Binding of Acylated Peptides and Fatty Acids to Phospholipid Vesicles: Pertinence to Myristoylated Proteins[†]

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ABSTRACT: We studied the binding of fatty acids and acylated peptides to phospholipid vesicles by making electrophoretic mobility and equilibrium dialysis measurements. The binding energies of the anionic form of the fatty acids and the corresponding acylated glycines were identical; the energies increased by 0.8 kcal/mol per number of carbons in the acyl chain (N_{carbon} = 10, 12, 14, 16), a value identical to that for the classical entropy-driven hydrophobic effect discussed by Tanford [*The Hydrophobic Effect* (1980) Wiley, New York]. The unitary Gibbs free binding energy, ΔG_u°, of myristoylated glycine, 8 kcal/mol, is independent of the nature of the electrically neutral lipids used to form the vesicles. Similar binding energies were obtained with other myristoylated peptides (e.g., Gly-Ala, Gly-Ala-Ala). The 8 kcal/mol, which corresponds to an effective dissociation constant of 10^{-4} M for myristoylated peptides with lipids, provides barely enough energy to attach a myristoylated protein in the cytoplasm to the plasma membrane. Thus, other factors that reduce (e.g., hydrophobic interaction of myristate with the covalently attached protein) or enhance (e.g., electrostatic interactions of basic residues with acidic lipids; protein–protein interactions with intrinsic receptor proteins) the interaction of myristoylated proteins with membranes are likely to be important and may cause reversible translocation of these proteins to the membrane. Finally, our results suggest that the mass-dependent entropy price paid by a molecule when it binds to a membrane and loses one translational and two rotational degrees of freedom is small: the membrane binding energy we measure for the neutral form of myristic acid, 12 kcal/mol, agrees with the value predicted from Tanford's measurements of the partitioning of the neutral form of fatty acids between water and a bulk organic phase (14 × 0.825 = 12 kcal/mol).

Numerous proteins have myristate chains attached covalently via an amide bond to their NH_2-terminal Gly[1] residues (Sefton & Buss, 1987; Towler et al., 1988; Gordon et al., 1991; Schmidt, 1989; Schultz et al., 1988; Simon & Aderem, 1992). For many of these proteins, the myristate chain is required for membrane binding, and membrane binding is required for proper cellular function. For example, when the NH_2-terminal Gly residue of pp60[v-src] is mutated to Ala, the protein is not myristoylated and it neither binds to membranes nor transforms cells, even though its tryosine kinase activity is unaffected (Kamps et al., 1985; Buss et al., 1986). Although the myristate chain is necessary for the attachment of many of these proteins to membranes, it often is not sufficient. For example, binding of the myristoylated alanine-rich C kinase substrate (MARCKS) protein to membranes can be reversed in many cells by PKC phosphorylation (Wang et al., 1989; Thelen et al., 1991; Sawai et al., 1993), binding of recoverin to membranes requires calcium ions (Dizhoor et al., 1992,

1993), and binding of ADP ribosylation factor (ARF) requires GTP (Serafini et al., 1991; Haun et al., 1993; Randazzo et al., 1993).

It clearly would be useful to know how strongly the myristate group attaches these proteins to the bilayer component of the biological membrane, but it is very difficult to answer this question by measuring the binding of myristoylated proteins to membranes, even simple phospholipid membranes. If, for example, the acyl chain folds back and binds to the protein, which appears to be the case for recoverin in the absence of calcium ions (Dizhoor et al., 1993), binding measurements would underestimate the membrane binding energy of the myristate. Alternatively, if basic residues on the protein interact with acidic lipids, which may be the case with the MARCKS protein (Kim et al., 1993), or if hydrophobic residues on the protein insert into the bilayer, which may be the case with the ARF protein (Kahn et al., 1992), binding measurements would overestimate the membrane binding energy of myristate. Thus, we studied the binding of simple acylated peptides to phospholipid vesicles. We used short hydrophilic peptides to rule out the possibility that the myristate chain interacts hydrophobically with the covalently attached peptide or that the peptide itself binds hydrophobically to the membrane. We used membranes formed from electrically neutral lipids to avoid any electrostatic attraction or repulsion of the peptides from the surface.

We know of no systematic study of the binding of acylated peptides to phospholipid vesicles, but there are several reports on the interaction of fatty acids with these vesicles (Ptak et al., 1980; Doody et al., 1980; Rooney et al., 1983; Hamilton & Cistola, 1986; Miyazaki et al., 1992; Anel et al., 1993). In an elegant study of the binding of both the neutral HA and

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[1] Abbreviations: Ala, alanine; ADP, adenosine diphosphate; ARF, ADP ribosylation factor; ^{14}C-PC, 1,2-di[1-^{14}C]oleoyl-L-3-phosphatidylcholine; CoA, coenzyme A; DTAB, dodecyltrimethylammonium bromide; ESR, electron spin resonance; Gly, glycine; GTP, guanosine triphosphate; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; ^3H-Myr-CoA, [9,10-$^3H_2$]myristoyl coenzyme A; ^3H-Myr, [9,10-$^3H_2$]myristic acid; MARCKS, myristoylated alanine-rich C kinase substrate; Myr, myristic acid; Myr-CoA, myristoylated coenzyme A; PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TTAB tetradecyltrimethylammonium bromide.

acidic A⁻ forms of a spin-labeled myristic acid to PC vesicles, Miyazaki et al. (1992) showed that HA binds about 100-fold more strongly than A⁻, an observation consistent (microscopic reversibility) with the observation that the surface pK is about 2 units higher than the bulk pK (Ptak et al., 1980). Thus, one cannot simply extrapolate studies with fatty acids to acylated peptides and myristoylated proteins because it is not clear whether one should use the partition coefficients for the HA or the A⁻ form of the fatty acids.

We report here the first systematic study of the binding of the A⁻ form of saturated fatty acids and acylated glycines (N_{carbon} = 10, 12, 14, 16) to lipid vesicles; we determined the binding from electrophoretic mobility (zeta potential) measurements. We also measured the binding of both the A⁻ and HA forms of myristic acid directly using equilibrium dialysis. We studied the interaction of several other myristoylated peptides with membranes to ensure that the binding did not depend on the structure of the small hydrophilic peptide, and we measured the binding of other myristoylated compounds (e.g., coenzyme A, tetradecyltrimethylammonium) to test our hypothesis that binding is independent of the charge and chemical nature of the polar end of the molecule. Finally, we studied the binding of myristoylated glycine to vesicles formed from mixtures of electrically neutral lipids to examine the dependence of binding on lipid composition.

MATERIALS AND METHODS

Materials. We purchased 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), egg PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), and sphingomyelin (SM) from Avanti Polar Lipids (Birmingham, AL). 1,2-di-[1-¹⁴C]oleoyl-l-3-phosphatidylcholine (¹⁴C-PC) was from Amersham (Arlington Heights, IL), and the fatty acids, capric (Cap), lauric (Lau), myristic (Myr), and palmitic (Pal) acids, were from both Sigma (St. Louis, MO) and NuChek Prep (Elysian, MN). Fatty acids from these two sources gave identical results. Myristoleic (14:1) acid was obtained from NuChek Prep. [9,10-³H₂]myristic acid (³H-Myr) was purchased from DuPont/NEN (Boston, MA). The acylated glycines, Cap-Gly, Lau-Gly, Myr-Gly, and Pal-Gly, were obtained from AminoTech. Myr-Gly-Ala and Myr-Gly-Ala-Ala, purity >99% by amino acid analysis and analytical HPLC, were synthesized by the Center for the Analysis and Synthesis of Macromolecules at SUNY—Stony Brook (supported by NIH Grant RP02427 and the Center for Biotechnology). [9,10-³H₂]myristoyl coenzyme A (Myr-CoA) was obtained from American Radiolabeled Chemicals (St. Louis, MO); dodecyltrimethylammonium bromide (DTAB), sodium dodecyl sulfate (SDS), and tetradecyltrimethylammonium bromide (TTAB) were from Sigma. Monoolein and cholesterol were from NuChek Prep, Tris Ultra Pure was from Calbiochem (La Jolla, CA); MOPS was from Pharmacia (Piscataway, NJ), Spectra/Por 2 dialysis tubing was from Fisher Scientific (Pittsburgh, PA), and 50-mL Teflon bottles with screwcaps were from Whatman (Hillsboro, OR).

Zeta Potential Measurements. We formed PC MLVs by drying a chloroform solution of lipids in a rotary evaporator, hydrating the lipids in a buffered salt solution, and then vortexing for 1 min (Bangham et al., 1974). We measured the electrophoretic mobility using a Rank Bros. Mark I instrument (Bottisham, Cambridge, UK), as described in detail elsewhere (Cafiso et al., 1989; McLaughlin et al., 1981). At least 20 measurements in two separate experiments were averaged for each data point in Figure 6. All lipids used for the electrophoretic mobility measurements were in the liquid-crystalline state. The concentrations of lipids were sufficiently

low that no significant loss of fatty acids or acylated compounds occurred upon addition of vesicles. In some experiments it was necessary to preequilibrate the electrophoresis tube and electrodes with the fatty acid or myristoylated compound before determining the electrophoretic mobility. We show an example of one set of these results in Appendix I and briefly explain how we calculated a partition coefficient from these electrophoretic mobility (zeta potential) measurements.

Large Unilamellar Vesicles (LUVs). We dried a mixture of PC and ¹⁴C-PC with a rotary evaporator and then hydrated the lipids in the solution used for the dialysis measurements: 100 mM KCl with 1 mM MOPS (pH ≤ 7) or 1 mM Tris (pH ≥ 8). Using the procedure of Hope et al. (1985), we prepared LUVs by vortexing the lipid solution for 1 min and then taking the resulting multilamellar vesicles (MLVs) through five cycles of freezing (liquid N₂) and thawing (40 °C water bath) followed by 10 extrusion cycles through a stack of two polycarbonate filters (0.1 μm diameter pore size) using a Lipex Biomembranes Extruder (Vancouver, BC, Canada). We determined the postextrusion PC concentration by comparing pre- and postextrusion ¹⁴C-PC readings using a Beckman LS 3801 scintillation counter; typical losses during extrusion were ≤20%.

Equilibrium Dialysis Measurements. A 0.5-mL sample of LUVs was placed in a flaccid sac of dialysis tubing, which was sealed by knotting the ends. The sealed sac was washed in the appropriate buffer solution and then placed in a Teflon bottle containing 20 mL of a ³H-Myr/Myr (or ³H-Myr-CoA/Myr-CoA) solution. The [³H-Myr] was <0.01[Myr], which was added to the solution to minimize the loss of ³H-Myr onto the container and dialysis bag; loss was typically <5%. (Preliminary experiments with different materials revealed that the loss of fatty acid was less for Teflon than for Pyrex glass, polyallomer, cellulose, polycarbonate, and stainless steel. To minimize the loss onto Teflon, we used relatively large volumes, 20 mL, to minimize the surface/volume ratio.) The sealed Teflon bottle was placed on a rocker plate at 22 °C for 18–22 h, sufficient time for myristic acid to equilibrate across the sac.

We determined the concentrations of [³H]myristic acid (or ³H-Myr-CoA) in samples from the bath and the dialysis sac by liquid scintillation counting. We determined the lipid concentration in the samples from both the ¹⁴C radioactivity and a phosphorus assay [a scaled-down version of the Lowry and Tinsley (1974) method]; in general, the two assays gave the same lipid concentration. We calculated the fraction of myristic acid bound to the LUVs (f_{bound}) from f_{bound} = 1 – ([³H$_{bath}$]/[³H$_{sac}$]). The leakage of lipid from the sac into the bath was negligible, but the percent lipid lost to the cellulose tubing increased as the lipid concentration decreased. Typically, the loss was 10%, but could range as high as 50% at the lowest lipid concentrations. Our calculation of f_{bound} is based on the lipid concentration in the sac at the end of the dialysis experiment, so that these losses should not affect the determination of the binding constant.

We selected myristic acid concentrations (10⁻⁷ or 10⁻⁶ M) to keep the number of bound myristic acid molecules low (≤1%) in comparison to the number of lipid molecules (see Appendix I). This prevented the accumulation of any appreciable surface charge, which would affect the binding.

Equations Used To Describe the Partitioning onto Membranes. Equation 1 defines a molar partition coefficient (Tamm, 1991), K_1, which is the proportionality factor between the mole fraction, χ, of the fatty acid or acylated peptide, P, that is bound to the membrane and the concentration (M) of

P, [P], in the bulk aqueous phase:

$$\chi = K_1[P] \tag{1}$$

where $\chi = [P]_m/[L]$, $[P]_m$ is the concentration (M) of peptide that is bound to the lipid membrane, and [L] is the concentration (M) of lipid that is *accessible* to the acylated peptide (or fatty acid). (For the experiments illustrated in Figure 7, the membranes are permeable to myristic acid and [L] is the total concentration of lipid in the dialysis bag. If the membranes are impermeable to the adsorbing compound, then [L] is the concentration of the lipid on the outer surface of the LUVs.) Note that K_1 has units of M^{-1} and that eq 1 may be written as $[P]_m = K_1[P][L]$. Thus, eq 1 has the same form as the limiting version of a mass action equation that assumes (incorrectly) the acylated peptide (P) forms a 1:1 complex with a lipid (L) with an association constant, K_1.

We can also define a partition coefficient, K_2, that relates the surface concentration of the peptide (number of peptides adsorbed to a unit area of the membrane's outer leaflet), {P}, to its concentration in the bulk aqueous phase, [P]:

$$\{P\} = K_2[P] \tag{2}$$

K_2 has units of distance. If a phospholipid occupies an area of 0.7 nm² (Huang & Mason, 1978), $K_1 = 420K_2$ when K_1 is expressed in M^{-1} and K_2 is in μm.

A dimensionless partition coefficient, K_3, can be defined by

$$\{P\}/d = K_3[P] \tag{3}$$

where d is the thickness of a monolayer of lipid and $\{P\}/d$ may be regarded as the concentration of acylated peptide within the membrane phase. (In contrast, $[P]_m$ in eq 1 is the concentration of bound peptide in the volume of the container.) $K_3 = K_2/d$ and d is about 2 nm (Huang & Mason, 1978).

Finally, we define a mole fraction partition coefficient as

$$\chi = K_4\chi_w \tag{4}$$

where χ_w is the mole fraction of the acylated peptide in the aqueous solution. The change in unitary Gibbs free energy, $\Delta G_u^o = \mu_m^o - \mu_w^o$, that occurs on adsorption is given by

$$\Delta G_u^o = -RT \ln (\chi/\chi_w) = -RT \ln K_4 \tag{5}$$

Tanford (1980, p 5) and Gurney (1953) discuss the advantage of using unitary units for the free energies (mole fraction units for the solute concentrations) when describing partitioning data. The change in the standard Gibbs free energy, $\Delta G^o = -RT \ln K_1$, differs from ΔG_u^o by the cratic contribution, about 2.4 kcal/mol at 300 K [Cantor and Schimmel (1980), pp 283–285].

RESULTS

Figure 1 illustrates three of our key findings about the binding of fatty acids and acylated peptides to phosphatidylcholine (PC) vesicles. First, the binding energy of the anionic form of fatty acids increases linearly with the number of carbons in the fatty acid. These data are well described by a line with a slope of 0.825 kcal/mol per carbon, which is identical to the value for the partitioning of the neutral form of these fatty acids from water into a bulk liquid *n*-heptane phase [see Tanford's (1980) Figure 3-1 and eq 3-2]. The simplest interpretation of our results is that the binding of fatty acids to these phospholipid vesicles is due mainly to the classical hydrophobic effect discussed in detail by Tanford (1980).

Second, Figure 1 shows that the neutral or HA form of myristic acid binds 3.5 kcal/mol more strongly to the PC vesicles than does the anionic or A⁻ form. Equivalently, the
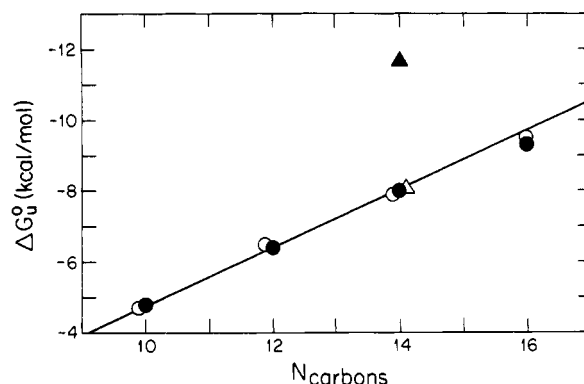

FIGURE 1: Change in the unitary Gibbs free energy, ΔG_u^o (eq 5), upon binding of fatty acids and acylated glycines to phosphatidylcholine (PC) vesicles plotted as a function of the number of carbons in the acyl chain. The data for the anionic forms of the fatty acids (O) and the acylated glycines (●) were deduced from zeta potential measurements on PC multilamellar vesicles (MLVs) similar to those illustrated in Figure 6. The binding energies of the neutral (▲) and anionic (△) forms of myristic acid were determined from the equilibrium dialysis measurements on PC large unilamellar vesicles (LUVs) illustrated in Figure 7. The line has a slope of 0.825 kcal/mol per carbon in the acyl chain.

partition coefficient of HA onto PC vesicles, K_{HA}, is 300 times that of A⁻, K_A, which qualitatively agrees with measurements of the binding of the HA and A⁻ forms of a spin-labeled myristic acid to PC vesicles (Miyazaki et al., 1992). Both ESR (Miyazaki et al., 1992) and fluorescence (Abrams et al., 1992) measurements suggest that the HA form of a fatty acid sinks more deeply into the membrane than the A⁻ form, and thus the hydrophobic binding energy is greater. A parsimonious interpretation of these results is that the Born repulsion (Parsegian, 1969) experienced by all ions adjacent to the low dielectric interior of the membrane keeps the charged carboxyl group of A⁻ outside the envelope of the polar head group of the phospholipids, whereas the neutral carboxyl moiety of HA does not experience this Born repulsion and can sink into the polar head group region of the membrane. As discussed by Miyazaki et al. (1992), other factors may also contribute to the difference between K_{HA} and K_A.

Third, Figure 1 shows that acylated glycines bind with the same energy as the A⁻ form of the homologous fatty acid. The binding energy of the acylated glycines also increases by about 0.8 kcal/mol per carbon in the acyl chain, which again suggests that the binding energy arises mainly from the insertion of the acyl chain into the bilayer (Tanford, 1980). Why is the binding energy of an acylated glycine equal to that of the A⁻ form of the corresponding fatty acid? The simplest explanation is that the glycine moiety does not penetrate the envelope of the polar head group region; thus, the hydrophobic chain on myristoylated glycine (Myr-Gly) penetrates the membrane to the same degree as the chain on the A⁻ form of myristic acid. (If this explanation is correct, about four CH_2 groups in the acyl chain of Myr-Gly would be required to span the $\approx$0.6 nm thick polar head group region.)

The binding energy of 8 kcal/mol for Myr-Gly or the A⁻ form of myristic acid corresponds to a partition coefficient of $K_1 \simeq 10^4 M^{-1}$ according to eq 1. The reciprocal of this number, $1/K_1 \simeq 10^{-4}$ M, can be regarded as an apparent dissociation constant that is equal to the lipid concentration at which one-half the A⁻ form of myristic acid in a solution is bound to membranes (e.g., Figure 7). Equivalently, $\Delta G_u^o = 8$ kcal/mol corresponds to a dimensionless partition coefficient, $K_3 \simeq 10^4$, according to eq 3, if we assume that the myristate chain partitions into one-half of a bilayer and the thickness of the monolayer is 2 nm. Finally, this binding energy
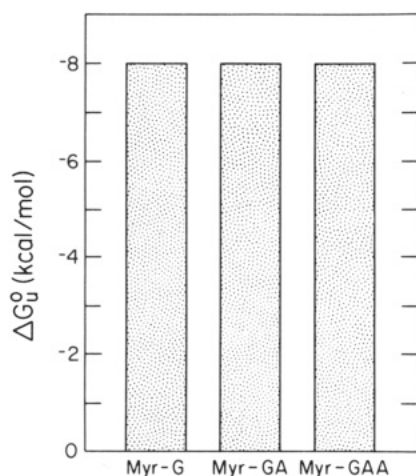
FIGURE 2: Binding energies of myristoylated peptides to PC multilamellar vesicles. The change in the unitary Gibbs free energy, ΔG_u^o, upon binding of myristoyl-Gly (Myr-G), myristoyl-Gly-Ala (Myr-GA), and myristoyl-Gly-Ala-Ala (Myr-GAA) was calculated from zeta potential measurements similar to those illustrated in Figure 6.

·FIGURE 3: Effect of lipid composition on the unitary Gibbs free binding energy, ΔG_u^o, of myristoylated glycine (Myr-G) to multilamellar phospholipid vesicles (MLVs). The binding energies of Myr-G to MLVs formed from the indicated mixtures of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and cholesterol (C) were determined using the zeta potential technique illustrated in Figure 6.

corresponds to a partition coefficient of $K_2 \simeq 20 \,\mu$m according to eq 2. (This corresponds to the distance one must move from a unit area of a membrane to obtain a volume of solution that contains the same number of peptides that are bound to the surface.) This distance is only slightly larger than that of a typical cell. As we discuss below, this 8 kcal/mol is probably the maximum a myristate chain could contribute to the membrane binding energy of a myristoylated protein. Thus, a covalently attached myristate chain provides, at best, barely enough energy to partition a protein onto the plasma membrane.

Myristoylated proteins, of course, contain amino acids besides the amino-terminal glycine residue covalently bound to myristate; the two amino acids following the terminal glycine usually are small and uncharged (Towler et al., 1988). We investigated whether adding one or two alanines to glycine would affect the binding of the myristoylated peptides. Figure 2 illustrates that the three myristoylated peptides bind to PC vesicles with identical energies.

The plasma membrane of a cell contains a mixture of lipids, not just PC. We studied the binding of myristoylated glycine to MLVs formed from mixtures of lipids that have no net charge. [Charged lipids produce surface potentials (McLaughlin, 1977, 1989) and equilibrium dialysis measurements similar to those illustrated in Figure 7 show that the binding of the A⁻ form of myristic acid to membranes containing 30% PG, an acidic lipid, is 5-fold weaker than that to electrically neutral PC membranes. This effect is consistent with the Boltzmann repulsion of A⁻ from the negatively charged vesicles.] The lipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM) are zwitterionic, and cholesterol (C) is electrically neutral. Figure 3 illustrates that Myr-Gly binds with energies of about 8 kcal/mol to MLVs formed from mixtures of these lipids. The simplest interpretation of Figure 3 is that the hydrophobic energy gained from the insertion of the myristate chain into the bilayer is essentially independent of the lipid composition of the vesicles. We also observed that the binding of Myr-Gly to dimyristoyl phosphatidylcholine vesicles is similar in the liquid-crystalline and gel states (data not shown), consistent with the fatty acid measurements of Anel et al. (1993).

The simple picture that emerges from the data in Figures 1–3 is that the negative charge on the anionic form of a fatty acid or the polar glycine residue on an acylated peptide remains
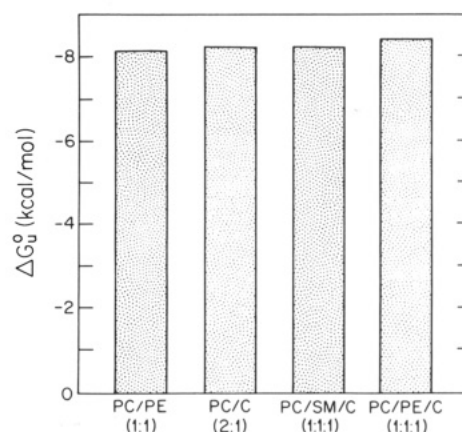
in the aqueous phase, probably immediately outside the envelope of the polar head group region, and that most of the CH_2 groups in the acyl chain penetrate the interior of the membrane: the binding energy derives mainly from the entropy-driven removal of the hydrophobic acyl chain from the aqueous solution.[2]

The charged acylated molecules we have considered so far all have a valence of −1, and the interior of a phospholipid bilayer has a large (several hundred millivolts) positive electrostatic potential (Honig et al., 1986; Flewelling & Hubbell, 1986). This potential could arise from oriented dipoles in carbonyl oxygens, water molecules, and polar head groups; the available evidence suggests that it does not extend into the aqueous phase outside the polar head group region. Thus, this potential should not affect the adsorption of charged, acylated compounds, and an acylated molecule with a net positive charge should adsorb to phospholipid membranes with about the same partition coefficient as both the A⁻ form of the corresponding fatty acid and the acylated glycine. Figure 4 illustrates a test of this prediction: DTAB (12-carbon acyl chain and a +1 charge) adsorbs to about the same degree as Lau-Gly (12-carbon acyl chain and a −1 charge), and TTAB (14-carbon acyl chain and a +1 charge) adsorbs to about the same degree as Myr-Gly (14-carbon chain and a −1 charge). Requena and Haydon (1985) reported similar results with DTAB and TTAB. Thus, the dipole potential does not appear to affect the adsorption of these acylated compounds.

The introduction of double bonds into the acyl chain decreases its hydrophobicity and its affinity for membranes. The partition coefficient of the A⁻ form of unsaturated myristoleic acid (14:1) onto PC vesicles (data not shown) is about 1 kcal/mol less than that of saturated myristic acid (14:0), consistent with the known ability of double bonds to

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[2] We determined the partition coefficient of the anionic form of myristic acid to PC MLVs at 10, 15, 25, 30, 35, and 40 °C by making electrophoretic mobility measurements. There was no significant dependence of binding on temperature, a result consistent with our working hypothesis that the binding is due to the classical entropy-driven hydrophobic effect discussed by Tanford (1980). This classical entropy-driven binding of myristate to vesicles differs from the nonclassical enthalpy-driven binding of several charged amphiphiles to bilayers (Huang & Charlton, 1972; Seelig & Ganz, 1991; Beschiaschvili & Seelig, 1992). We should also note that, although the data obtained by Tanford (1980) remain valid, the interpretation of the classical hydrophobic effect has changed in the past decade (Baldwin, 1986; Dill, 1990a,b; Murphy et al., 1990; Privalov et al., 1990; Sharp et al., 1991; Spolar et al., 1992; Wimley & White, 1993).
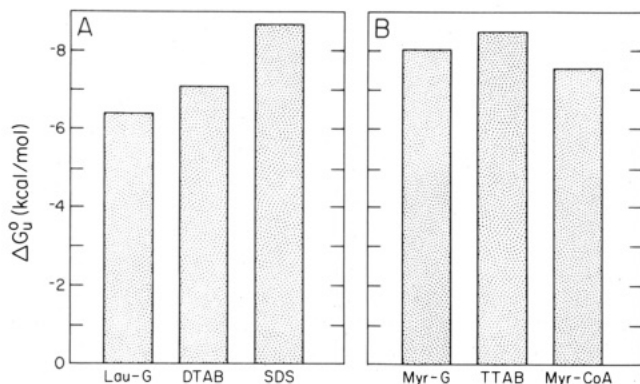
FIGURE 4: Unitary Gibbs free binding energies, ΔG_u^o, of acylated molecules with 12-carbon chains (A) or 14-carbon chains (B). (A) Lauryl-Gly (Lau-G) has a charge of −1, dodecyldimethylammonium bromide (DTAB) has a charge of +1, and sodium dodecyl sulfate (SDS) has a net charge of −1. (B) Myristoyl-Gly (Myr-G) has a charge of −1, tetradecyldimethylammonium bromide (TTAB) has a charge of +1, and myristoylated coenzyme A (Myr-CoA) has several negative charges in these pH 7.0 solutions. The binding energies of the first five molecules were determined using the zeta potential technique illustrated in Figure 6; the binding energy of Myr-CoA was determined using the equilibrium dialysis approach illustrated in Figure 7 and is probably an underestimate.[3]

decrease the partition coefficient of acyl chains into bulk organic phases (Tanford, 1980) and vesicle partitioning experiments with saturated and unsaturated 18 carbon fatty acids (Anel et al., 1993). The chemical nature of the polar compound to which the myristate chain is attached, however, does not necessarily affect its binding to phospholipid vesicles. For example, myristoylated coenzyme A, Myr-CoA (Figure 4B), binds with about the same energy as the small acylated peptides we have investigated.[3] SDS (Figure 4A), however, binds with about 2 kcal/mol greater energy than Lau-Gly for reasons that we do not understand. Thus the simple picture we have elaborated for acylated peptides cannot be extrapolated to all acylated compounds. However, the data we report here can be extrapolated to larger myristoylated peptides: a myristoylated peptide corresponding to the first 11 amino acids of pp60[v-src] binds to PC vesicles with about the same energy or partition coefficient as the peptides illustrated in Figure 2 (C. Buser, M. Strzelczyk, C. Sigal, M. Resh, and S. McLaughlin, unpublished data).

DISCUSSION

Our most important conclusions are that small myristoylated peptides bind to phospholipid bilayer membranes with a unitary Gibbs free energy of about 8 kcal/mol and that the binding is driven mainly by the hydrophobic interaction of the acyl chain with the interior of the membrane (Figure 1). The latter conclusion follows from our observations that the membrane binding energy of acylated glycines increases by 0.8 kcal/mol for each carbon added to the acyl chain (Figure 1) and that the binding is independent of the chemical nature of the three peptides we investigated (Figure 2), the lipid composition of the membrane (Figure 3), the net charge of the molecule (Figure 4), and the temperature.[2]

What is the biological significance of our results? The 8 kcal/mol contribution of myristate to the membrane binding energy is barely enough to partition a myristoylated protein onto the plasma membrane. For example, if we consider the partition coefficient $K_1 = 10^4$ M^{-1} and note that the effective concentration of lipids in a spherical shell of radius 10 μm is about 10^{-3} M, 90% of the myristoylated protein should be bound to the membrane if no other factors contribute to or
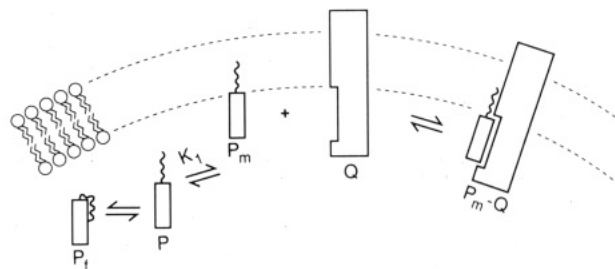

FIGURE 5: Sketch of two factors that could influence the binding of myristoylated proteins, P, to membranes. The results illustrated in Figures 1–4 show that the acyl chain on the polypeptide can bind to the membrane with an energy of 8 kcal/mol or a partition coefficient of $K_1 = 10^4$ M^{-1}. If the acyl chain can fold back and bind to the protein to form the complex represented by P_f, this interaction will reduce the overall binding to the membrane by decreasing the free concentration of P in the cytoplasm. If the membrane-bound form of the myristoylated peptide, P_m, can interact laterally with membrane-bound receptor proteins, Q, this interaction will drive the illustrated reactions to the right and increase the overall binding of protein to the membrane. Other factors that could decrease or increase the binding of myristoylated proteins are discussed in the text.

detract from the binding. Only 40% of a protein with an acyl chain of 12 carbons would be bound, and 99% of a protein with an acyl chain of 16 carbons would be bound. Thus, the overall partitioning of myristoylated proteins onto cellular membranes will be exquisitely sensitive to any additional factors that can affect the binding of these proteins to membranes, and these factors could control the reversible translocation of myristoylated proteins onto and off of membranes.

Several factors could reduce the binding. If, for example, the acyl chain can fold back and bind to the protein form to form P_f (Figure 5), the [P] available to bind to the membrane will be reduced. Even a weak (e.g., 2 kcal/mol) interaction of the acyl chain with the protein will produce a large (e.g., 10-fold) reduction in the partitioning of the protein onto the membrane. If this interaction depends on an intracellular signal, such as [Ca^{2+}], the partitioning of the protein onto the membrane will also depend on the signal. For example, Dizhoor et al. (1993) showed that recoverin binds to photoreceptor membranes when the [Ca^{2+}] is greater than 1 μM; they interpret this to mean that the "hydrophobic NH$_2$-terminus is constrained in Ca^{2+}-free recoverin and liberated by Ca^{2+} binding". Serafini et al. (1991) showed that the ADP ribosylation factor binds to membranes in a GTP-dependent manner and suggested that GTP weakened the hydrophobic interaction of myristate with ARF. Negative charges (e.g., acidic residues or phosphate groups) on the protein will be repelled electrostatically from the negatively charged phospholipids on the cytoplasmic surface of the plasma membrane, which could also reduce the binding. [n.b. Only those charges within a Debye length, about 1 nm, of the surface will be affected. A reasonable estimate for the electrostatic potential at the cytoplasmic surface of a plasma membrane is −30 mV (McLaughlin, 1977, 1989), so that this factor could decrease the binding by a factor of 100 if the effective valence of this region of the protein is −4.] Finally, any mass-dependent entropy price that must be paid upon membrane binding could lower the partitioning of a myristoylated protein onto a membrane, but we argue below that this price is negligible.

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[3] As discussed in Appendix I, the binding energy illustrated in Figure 4B for Myr-CoA is a minimal value that was calculated by assuming that the lipids on both monolayers of the vesicles in the dialysis sac were available to bind Myr-CoA. The binding energies for the other compounds in Figure 4 were determined from zeta potential measurements, which require no assumptions about the permeability of the membrane to the compound.

There also are factors that could enhance the binding of the myristoylated proteins to membranes. First, many proteins contain clusters of basic residues that could bind to acidic lipids in the membrane. For example, a peptide corresponding to the calmodulin-binding domain of the MARCKS protein binds strongly to acidic lipids in membranes: for membranes with >10% acidic lipids, this electrostatic interaction could contribute more binding energy than the hydrophobic insertion of the myristoyl chain (Kim et al., 1993).[4] Phosphorylation of this domain causes the MARCKS protein to move reversibly off the plasma membranes of several cells (Wang et al., 1989; Thelen et al., 1991; Sawai et al., 1993) and phospholipid vesicles (Taniguchi & Manenti, 1993). Thus phosphorylation provides another mechanism to control the reversible binding of myristoylated proteins to membranes. Second, hydrophobic residues could contribute to the interaction of a myristoylated protein with a membrane (Kahn et al., 1992). Third, the membrane-bound form of a myristoylated protein, P_m (Figure 5), could interact laterally with a membrane-bound receptor protein, Q, to form a complex. This interaction would be facilitated about 1000-fold by the reduction of dimensionality that occurs when P binds to the membrane. (Equivalently, there is a large increase in the effective concentration when a myristoylated protein binds to a plasma membrane because it is restricted to a shell of thickness r, where $r \approx 1$ nm. For a spherical cell of radius R, where $1 < R <10$ μm, the ratio of the volume of this shell to the volume of the cell is $3r/R \approx 10^{-3}$.) Thus, relatively weak interactions between P_m and Q could significantly increase the overall partitioning of P onto the membrane. The binding of pp60[v-src] to membranes is probably enhanced both by this mechanism (Resh, 1989) and by the interaction of basic residues in the NH_2-terminal region with acidic lipids.[4] Interactions with a receptor protein are presumably required to direct both pp60[v-src] to the plasma membrane (Resh, 1989) and ARF to the Golgi membrane (Serafini et al., 1991).

Our results help answer a question posed in previous reviews of myristoylated proteins. Sefton and Buss (1987) note that, "if a hydrophobic lipid is all that is required for membrane binding, it is not clear why only this nonabundant fatty acid is used as an amino-terminal anchor", and Towler et al. (1988) note that, "it remains to be explained what properties of the 14-carbon fatty acid led to its unique selection by the cell over other more abundant fatty acids". Our results support the explanation proposed by Towler et al. (1988) that "the lower hydrophobicity of myristic acid, when compared to the longer acyl chains of palmitic or stearic acids, may be compatible with reversible association of myristoylated proteins with various membrane organelles".

We now consider the mass-dependent entropy price that must be paid when a protein binds to another protein or to a membrane. When a myristoylated protein binds to a membrane, it loses approximately one translational and two rotational degrees of freedom, which reduces the entropy of the system. If one assumes the protein is a rigid rotator in a vacuum (Dwyer & Bloomfield, 1981; Janin & Clothia, 1978), this mass-dependent entropy price is calculated theoretically to be about 8, 10, and 15 kcal/mol for 0.1-, 1-, and 100-kDa molecules, respectively. This calculation, however, overes-

FIGURE 6: Effect of myristic acid on the zeta potential of multilamellar vesicles (MLVs) formed from palmitoyloleoylphosphatidylcholine (PC). The MLVs were formed in 1 (●), 10 (○), or 100 mM (■) KCl buffered to pH 7.0 with 0.1, 1, and 1 mM MOPS, respectively, at 25 °C. The theoretical curves are the least-squares best fits of the Gouy–Chapman–Stern theory (see text) to the data; the single adjustable parameter in this equation is the binding energy or partition coefficient of the anionic form of myristic acid onto the vesicles. A best fit was obtained for a partition coefficient of $K_1 = 8 \times 10^3$ M^{-1} (see eq 1) or $\Delta G_u^o \simeq 8$ kcal/mol. This measurement is represented by ○ in Figure 1.

timates the entropy price (Finkelstein & Janin, 1989; Searle & Williams, 1992), and it is generally acknowledged that there is no adequate theoretical description of this phenomenon for flexible molecules in solution. Our result (Figure 1) with the HA form of myristic acid gives us a unique opportunity to estimate the entropy price when this $\approx$200-Da molecule binds to a membrane. By studying the partitioning of a large number ($8 \leq N_{carbon} \leq 22$) of fatty acids into n-heptane, Tanford (1980) showed that the unitary Gibbs free energy for this process is $\Delta G_u^o = 4.26 - 0.825 N_{carbon}$ where the units are kcal/mol. The constant term on the right-hand side reflects the preference of the COOH group for water. When the HA form of myristic acid adsorbs to a membrane, the COOH group should remain in the aqueous phase, and the partition coefficient should thus be $14 \times 0.825 = 12$ kcal/mol if the entropy price is negligible. This agrees, within experimental error, with the value we measure (Figures 1 and 7). Thus, we conclude that a negligible entropy price is paid when the HA form of myristic acid binds to a membrane. Horton and Lewis (1992) concluded that the entropy price is also small for protein–protein interactions.

Several other factors could reduce the membrane binding of large myristoylated proteins. Silvius and Zuckermann (1993) suggest that the conformational entropy of a flexible protein decreases upon binding to a membrane and thus lowers the partition coefficient. Finally, fluctuations in the bilayer could exert a repulsive force on large proteins (Evans & Parsegian, 1986).

ACKNOWLEDGMENT

APPENDIX I. CALCULATION OF PARTITION COEFFICIENTS.

Zeta Potential Measurements. Figure 6 illustrates how zeta potential, ζ, measurements can be used to determine the partition coefficient of the anionic form of myristic acid to multilamellar PC vesicles. The zeta potential is calculated from the measured value of the electrophoretic mobility, u,

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[4] Basic residues in the amino terminal region of the src protein also probably contribute to the binding of this protein to the plasma membrane. A myristoylated peptide consisting of the first 11 amino acids of pp60[v-src] binds with about the same partition coefficient as Myr-Gly to PC vesicles, but 30-fold more strongly to vesicles containing 30% acidic lipid (C. Buser, M. Strzelczyk, C. Sigal, M. Resh, and S. McLaughlin, unpublished data).
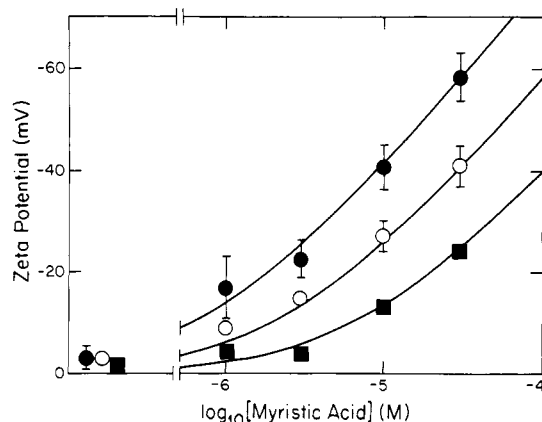
the velocity of the vesicle in a unit electric field, by using the Helmholtz–Smoluchowski equation (Hunter, 1981):

$$\zeta = u\eta/\epsilon_r\epsilon_0 \tag{6}$$

where η is the viscosity of the aqueous solution, ϵ_r is the dielectric constant of the aqueous phase, and ϵ_0 is the permitivity of free space.

For a large smooth particle with charges at the interface (e.g., a phospholipid vesicle with adsorbed myristic acid anions), ζ is the electrostatic potential, ψ, at the hydrodynamic plane of shear (Overbeek & Wiersema, 1967; O'Brien & White, 1978), which is located about 0.2 nm from the surface (Eisenberg et al., 1979; Alvarez et al., 1983; Rooney et al., 1983; Ermakov, 1990): $\zeta = \psi(0.2 \text{ nm})$.

We have discussed in detail elsewhere how zeta potential measurements can be used to calculate the partition coefficients of charged molecules onto membranes (McLaughlin & Harary, 1976; McLaughlin, 1977; Kim et al., 1991). Specifically, the theoretical curves that describe the data in Figure 6 were drawn by combining eqs 1, 2, 4, and 6 of Kim et al. (1991) with eq 1 of this report; we call this combination of equations the Gouy–Chapman–Stern theory. We give here only a qualitative description of the phenomenon rather than repeating these equations. The zeta potential is approximately equal to the surface potential, $\zeta = \psi(0.2 \text{ nm}) \simeq \psi(0)$, which can be calculated from the Gouy–Chapman theory of the diffuse double layer (McLaughlin, 1977, 1989). When the potential is small, this theory predicts that

$$\psi(0) = \sigma/(\epsilon_r\epsilon_0\kappa) \tag{7}$$

where σ is the surface charge density or charge per unit area of surface and $1/\kappa$ is the Debye length, which is about 1 nm in a 0.1 M solution and 10 nm in a 0.001 M solution. Note that this simple equation also describes the potential difference between the two parallel plates of a capacitor separated by a distance $1/\kappa$.

The PC vesicles illustrated in Figure 6 are electrically neutral: $\sigma = 0$. Thus ζ should be zero, as we observe experimentally. When myristate anions adsorb to the vesicles, they produce a negative surface charge density, σ, and a negative zeta potential (Figure 6). Equations 6 and 7 illustrate that, to a first approximation, the zeta potential is proportional to the number of adsorbed myristate anions, A^-, per unit area. The number of adsorbed anions is equal to the concentration of anions in the aqueous phase immediately adjacent to the membrane, $[A^-]_0$, multiplied by a partition coefficient (eq 1). Finally, the Boltzmann equation relates $[A^-]_0$ to the concentration of A^- in the bulk aqueous phase. As all of the parameters in the Gouy–Chapman–Stern theory are known, a zeta potential measurement at a single myristate concentration would suffice to determine the partition coefficient. We made measurements at several different myristate concentrations and three different salt concentrations (different Debye lengths) to ensure that the theory does adequately describe the data (Figure 6). It is apparent from Figure 6 that the theory can describe all of the data with a single partition coefficient of $K_1 = 8 \times 10^3$ M⁻¹. We made measurements similar to those illustrated in Figure 6 for each of the molecules represented by open and filled circles in Figure 1. Comparable fits of theory to the experimental data were obtained in all cases.

Equilibrium Dialysis Measurements. Figure 7 shows the equilibrium dialysis results we obtained with myristic acid at pH 6, 7, 8, and 9. As the pH decreases, the ratio of the neutral, HA, to the anionic, A^-, forms of myristic acid increases in the aqueous phase ($pK_a = 5$), and the binding curves shift
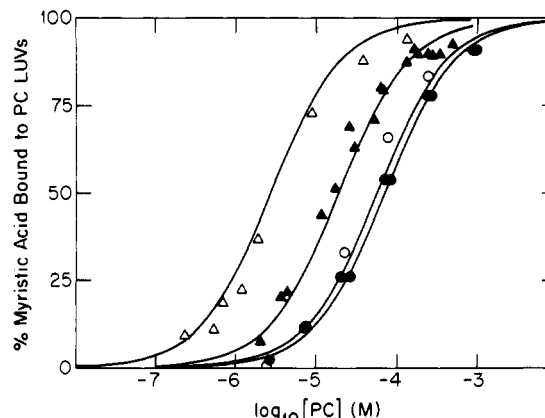

FIGURE 7: Equilibrium dialysis measurements of the percent of myristic acid bound to palmitoyloleoyl phosphatidylcholine (PC) large unilamellar vesicles (LUVs) plotted as a function of the concentration of PC in the sac of dialysis tubing using solutions containing 100 mM KCl (and MOPS or Tris). Measurements were made at pH 9 (●), 8 (○), 7 (▲), and 6 (△) at 22 °C. The curves illustrate the predictions of eq 8, assuming that $K_A = 1.4 \times 10^4$ M⁻¹ and $K_{HA} = 4 \times 10^6$ M⁻¹.

toward lower lipid concentrations, which implies that HA binds more strongly than A^-. (We could not obtain reliable data for pH < 6.)

We describe the binding by defining partition coefficients (eq 1) for the HA and A^- forms (K_{HA} and K_A) and using the Henderson–Hasselbach equation to give the relative concentrations of HA and A^- in the aqueous phase. The total fraction of myristic acid bound to the PC vesicles in the dialysis sac, f_{bound}, is given by the combination of these equations:

$$f_{bound} = \frac{(K_{HA} + K_A 10^{pH-pK})[L]}{1 + 10^{pH-pK} + (K_{HA} + K_A 10^{pH-pK})[L]} \tag{8}$$

where [L] (M) is the concentration of lipid in the sac accessible to myristic acid and pK is the pK_a of myristic acid in the bulk aqueous phase, which is 5.0 (Ptak et al., 1980). We assume that myristic acid equilibrates across the PC vesicles during 24 h, an assumption for which there is good experimental support (Gutknecht, 1988), and thus [L] in eq 8 is the total concentration of lipid in the dialysis sac. This equation is valid in the limit that the number of bound myristic acid molecules is much less than the number of lipid molecules (because the adsorption of A^- produces a surface potential): the ratio of these numbers is ≤ 0.01 in our experiments.

Since f_{bound} increased only slightly when we increased the pH from 8 to 9 (Figure 7), we assume that bound HA is negligible at pH 9. We obtain K_A from a best fit of the pH 9 data in Figure 7 to the limiting form of eq 8, $f_{bound} = K_A[L]/(1 + K_A[L])$: $K_A = 1.4 \times 10^4$ M⁻¹. This number qualitatively agrees with the value of $K_A = 8 \times 10^3$ M⁻¹ we obtained from the independent zeta potential measurements illustrated in Figure 6.

Keeping K_A fixed, we used eq 8 to determine K_{HA} from the data at pH 6, 7, and 8, assuming $pK_a = 5.0$. (We obtained a least-squares best fit of eq 8 to our most reliable data points, those obtained for $0.2 < f_{bound} < 0.8$, with $K_{HA} = 4 \times 10^6$ M⁻¹.) The curves in Figure 7 were drawn with $K_A = 1.4 \times 10^4$ M⁻¹ and $K_{HA} = 4 \times 10^6$ M⁻¹. Our conclusion from the data in Figure 7 that $K_{HA}/K_A = 300$ qualitatively agrees with the value (140) obtained by Marsh and colleagues for spin-labeled myristic acid (Miyazaki et al., 1992).

We made equilibrium dialysis measurements similar to those illustrated in Figure 7 with radioactive myristoylated-coenzyme A (Myr-CoA) and obtained identical data at pH 4 and 7 (data not shown). Using eq 1 and the limiting form of eq

8 given above, we obtained a best fit to the Myr-CoA data with $K_1 = 5 \times 10^3$ M^{-1}, assuming Myr-CoA permeates the membranes. We show the corresponding binding energy in Figure 4. If, as seems more likely, Myr-CoA does not equilibrate across the PC membranes in 24 h, K_1 is twice as large (binding energy increases by 0.4 kcal/mol) and is identical to the value obtained for the A$^-$ form of myristate, 10^4 M^{-1}.

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