules are bound to lipid membranes. By using eq 4, it is possible to determine

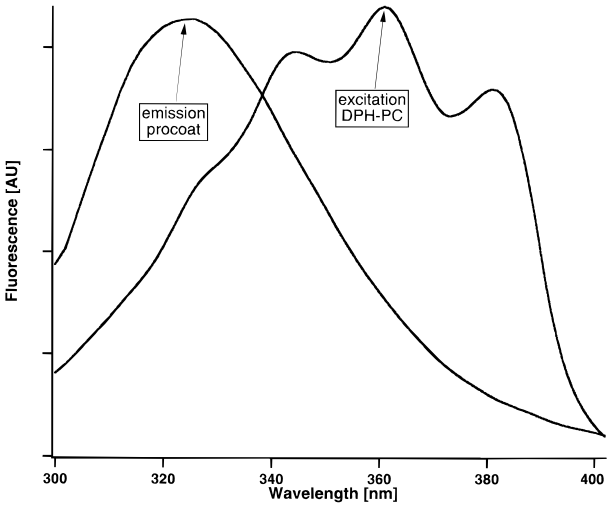

FIGURE 2: Trp/Tyr emission spectrum of M13 procoat protein (excitation at 280 nm) and diphenylhexatriene (DPH) excitation spectrum of DPH-PC (emission at 430 nm), both compounds incorporated into lipid vesicles of POPC. Experimental conditions: 1 μM procoat protein in 0.3 mM POPC vesicles; 2 μM DPH-PC in 0.1 mM POPC vesicles; 1 mM Tris-HCl, pH 7.4, in both cases. Due to the overlap between the two spectra, fluorescence energy transfer occurs between the Tyr and Trp residues of vesicle-associated proteins which act as donors and lipid-anchored DPH moieties which act as acceptors.

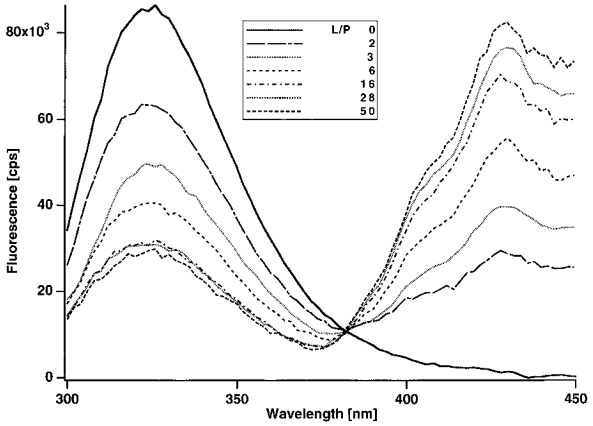

FIGURE 3: Emission spectra of M13 procoat protein (0.5 μM in 1 mM Tris buffer, pH 7.4, 23 °C) excited at 280 nm in the absence and presence of POPC vesicles doped with 2 mol % DPH-PC at increasing lipid to protein ratios (L/P). All spectra are corrected for DPH fluorescence in the absence of protein by blank subtraction. Thus, the DPH fluorescence in the samples containing lipid vesicles is due only to FET between the Trp/Tyr residues of vesicle-associated proteins and lipid-bound DPH moieties. For comparison, the Trp/Tyr emission spectrum of M13 procoat protein bound to pure POPC membranes (L/P = 100) in the absence of DPH-PC was determined to have the same appearance as in buffer, but showing a 3 times higher quantum yield.

c_f for each known value of c_{as}. The apparent partition coefficient can be determined according to eq 1 from the association isotherm of r versus the free protein concentration c_f if $f(r)$ is known. For the ideal case of $f(r) = 1$, a simple linear relation exists between r and Γ_{app}.

RESULTS

Figure 2 shows a fluorescence emission spectrum of procoat protein arising from the intrinsic single tryptophan and the two tyrosine residues, together with an excitation spectrum of DPH-PC in POPC lipid vesicles. The intrinsic fluorescence spectrum of the coat protein under identical experimental conditions is comparable to that of procoat protein, since the three chromophores mentioned above are located in the mature part of the protein (see Figure 1). The overlap of both spectra makes DPH-PC-doped membranes ideally suited for the detection of the membrane binding of coat and procoat proteins by FET by measuring either the concomitant decrease of the intrinsic protein fluorescence or the corresponding increase of the DPH fluorescence. The Förster distance of the couple Trp/DPH was determined to be 4 nm (Le Doan et al., 1983). In the present case of procoat and coat proteins, which both contain one Trp and two Tyr residues in close proximity, we estimated from the optical properties a formal Förster distance of 5−6 nm for the coat or procoat proteins and DPH-PC in lipid bilayers assuming an orientational factor of $\kappa^2 = {}^2/_3$ in the standard equations (Förster, 1948). However, the exact Förster distance is not important in the present case because we use FET only to distinguish between membrane-bound and "free", nonbound protein molecules. Membrane-bound proteins include membrane insertion into the interfacial region and the transmembrane insertion. The present fluorescence assay does not allow to distinguish directly between the two cases.

A typical example of such an experiment is demonstrated in Figure 3. Here a small volume of procoat protein, dissolved at relative high concentrations in 2-propanol (50 μM−300 μM), was diluted into various samples comprising a large volume of an aqueous dispersion of DPH-PC-doped SUV of POPC at different concentrations. There are the following important results to be mentioned in particular: (i) At a given protein concentration, an increase of the lipid vesicle concentration both reduces the intrinsic protein fluorescence intensity and increases the DPH fluorescence intensity, as is shown in Figure 3. (ii) In the presence of 2 mol % DPH-PC, the Trp/Tyr fluorescence intensity of procoat protein (and similarly of coat protein) in the membrane-bound state, i.e., at high lipid to protein ratios, is less than 10% of the intensity when the proteins are bound to pure POPC membrane vesicles without DPH-PC. This considerable quenching of the Trp/Tyr fluorescence due to FET is in good agreement with theoretical considerations of Kwong and Stryer (1978) and Wolber and Hudson (1979). These authors have shown that the fluorescence transfer efficiency between a donor−acceptor pair in a lipid bilayer depends only on the membrane surface density of the acceptor and is independent of the surface density of the donor, i.e., of the protein bound to the lipid membrane. Adapting their considerations to our present case for a Förster distance of 5−6 nm and an acceptor density of 2 mol % in the membrane, a FET quenching of more than 90% is calculated.

The fluorescence data are evaluated in Figure 4 in the form of a graph showing c_{as}/c_p as a function of the molecular lipid to protein ratio, L/P.[2] The titration experiments demonstrate,

[2] A prerequisite to determine the amount of membrane-associated proteins, c_{as}, by FET measurements according to eq 3 is a linear dependence between the FET-induced increase of the DPH fluorescence, F, and c_{as}. This was tested to be actually fulfilled by measuring the fluorescence signal F for coat protein bound to lipid vesicles (lipid/protein molar ratio of 300) at 0.5, 1, 1.5, and 2 μM protein concentration. One would also expect such a linear relationship from theoretical considerations (Kwong-Keung & Stryer, 1978; Wolber & Hudson, 1979).

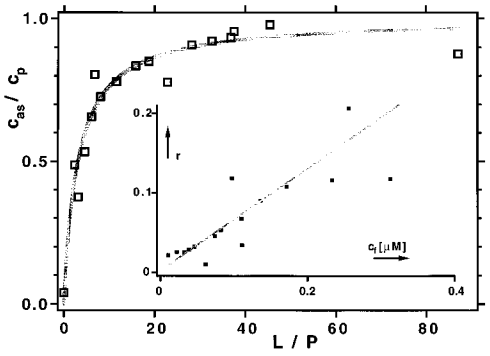

FIGURE 4: Fraction of procoat protein associated to POPC lipid vesicles relative to the total peptide concentration, c_{as}/c_p, plotted as a function of increasing lipid to protein ratios L/P. c_{as} was determined from the FET-induced increase of the DPH-PC fluorescence of the data shown and described in Figure 3, according to eqs 3 and 4. The insert shows a plot of r, the molar ratio of lipid-associated protein molecules per total lipid molecules, as a function of the free protein concentration c_f. The straight line corresponds to a least-squares linear fit of an ideal protein partitioning into the lipid bilayers according to eq 1, yielding a partition coefficient $\Gamma = 6.5 \times 10^5\ M^{-1}$.

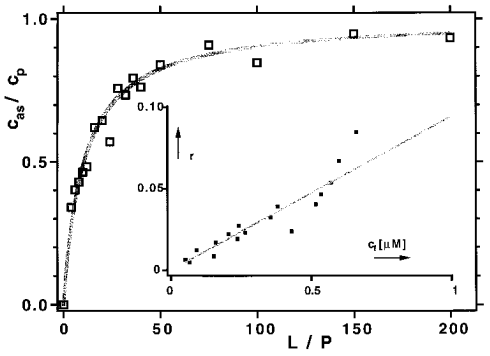

FIGURE 5: Association of M13 coat protein with POPC vesicles containing 2 mol % DPH-PC, determined by FET as described for M13 procoat protein in Figures 2, 3, and 4. Experimental conditions: 1 μM coat protein in 1 mM Tris buffer, pH 7.4; 25 °C; excitation at 280 nm. Fitting as shown in the insert yielded a partition coefficient $\Gamma = 1.0 \times 10^5\ M^{-1}$.

first, that the procoat protein binds to POPC lipid membrane vesicles and, second, that the changes of the corresponding fluorescence signals continuously approximate a limiting value at high L/P ratios which reflect the decrease of the concentration of free procoat protein in the aqueous phase of the corresponding samples. As outlined under Experimental Procedures, such a titration curve can be described by a thermodynamic equilibrium partitioning of the protein between the aqueous phase and the lipid bilayer. The solid line in the insert of Figure 4 (plot of r versus c_f) corresponds to a fit of the experimental data to eq 1 with an apparent partition coefficient $\Gamma_{app} = 6.5 \times 10^5\ M^{-1}$, the activity coefficient being taken to be $f(r) = 1$; we believe this is a reasonable approximation, although cooperative binding effects cannot be excluded totally, due to scattering data at higher values of c_f.

For the association of M13 coat protein to POPC vesicles, performed under identical conditions as for M13 procoat protein, we found a distinctly lower value for the apparent partition coefficient, $\Gamma_{app} = 1.0 \times 10^5\ M^{-1}$ (Figure 5). Again the binding isotherm can be described using an activity coefficient of $f(r) = 1$.

In order to investigate whether the vesicle size influences the partition coefficients of either M13 procoat or coat protein, experiments were also performed using extruded vesicles with an average diameter of 1000 Å. No difference in membrane association of either protein was observed when compared with the experiments with sonicated SUVs with an average diameter of about 30 nm.

For comparison, a different reconstitution protocol was used; instead of injecting the proteins into a dispersion of preformed vesicles, the proteins were cosolubilized with POPC lipids in organic solvent. This solution was injected into an excess volume of aqueous buffer, thereby spontaneously forming lipid bilayers. It is assumed that, during this process, the hydrophobic protein segments are integrated into the growing lipid bilayers (see below). Although a clear binding of the proteins could be detected by this assay, the high turbidity of the thus-formed vesicle dispersions as well as the necessity to work with an extra lipid blank sample led to relatively large uncertainties in the data related to the final partition isotherms. This made it impossible to obtain a reliable partition coefficient for comparison with corresponding data from the preformed lipid vesicle assay.

Additional titration experiments with M13 coat and procoat proteins were performed to gain information about the influence of membrane surface charges and the physical state of the membrane on the association of M13 coat protein to lipid membranes. The results are summarized in Table 1. Interestingly, the apparent binding of M13 coat and procoat proteins distinctly increased if lipid membranes with negative surface charges were used. In the present work, the negative surface charges were provided by inclusion of POPG in the applied lipid vesicles at a molar composition of POPC/POPG = 1/1.

In order to investigate the role of the phase state of the lipids in the membrane, experiments were performed with vesicles composed of DPPC, which show an ordered ↔ fluid phase transition at $T_m = 41$ °C. In contrast to the fluid bilayers of POPC, at room temperature DPPC membranes are in an ordered state in which the lipid acyl chains form a densely packed two-dimensional crystal. Under these conditions, one would expect the membrane insertion of proteins to be considerably hindered. Indeed, no association with preformed DPPC vesicles below the phase transition temperature could be measured. In a control experiment, a mixed solution of M13 coat protein, DPPC, and DPH-PC in organic solvent was injected into buffer. During this procedure, the protein is thought to incorporate in the membrane at the moment of bilayer formation, and thus does not need to penetrate the bilayer from the water phase. This experiment yielded vesicles in which the M13 coat protein was incorporated into the lipid bilayer, as judged by a marked decrease in the Trp fluorescence and a concomitant increase in the DPH fluorescence which was comparable to the changes observed in analogous experiments performed with POPC vesicles as reported before. These results show that M13 coat protein needs a fluid bilayer to be able to spontaneously insert into membranes from the aqueous phase.

The topology of the membrane-bound proteins was analyzed by protease digestion experiments. The proteolytic fragments were separated by SDS−PAGE and probed for the presence of the intact mature N-terminal coat sequence by binding of an antibody against this protein sequence, followed by an immunoblot assay. After binding of procoat protein to preformed vesicles, proteinase K was added to the outside. We found that 20% of the protein was protected

Table 1: Apparent Partition Coefficients (Γ_{app}) and Values for the Free Energy of Transfer ($\Delta G°$) of Coat and Procoat Proteins from Water to Different Lipid Membranes at 23 °C[a]

protein	lipid	$\Gamma_{app} \times 10^{-5}$ M^{-1}	$-\Delta G°$ (kcal/mol)
H5 procoat protein	POPC	6.5	10.4
	POPC/POPG, 1/1	>10	>10.7
OM30R procoat protein	POPC	0.3	8.6
M13 coat protein	POPC	1.0	9.3
	POPC/POPG, 1/1	3.5	10.1
	DPPC	no spontaneous insertion	

[a] The partition coefficients Γ_{app} are defined using the protein/lipid molar ratio as the concentration variable in the membrane. Following the considerations of Tanford (1980), the standard free energy change upon transfer of the protein from water to the lipid bilayer is calculated as $\Delta G° = -RT \ln \gamma_{MF}$ using the mole fraction partition coefficient γ_{MF} which for sufficiently high dilution of the proteins in the membranes is given by $\gamma_{MF} = \Gamma_{app}/V_W$ with the partial molar volume of water $\bar{V}_W = 18$ mL/mol[3]. This form has been recently applied for the calculation of the partitioning of small peptides to lipid bilayers (Terzi et al., 1994).

from degradation (data not shown). When the vesicles were first dissolved by a detergent solution of 1% octyl glucoside and subsequently treated with proteinase, the procoat protein was entirely digested. We conclude from this experiment that a proportion of the inserted procoat protein is transmembrane (i.e., with the mature coat N-terminus in the lumen of the vesicles) with the remainder not being translocated (i.e., with the mature N-terminal segment located on the vesicle surface and thus accessible to the proteinase K). In the case of coat protein bound to preformed lipid vesicles, the N-termini of all molecules were accessible to proteinase K digestion from the outside. We cannot exclude membrane translocation of the C-termini, although it appears to be unlikely that four positive lysine residues would cross the lipid bilayer.[3]

Using OM30R protein, the procoat mutant protein containing a positively charged amino acid (position 30) in the center of the hydrophobic segment (amino acids 21−39) of the mature region, it is possible to identify the contribution of this particular segment to the overall free energy change of the M13 procoat protein interaction with the lipid membranes. The partition coefficient of OM30R procoat mutant protein was $\Gamma_{app} = 2.6 \times 10^4$ M^{-1}, more than an order of magnitude lower than that of H5 procoat protein.

DISCUSSION

In the present work, the membrane insertion process of the M13 procoat protein is analyzed thermodynamically, focusing on three aspects: the spontaneous membrane insertion of the procoat and coat proteins; the effect of membrane surface charges; and, finally, the effect of point mutations in the mature coat protein segment in order to distinguish from thermodynamic data between transmembrane and interfacial insertion.

(i) Spontaneous Membrane Insertion of M13 (Pro-)Coat Proteins. Both proteins associate to lipid vesicles comprising bilayers in a fluid lipid state but not to those which are composed of ordered lipid membranes such as DPPC at room temperature. Interestingly, the binding of both proteins to fluid lipid membranes can be described within experimental accuracy by an ideal partitioning of the particular protein between the aqueous and the membrane phase. Neither aggregation nor electrostatic protein−protein interaction phenomena are observed as is shown by the corresponding linear partition isotherms obtained by FET measurements. The partition coefficients for pure POPC membranes are clearly higher for procoat than for coat proteins under identical conditions, but still of the same order of magnitude.

For the determination of the partition coefficients in the present paper, a given protein was mixed with lipid vesicles with which it spontaneously associates. In principle, the protein might either integrate only into the interfacial region of the membrane or insert into the lipid bilayer in a transmembrane conformation. A further mechanistic interpretation of the partition coefficients demands knowledge of the structure of the membrane-assembled (pro-)coat proteins obtained under the present experimental conditions.

With this in mind, the topology of the procoat protein bound to preformed vesicles was analyzed by the subsequent addition of proteinase K to the outside. It was found that 20% of the procoat protein was protected by the vesicles and could only be digested when detergent was added prior to the proteinase treatment. This strongly suggests that a fraction of the procoat protein molecules inserts into the bilayer by forming two transmembrane segments, with the loop region translocated across the bilayer. The translocation efficiency of 20% corresponds to earlier *in vitro* procoat expression experiments where the insertion into *E. coli* membrane vesicles was tested (Wickner et al., 1978; Silver et al., 1981; Ohno-Iwashita & Wickner, 1983; Geller & Wickner, 1985). Our finding in this respect is important because it demonstrates that our isolated H5 procoat mutant protein behaves in the reconstitution experiments in an identical manner to the wild-type protein.

Biochemical experiments have shown that membrane insertion of the procoat protein requires the electrochemical membrane potential (Date et al., 1980). Recently, it was demonstrated that the electrochemical potential promotes membrane translocation if negatively charged residues are present in the translocated protein domain (Cao et al., 1995). In the absence of the electrochemical potential, however, the translocation of the wild-type procoat protein was only partially inhibited, suggesting that there is no absolute requirement for a membrane potential. This might explain why procoat protein can to a certain extent spontaneously integrate into and translocate a lipid vesicle membrane. In addition, membrane insertion into lipid vesicles might be different from the *in vivo* situation.

In the case of coat protein, the digestion experiments performed with proteinase K show that the N-termini of all protein molecules are accessible, indicating an insertion of

[3] A detailed mass spectroscopic investigation of the proteolytically digested proteins is presently being performed in our laboratories. Preliminary experiments indicate that even in detergent solution, the hydrophobic central segment in the sequence of the coat protein is protected against proteolysis.

coat protein only into the membrane interfacial region (if one excludes the energetically unfavorable membrane translocation of the positively charged C-terminus). In contrast, the hydrophobic parts of the coat and the procoat proteins span the lipid bilayer in a transmembrane helical conformation when the proteins are reconstituted into artificial lipid bilayers by the mixed injection method; this is the conclusion drawn from our present digestion experiments and recent biophysical investigations (Thiaudière et al., 1993).

Obviously, the two different reconstitution protocols applied in the present work (protein binding to preformed vesicles versus protein binding during vesicle formation) result in differing membrane insertion of the two proteins. It is difficult to predict the partition coefficients for the two cases. The contribution of the hydrophobic effect to the standard free energy change $\Delta G°$, which occurs during the transfer of a protein from water to the membrane phase (either by insertion into the membrane interfacial region or into a transbilayer form), is directly proportional to the surface of the hydrophobic part of the protein in contact with the lipid membrane.[4] In the present work, one has to compare the situation of the (pro-)coat proteins inserted into the interfacial region of a lipid bilayer with the transbilayer configuration of procoat protein. As discussed in detail by White and colleagues (Jacobs & White, 1989; White & Wimley, 1994), the interfacial region of a lipid bilayer, which actually accounts for about 50% of the total time-averaged thickness of the membrane, is large enough to accommodate a helical protein oriented parallel to the membrane plane.

As described in detail elsewhere (Engelman & Steitz, 1981; Jähnig, 1983; Engelman et al., 1986; Lemmon & Engelman, 1994), the formation of a hydrophobic transmembrane helix can be thermodynamically described by the standard free energy change, $\Delta G°$, for the insertion of this α-helix into a lipid bilayer. The overall driving force for the insertion is the hydrophobic effect. But, in addition, unfavorable terms must be considered, for example, the fact that part of the helix surface is polar. Even in the case where only hydrophobic amino acid side chains are involved, negative entropic terms arise from the restricted mobility of the polypeptide in the membrane and the effect of disturbing the lipid bilayer itself. Of course, all these effects also hold for the insertion of a protein into the interfacial region of a lipid bilayer. However, one has to take into account that the contribution of the hydrophobic interactions might be different in the transmembrane form compared to that of the interfacial membrane form.

Taking the arguments from the work of Jähnig (1983), an estimated value of $-\Delta G°$ for the partitioning of a transmembrane hydrophobic α-helix of 15 (procoat leader helix segment) or 19 (mature coat helix segment) amino acids would be between 10 and 20 kcal/mol for the leader sequence helix and 12−24 kcal/mol for the coat protein helix, depending on the applied scale for the strength of the hydrophobic effect.

In order to compare these values with our actual experimental data, we have furthermore to consider the following. If the procoat protein spans the lipid bilayer with two helices, which due to their close neighborhood are already associated, then the actual hydrophobic surface of the helices in contact with the lipid hydrocarbon core of the membrane is only about 30−40% larger than in the case of a single, transbilayer helix. In this respect, the structure of the membrane-inserted part of the procoat protein seems to be similar to that of leader peptidase, where about 30% of the surface areas of two adjacent transmembrane helices are in direct contact, leaving 70% of the total helix surface area to interact with the lipid hydrocarbon chains (Whitley et al., 1993). In addition, it should be considered that for procoat protein, the hydrophobic mature part and the leader sequence contribute differently to $\Delta G°$ of the membrane insertion process. This is actually demonstrated by preliminary experiments performed with chemically synthesized M13 leader peptide. Surprisingly, the peptide does not incorporate to a detectable extent into electrically neutral POPC membranes but shows only electrostatic binding to the surface of negatively charged lipid membranes of POPC/POPG (see also below). A detailed analysis of this effect will be published in a forthcoming paper.

On the basis of these considerations, it is now possible to estimate the $(\Delta G°)_{tm}$, the standard free energy change for the transmembrane insertion of procoat protein. Here we assume that the experimentally determined $\Delta G°$ of −10.4 kcal/mol for the binding of procoat protein to POPC vesicles is composed of 20% by transmembrane insertion and 80% by interfacial membrane insertion. For the latter case, we furthermore assume no contribution from the leader sequence; i.e., we can adopt the experimental value of $\Delta G° = -9.3$ kcal/mol for the coat protein binding to the POPC vesicles. Simple algebra yields $(\Delta G°)_{tm} = -14.8$ kcal/mol. [A realistic uncertainty of 20 ± 5% in the fraction of membrane-translocated procoat protein would give a range of −13.7 to −16.6 kcal/mol for $(\Delta G°)_{tm}$.] This estimated value of $(\Delta G°)_{tm}$ corresponds to an upper limit because a possible contribution of the leader sequence to membrane binding would necessarily decrease $(\Delta G°)_{tm}$. If one furthermore takes into account the shielding effect of neighboring transmembrane helices as discussed above, the $(\Delta G°)_{tm}$ of an individual coat protein would be about −12 kcal/mol, i.e., an additional contribution of only −2.7 kcal/mol for the transmembrane insertion compared to the interfacial insertion. Our results imply that the major part of the free energy

[4] At present, there are several published propositions for the calculation of the thermodynamic parameters of membrane partitioning processes. Tanford (1980) suggested the use of mole fraction (MF) based partition coefficients, γ_{MF}, to calculate the free energy change, $\Delta G° = -RT \ln \gamma_{MF}$, for the transfer of a substance from water to a lipid bilayer. In this way, the cratic contribution arising from the entropy of mixing in the partition equilibrium is considered as in the case of partitioning between two bulk phases. However, recent papers suggested that Flory−Huggins-corrected (FH) volume fraction units are more appropriate (Sharp et al., 1991; White & Wimley, 1994): $\Delta G° = -RT \ln \gamma_v + RTV_S(1/\bar{V}_L - 1/\bar{V}_W)$. Here γ_v is the volume fraction partition coefficient and $\bar{V}_S$, $\bar{V}_L$, and $\bar{V}_W$ are the partial molar volumes of solute, lipid bilayer, and water, respectively. As pointed out by White and Wimley (1994), the critical point in membrane partitioning is how to consider the cratic entropy contributions. MF units assume that solute and solvent molecules have the same molecular volumes, which certainly is not the case for the incorporation of proteins into a lipid bilayer membrane. The Flory−Huggins correction term takes this difference into account. In the case of membrane insertion of proteins, the FH correction term is considerably higher than expected from MF units. For example, in the case of a 19 amino acid long hydrophobic α-helix inserted into a lipid bilayer, the second term in the FH calculation is about 50 kcal/mol which is about 5 times larger than the MF term. The applicability of the FH formalism for protein insertion into membranes is still under debate (Holtzer, 1992; White & Wimley, 1994). In our present paper, we calculate $\Delta G°$ values on the basis of simple MF units which, however, does not influence the general conclusions drawn from our results.

change during membrane interaction occurs already at the membrane interface. It is the additional energy gain from the interaction of the hydrophobic portion of the folded preprotein with the lipid chains which drives the final transmembrane insertion of the M13 procoat protein. Neither the leader sequence nor the mature coat protein alone yields this free energy gain. This model is in accord with propositions made by Jacobs and White (1989) for the insertion of proteins into the membrane interfacial region, based on binding experiments of tripeptides to lipid bilayers. Our values of $-\Delta G°$ are the first experimental data for transmembrane proteins that have a signal sequence, and they are on the lower limit of the proposed estimates of Engelman and Steitz (1981) and Jähnig (1983).

In this context, it is quite interesting to compare our data with published ones of other membrane-associated proteins. Our experimentally determined partition coefficients Γ for the M13 coat and the H5 procoat proteins are more than 1−2 orders of magnitude larger than those published for small (about 20−30 amino acids), amphipathic membrane-active polypeptides which show typical values in the range of $\Gamma = 10^3-10^4$ M^{-1} corresponding to $-\Delta G° = 6.5-7.9$ kcal/mol (Schwarz et al., 1986; Schwarz & Beschiaschvili, 1989; Peled & Shai, 1993; Ben-Efraim et al., 1994).[5] Recently, the membrane association of a semisynthetic protein was investigated (Moll & Thompson, 1994): The water-soluble bovine pancreatic trypsin inhibitor was attached to a hydrophobic Ala_{20} peptide segment, which in the membrane-bound form was assumed to adopt a transmembrane helix. The partition coefficient of this artificial protein to lipid vesicles was determined as $\Gamma = (2-3) \times 10^3$ M^{-1} (value recalculated in order to have identical dimensions as the data in the present paper). A considerably higher value of $\Gamma = 1.5 \times 10^9$ M^{-1} ($-\Delta G° = 15$ kcal/mol) was determined for template-assembled melittin, which is supposed to span the lipid membrane by four helical segments (Pawlak et al., 1994b). The partitioning of procoat protein to lipid bilayers is distinctly higher compared to simple amphipathic helical polypeptides and lower than that of a four-transmembrane helix protein.

It is interesting to note in this context a recent paper by Lee and Manoil (1994). The authors showed that mutations eliminating the protein export function of a membrane-spanning sequence can be explained by a model where a minimum hydrophobicity is required for membrane insertion.

(ii) Dependence of Membrane Insertion on Negative Membrane Surface Charges. The apparent partition coefficient for coat and procoat proteins is larger when net negative charges are introduced to the surface of the membrane as shown for mixed POPC/POPG membranes, but it is still of the same order of magnitude as for POPC membranes. This can be explained qualitatively by a preferential electrostatic interaction between the positively charged amino acid residues of the protein and the negative charges of POPG. Such an interaction would lead to an increased concentration of the corresponding protein near the membrane surface. For a qualitative discussion of this electrostatic effect, we assume that the intrinsic coefficient Γ for the protein partitioning to POPC and mixed POPC/POPG membranes is identical. In the presence of a surface potential Ψ_o, induced by negatively charged lipids, the increase (decrease) of the free protein concentration at the membrane surface is determined by the protein's effective positive (negative) net charge. For POPC/POPG = 1/1 (mol/mol) mixed vesicles, the membrane surface potential, Ψ_0, exerted by the negative charges of the POPG molecules can be calculated for the experimental buffer conditions (1 mM Tris-HCl) using the Gouy−Chapman theory (Träuble et al., 1976; McLaughlin, 1977) which gives $\Psi_0 \approx -200$ mV.

It is the Ψ_0 of the membrane and the effective positive charge z_{eff} of the protein which determine the concentration of the protein at the membrane surface. Under these considerations, the following relationship between the apparent and the intrinsic partition coefficients of the protein holds [see Swanson and Roise (1992) and Roise (1993) and references cited therein]:

$$\Gamma_{app} = \Gamma \exp(-z_{eff} e_o \Psi_o / kT) \qquad (5)$$

with e_o the elementary electrical charge and k the Boltzmann constant. The measured Γ_{app} yields a formal (effective) positive charge of $z_{eff} \approx 0.2$ for the M13 coat protein. The protein has one positive and four negative charges at the hydrophilic N-terminal segment and a further four positive charges at the hydrophilic C-terminal segment. It should be noted that, according to eq 5, one effective positive charge on the protein increases the partitioning by a factor of $\Gamma_{app}/\Gamma \approx 3 \times 10^3$. Although, formally, only one positive excess charge is present in the coat protein at neutral pH, the asymmetrical distribution of the negative and positive charges creates a rather high charge density at the hydrophilic protein termini which might play a role in the organization of the protein on and in the lipid bilayer. The present experiments indicate that only 0.2 positive charge is effective during the membrane association of M13 coat protein. There are at least two possible explanations why the electrostatic effects are small in our experiments. First, in the case of relative large proteins, the finite size of the molecule might reduce the formal charges considerably as has been shown theoretically (Stankowski, 1991) and experimentally for other polypeptides (Schwarz & Beschiaschvili, 1989) and for synthetic proteins [Pawlak et al. (1994b) and references cited therein]. Second, the M13 coat protein might form, both in water and in the lipid membrane-bound state, a head-to-tail dimer matching the negative and positive charges at the opposite termini. The existence of a protein dimer would result in linear association isotherms (Figure 4) as for monomers.

Indeed, stable dimers of M13 coat protein as well as certain mutant coat proteins have recently been observed by Deber et al. (1993). Whether such structures are present in our reconstitution system will be a topic of future investigation. At least under *in vivo* conditions the aggregation of coat proteins in the membrane must occur during the phage assembly process (Marvin, 1989; Russel, 1991).

[5] The partition coefficients of Jones and Gierasch (1994) cannot directly be compared to our values because these authors apply a different description of the binding of the polypeptides to lipid membranes. Their values of partition coefficients, even for polypeptides with only 12 consecutive hydrophobic amino acids, are orders of magnitude higher than all other published values for either amphipathic or hydrophobic polypeptides of comparable structure. We assume that this is due to the fact that the evaluation procedure of Jones and Gierasch (1994) does not allow to distinguish between electrostatic binding and hydrophobic partitioning of the polypeptides.

The influence of electrical surface charges in the case of membrane insertion of procoat protein is less clear than for coat protein, but the simulation seems to be similar to that of the coat protein. The partition coefficient of procoat protein for POPC/POPG membranes is higher than 10^6 M^{-1} compared to 6.5×10^5 M^{-1} in POPC, which corresponds to a $z_{eff} \geq 0.06$. Unfortunately, it was not possible under the present experimental conditions to determine $\Delta G°$ more precisely because preparations at low L/P molar ratios always resulted in highly turbid samples. Similar difficulties arose if buffers at higher ionic strength than 5 mM were used, both for procoat and for coat proteins, because, under such conditions, the proteins start to aggregate in the aqueous phase.

In contrast to this relatively small electrostatic effect in the case of M13 (pro-)coat proteins, large contributions of charged residues to membrane binding were recently observed in the case of *E. coli* LamB signal sequence peptides (Jones & Gierasch, 1994). It is interesting to note in this context that preliminary measurements with synthetic M13 leader sequence peptide performed in our laboratory show a strong electrostatic binding to negatively-charged lipid membrane surfaces but no interaction with electrically-neutral POPC membranes. This finding is consistent with the observation that the arginine mutation in the hydrophobic segment of the leader sequence has only a weak effect in the transmembrane insertion of procoat protein (Kuhn et al., 1986b). Further, the minor contribution of the leader sequence is also evident if one compares the conformation and solubility of the leader sequence peptide (−1 to −23) with those of the M13 coat protein. Shinnar and Kaiser (1984) have shown that the synthetic M13 leader sequence peptide has a remarkable solubility in water, up to 1 mg/mL. At a concentration of 4×10^{-5} M in 0.02 M phosphate buffer of pH 2.8, the M13 leader sequence peptide adopts a nonregular structure, according to CD measurements. In contrast, M13 coat protein is much less soluble in water, and even at a concentration of 10^{-7} M in aqueous buffer, the protein shows a considerable helix content of 50% (Thiaudière et al., 1993). Taken together, these findings indicate that the isolated signal peptide behaves differently from the entire procoat protein. In consequence, one should be cautious to draw conclusions on biological functions of newly synthesized proteins based simply on results obtained from experiments with isolated leader peptides. The properties of an entire pre-protein seem not in every respect to be simply a sum of the properties of the mature protein and the leader sequence itself.

(iii) Membrane Association of OM30R Mutant Procoat Protein. The OM30R mutant protein, with an additional positively charged amino acid residue within the hydrophobic mature coat protein segment, shows a distinctly lower partitioning ($\Gamma_{app} = 0.3 \times 10^5$ M^{-1}; $\Delta G° = -8.6$ kcal/mol) for POPC membranes than the H5 procoat protein ($\Gamma_{app} = 6.5 \times 10^5$ M^{-1}; $\Delta G° = -10.4$ kcal/mol). Clearly, the hydrophobic mature part plays an important role for insertion of the procoat protein into the lipid bilayer. Yet, the affinity to lipid bilayers of the OM30R mutant protein is still relatively high. This is in total agreement with *in vivo* experiments from which it is known that the OM30R mutant protein is not translocated across the *E. coli* plasma membrane, but still interacts with and binds to these membranes (Kuhn et al., 1986b, 1990). According to the arguments presented before, i.e., that the leader sequence does not play an important role for interfacial membrane insertion, one should compare the $\Delta G°$ values of OM30R and coat protein. Clearly, a positive charge in the central hydrophobic segment decreases the affinity for interfacial membrane insertion, although only to a relatively small extent. Therefore, the major barrier for preventing a transmembrane structure in the case of OM30R seems to be the energetically unfavorable insertion of a positive charge into the center of a lipid bilayer.

In conclusion, we believe that the presented procedure might be generally applicable to probe the membrane insertion process of hydrophobic proteins into lipid bilayers. The thermodynamic data obtained are independent of the particular structure of the protein at or in the lipid membrane. They hold for a partitioning of a protein to the polar lipid headgroup region, which has been shown to establish about 50% of a lipid bilayer (White & Wimeley, 1994), as well as for a real transmembrane insertion. In the case of M13 procoat protein, future experiments are necessary to reveal details of how an electrochemical membrane potential influences the membrane insertion, helix formation, and membrane translocation of the protein.

Partitioning on the membrane interfacial region might also be an important first step of membrane interaction of proteins which are imported into or translocated across membranes by a more complex signal−receptor mechanism. A first partitioning of such a protein to the membrane surface region would increase the efficiency to recognize a corresponding receptor and translocation machinery on the membrane level as was proposed as a possible model for hormone−receptor interactions in the case of membrane neuroreceptors (Schwyzer, 1977; Sargent & Schwyzer, 1986).

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