Uptake of basic amino acids and peptides into liposomes in response to transmembrane pH gradients

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ABSTRACT The uptake of derivatives of lysine and a pentapeptide (ala-met-leu-trp-ala) into large unilamellar vesicle (LUV) systems in response to transmembrane pH gradients has been examined. In these derivatives, the C-terminal carboxyl functions have been converted to methyl esters or amides. It is shown that the presence of a pH gradient (interior acidic) results in the rapid and efficient accumulation of these weak base amino acid and peptide derivatives into LUVs in a manner consistent with permeation of the neutral (deprotonated) form. It is suggested that this property may have general implications for mechanisms of transbilayer translocation of peptides, such as signal sequences, which exhibit weak base characteristics.

INTRODUCTION

The ability of the neutral forms of certain weak acids and weak bases to permeate through membranes has long been recognized. Early studies in this area included that of Jacobs (1940) on red cells; they were followed by studies using mitochondria (Chappell and Crofts, 1966) and chloroplasts (Crofts, 1967) to demonstrate the rapid transbilayer movement of the neutral form of amines, such as ammonia, and carboxylic acids, such as acetate. These observations have led to the use of weak acids and bases as probes for pH gradients (ΔpH) in cells and organelles (Rottenberg, 1979) and have also stimulated studies demonstrating the uptake of weak bases (Deamer et al., 1972; Nichols and Deamer, 1976) into simple liposomal systems exhibiting a ΔpH.

More recently, it has been shown that a variety of amino-containing drugs can be accumulated into large unilamellar vesicles (LUVs) with an acidic interior (Madden et al., 1990) and that the transbilayer distributions of phospholipids such as phosphatidylglycerol (PG; Redelmeier et al., 1990) or phosphatidic acid (PA; Eastman et al., 1991) in LUVs can also be readily modulated by transbilayer ΔpHs. All these processes rely on the fact that the neutral forms of the transported compounds are considerably more permeable than the charged forms, which can result in net transport of weak bases into acidic compartments and weak acids into basic compartments. The concentration gradients that can be achieved at equilibrium are considerable. Under appropriate conditions, a lipophilic amine, for example, will redistribute across a membrane to reflect the proton gradient. A ΔpH of three units (inside acidic) across an LUV membrane can, therefore, result in a 1,000-fold higher concentration of the amine inside than outside.

In this work, we extend these studies to those amino acids and peptides for which carboxyl groups have been modified to methyl ester or amide forms. It is shown that such compounds are readily accumulated into LUVs with an acidic interior, and a detailed kinetic analysis indicates that transport proceeds via the neutral form.

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MATERIALS AND METHODS

Egg phosphatidylcholine (EPC) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). [^{14}C]methylamine and [^{3}H]-tetraphenylphosphonium bromide were purchased from New England Nuclear (Boston, MA). Lysine methyl ester and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO). The hydrophobic pentapeptide (ala-met-leu-trp-ala; de Kroon et al., 1989) was synthesized as a carboxyl amide or free acid by use of solid phase methods (Clark-Lewis and Kent, 1989). The carboxylic acid form was converted to a methyl ester as described by de Kroon et al. (1989).

Multilamellar vesicles (MLVs) were produced by hydrating 50 mg EPC in 1.0 ml of 300 mM citrate buffer, pH 4.0. The MLVs were frozen in liquid nitrogen and thawed at 60°C (in water) for five freeze-thaw cycles. This treatment increases the trapped volume of the vesicles and promotes equilibrium transmembrane solute distributions (Mayer et al., 1985). Extrusion of the frozen and thawed MLVs through two stacked polycarbonate filters (Nuclepore Corp., Pleasanton, CA) (100-nm pore size) was performed 10 times at 20°C using an extrusion device obtained from Lipex Biomembranes Inc. (Vancouver, BC, Canada), as described by Hope et al. (1985). The resulting LUVs were ~110 nm diam, as determined by quasielastic light scattering using a particle sizer (Nicomp Instruments, Santa Barbara, CA).

To generate the transmembrane ΔpH, the LUVs in the pH 4.0 media were passed down a 10-cm Sephadex G-50 (G50-150) column previously equilibrated with 150 mM NaCl and 20 mM HEPES, pH 7.5 (Hepes-buffered saline, HBS). Uptake of the lysine methyl ester and pentapeptides was performed by first dissolving them in 1 ml HBS media, to which 0.25 ml LUVs (final lipid concentration of 1–5 mM) exhibiting a ΔpH (pH_o = 7.5, pH_i = 4.0) were added. Entrapment

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levels were monitored using 0.1-ml aliquots, which were removed at selected times from this incubation mixture and passed through 1.0-ml Sephadex G-50 columns (prespun) by centrifugation for 1 min at 2,500 rpm to remove exterior (untrapped) material. All experiments were conducted at 20°C unless otherwise indicated.

Transmembrane Na^+/K^+ (external/internal) gradients were created using a modification of the technique of Hope et al. (1985). Vesicles were prepared in 150 mM K_2SO_4 and 300 mM Hepes (pH 7.5), after which the external K^+-buffer was replaced by 150 mM Na_2SO_4 and 300 mM Hepes (pH 7.5) by passing the vesicles down a 10-ml Sephadex G-25 column. In some cases (see Results) a Hepes concentration of 20 mM in the internal and external buffers was employed. Uptake of the hydrophobic pentapeptide in response to membrane potential ($\Delta\Psi$) was carried out at 20°C by dissolving the pentapeptide in the Na^+-buffer and adding EPC vesicles bearing a Na^+/K^+ gradient (final lipid concentration 2–3 mM) and 1 μM valinomycin. Aliquots were taken at indicated intervals and untrapped material removed via passage through 1.0-ml Sephadex G-50 columns (prespun) as indicated above.

Lysine methyl ester concentrations were determined by a modification of the technique employed by Hope and Cullis (1987), using trinitrobenzenesulfonic acid (TNBS) to label the primary amino groups of lysine methyl ester. The buffer used for the labeling was 100 mM $NaHCO_3$, 50 mM H_3BO_3, pH 10.0. A reference cuvette containing 2.5 ml of buffer, pH 10.0, was placed in the reference beam. The sample cuvette contained 2.5 ml of buffer, pH 10.0, with 0.5 mM TNBS. 50-μl aliquots of vesicles containing lysine methyl ester were then added. The resulting change in absorbance was measured at 420 nm after incubation for 1 h (in the dark). 200 μl, 0.5% Triton X-100 was added to both cuvettes to solubilize the vesicles and thus expose all primary amino groups to the TNBS. The absorbance in the presence of detergent was taken to represent 100% labeling (Hope and Cullis, 1987).

The amount of hydrophobic pentapeptide trapped was quantified by measuring the tryptophan fluorescence (λ_{ex} = 280 nm, λ_{em} = 354 nm) with a luminescence spectrometer (model LS 50, The Perkin-Elmer Corp., Instrument Division, Norwalk, CT) in 0.5% (w/v) sodium cholate–containing buffer (300 mM Hepes and 150 mM Na_2SO_4, pH 7.5). The sample volumes used were adjusted so that the resulting emission intensity was directly proportional to the amount of peptide present.

The magnitudes of the ΔpHs and membrane potentials present were measured using [^{14}C]methylamine and [^{3}H]-tetraphenylphosphonium bromide (^{3}H-TPP), respectively, as indicated elsewhere (Hope et al., 1985; Madden et al., 1990). The concentrations used were 1 μCi/ml. The amount of probe accumulated was determined via liquid scintillation counting after removing untrapped label. Transmembrane ΔpH could then be calculated using the relationship $\Delta pH = \log([\text{methylamine}]_{in}/[\text{methylamine}]_{out})$, as indicated in Mayer et al. (1988). Membrane potentials were calculated similarly for ^{3}H-TPP using the relationship $\Delta\Psi = -59 \log\{[TPP]_{in}/[TPP]_{out}\}$ (see Hope et al., 1985).

Phospholipid concentrations were determined by a modification of the method of Fiske and Subbarow (1925). Typical phospholipid concentrations were ~3 mM. All data are means ± SEM (n = 3). Where error bars are not shown on figures indicating amino acid or peptide uptake, the SEM values were within the dimensions of the symbols used.

The kinetics of the uptake process were analyzed assuming that only the neutral (deprotonated) form of the amino acid or peptide could move across the LUV bilayer. As developed more completely elsewhere (Cullis, P. R., et al., manuscript in preparation), the accumulation process should then obey the relation

$$[A(t)]_i = [A(eq)]_i(1 - e^{-kt}),$$

Where $[A(t)]_i$ is the interior concentration of the amino acid or peptide at time t, $[A(eq)]_i$ is the equilibrium interior concentration at $t = \infty$, and k is the rate constant associated with the uptake process.

We consider the analysis for the lysine methyl ester, which contains two ionizable (primary) amino functions with corresponding dissociation constants K_1 and K_2. Under the assumption that only the neutral (fully deprotonated) form is able to translocate the membrane, it is readily shown that

$$k = (PA_m/V_0)(1 + [H^+]_o/K_1 + [H^+]_o^2/K_1K_2)^{-1},$$

where P is the permeability coefficient for the neutral form, A_m is the area of the LUV membrane, V_0 is the aqueous volume of the lipid dispersion, and $[H^+]_o$ is the exterior proton concentration. Thus, if K_1, $K_2 \ll [H^+]_o$, we obtain $k = PA_m K_1K_2/V_0\,[H^+]_o^2$.

Alternatively, if the peptide contains only one amino function, as is the case for the pentapeptide investigated here, we obtain $k = PA_mK_1/V_0\,[H^+]_o$. It should be noted that P is an effective permeability coefficient that is related to the actual membrane permeability coefficient P_m by the relation $P_m = KP$, where K is the membrane-water partition coefficient of the charged (protonated) species of the amine. Also, this is an initial rate analysis and assumes that the concentration of the neutral amine on the outer surface of the LUVs is much greater than that on the inner surface. The rate constant k can be calculated from the slope of a plot of $\ln\{([A(eq)]_i - [A(t)]_i)/[A(eq)]_i\}$ versus t, and a subsequent plot of $\log k$ versus the external pH should exhibit a slope of two if the uptake actually proceeds via the neutral form for the lysine methyl ester or a slope of one for the pentapeptide methyl ester. The rate constant k was determined by applying a linear least-square analysis to the uptake data using a commercially available plotting program (Sigma-Plot, Jandel Scientific, Corte Madera, CA; 1986), with k and $[A(eq)]_i$ as variables.

RESULTS

The first set of experiments was aimed at demonstrating that a basic amino acid derivative could respond to transmembrane ΔpHs in a manner similar to that observed for other amines (Mayer et al. 1986; Madden et al. 1990). As shown in Fig. 1, lysine methyl ester was rapidly and efficiently accumulated into EPC LUVs with an acidic interior (pH_i = 4.0, pH_o = 7.5). Maximal entrapment levels were reached within 5–10 min. A corresponding decrease in the ΔpH using [^{14}C]methylamine was observed (the gradient dropped from 3.2 to 1.2 pH units). The maximal concentrations entrapped under these conditions were ~65 nmol lysine methyl ester/μmol phospholipid (corresponding to an interior concentration of 43 mM). This high level of internalized lysine methyl ester was maintained for ≥24 h with no measurable leakage. It should be noted that the drop in the ΔpH on accumulation of lysine methyl ester is larger than can be accounted for by the uptake of lysine. It is possible that this results from reduced trapped volumes that can be observed at high citrate concentrations. The control vesicles exhibiting no pH gradient (7.5/7.5; 4.0/4.0) showed little uptake of the lysine methyl ester (≤10% of that observed for the vesicles with a ΔpH).

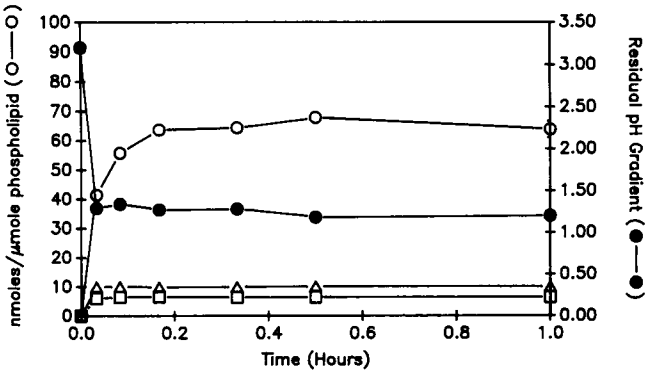

FIGURE 1 Time course (○) of uptake of lysine methyl ester into 100 nm EPC vesicles (3.8 mM) exhibiting a ΔpH (pH_i = 4.0; pH_o = 7.5). Control vesicles with no ΔpH (△, pH_i = pH_o = 7.5; □, pH_i = pH_o = 4.0) were also tested. Uptake was conducted at 20°C, and the initial external concentration of lysine methyl ester was 1.3 mM.

Lysine (unmodified) was not accumulated in vesicles bearing a ΔpH (data not shown). This may be attributed to the presence of the negatively charged carboxylic acid group, which would be expected to inhibit transbilayer diffusion (see, e.g., Gutnecht and Walter, 1981). Control experiments using TNBS revealed that lysine methyl ester accumulated into these LUVs did not react with the TNBS. Labeling occurred only after the vesicles had been solubilized with Triton X-100. Hence it was concluded that the primary amino groups of accumulated lysine methyl ester were not accessible to the TNBS reagent (e.g., not surface associated) in the intact vesicles.

The next set of experiments was designed to clarify the mechanism of uptake, specifically to show that the lysine methyl ester was accumulated in the neutral form. As outlined in the Materials and Methods, if this is the case, the slope of a plot of log k versus pH_o should exhibit a slope of two. As shown in Fig. 2 A, the initial rate of uptake of the lysine methyl ester into these LUVs increased considerably as the exterior pH was raised from 6.40 to 7.25. The absolute value of ΔpH was maintained at 3.5 units by varying the pH of the internal (citrate) buffer used and the external (HBS) buffer in unison. Rate constants (k) for the different pH_o values were extracted from this data employing the plots of Fig. 2 B, and a subsequent plot of log k versus pH_o (Fig. 2 C) reveals a straight line with a slope of 1.85. This supports a mechanism involving the transport of the neutral (deprotonated) form of the lysine methyl ester.

Previous studies from this laboratory have shown that acidic phospholipids, such as PG and PA, can also experience transmembrane transport in response to ΔpHs, again involving transport of the neutral form

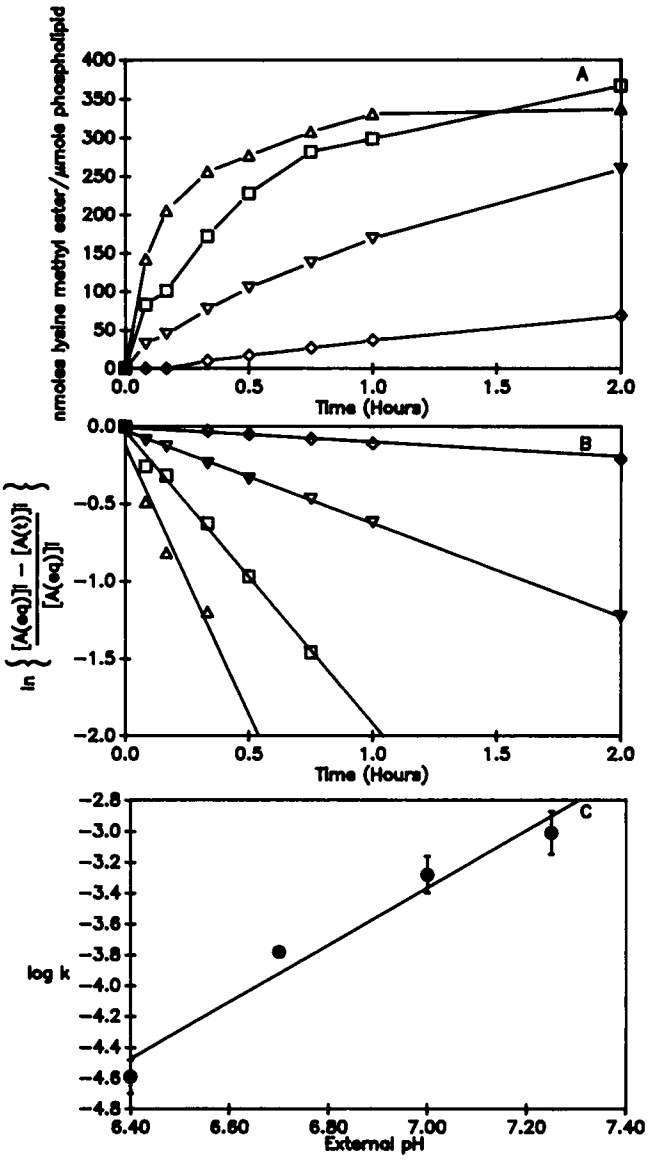

FIGURE 2 (A) Time course of uptake of lysine methyl ester into 100 nm EPC vesicles (~1.2 mM) for different external pH values. The exterior/interior pH values of the samples were 6.4/2.9 (◇), 6.7/3.2 (▽), 7.0/3.5 (□), and 7.25/3.75 (△). The initial external concentration of lysine methyl ester used was 1.8 mM. (B) Plot of ln $\{([A(eq)]_i - [A(t)]_i)/[A(eq)]_i\}$ vs. time, where $[A(t)]_i$ is the interior concentration of the accumulated amine at time t and $[A(eq)]_i$ is the interior concentration at equilibrium. For details see Materials and Methods. The slopes of the lines give the rate constant (k) for the transbilayer transport of lysine methyl ester. (C) Plot of log k vs. external pH. The slope of this line is 1.85.

(Hope et al., 1989; Redelmeier et al., 1990). Such transport exhibits high activation energies, in the range of 30 kcal/mol. As shown in Fig. 3 A, the uptake of the lysine methyl ester also increased considerably as the temperature was raised from 25°C to 45°C for an EPC

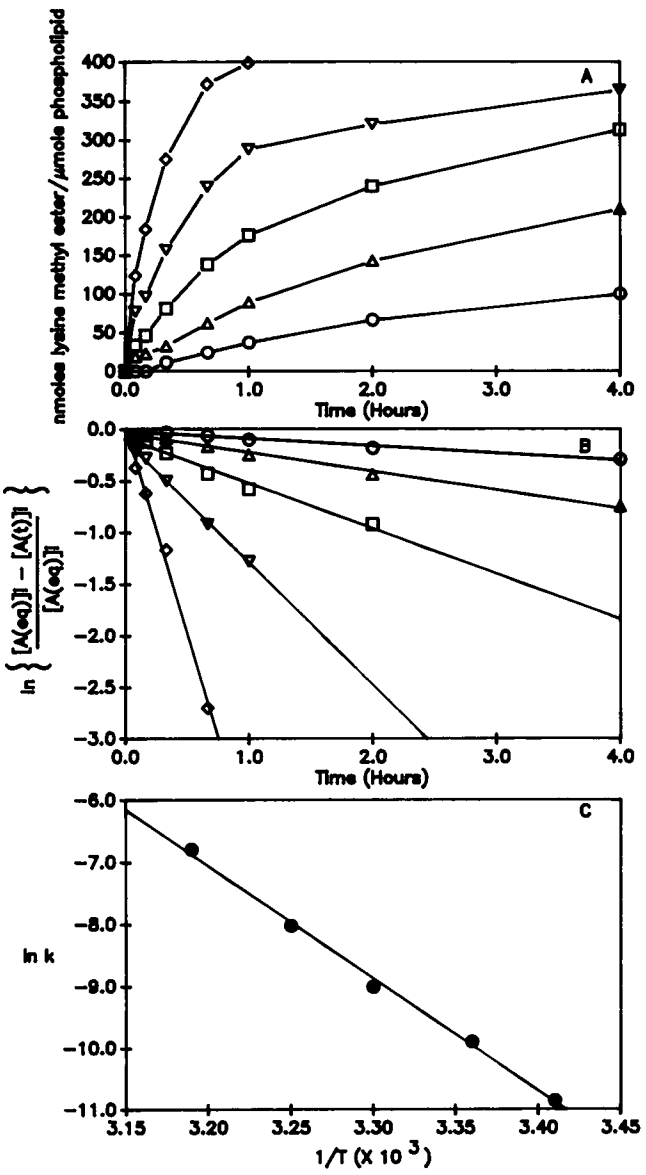

FIGURE 3 (*A*) Time course of uptake of lysine methyl ester into 100 nm EPC vesicles (2.2 mM) exhibiting a ΔpH (pH_i = 3.0; pH_o = 6.5). Uptake was conducted at 20 (○), 25 (△), 30 (□), 35 (▽), and 40°C (◇). The external concentration of lysine methyl ester was 1.8 mM. (*B*) Plot of ln $\{([A(eq)]_i - [A(t)]_i)/[A(eq)]_i\}$ vs. *t*, where $[A(t)]_i$ and $[A(eq)]_i$ have the same meanings as indicated in the legend to Fig. 2. (*C*) Arrhenius plot of the rate constants (*k*) for lysine methyl ester uptake. The activation energy calculated from the slope of this plot is E_a = 36 kcal/mol.

system where pH_o = 6.5 [20 mM MES [2-(*N*-morpholino)ethanesulfonic acid] and 150 mM NaCl] and pH_i = 3.0. An analysis of the rate constants (Fig. 3 *B*) and subsequent calculation of the activation energy from the Arrhenius plot of Fig. 3 *C* revealed an activation energy of 36 kcal/mol.

In a previous study, de Kroon et al. (1989) showed that the methyl ester of a hydrophobic, basic pentapeptide (ala-met-leu-trp-ala-(MeO)) can be accumulated into LUVs exhibiting a membrane potential (ΔΨ). As emphasized above, weak bases generally permeate through bilayer membranes in the neutral (deprotonated) form. In this regard, the presence of ΔΨ (inside negative) results in the induction of a ΔpH (inside acidic) at electrochemical equilibrium (Cafiso and Hubbel, 1983). It is therefore likely that the behavior observed by de Kroon et al. (1989) reflects uptake in response to an induced ΔpH than to the ΔΨ per se. As shown in Fig. 4 *A*, the pentapeptide methyl ester employed by de Kroon et al. (1989) is very rapidly accumulated into LUVs exhibiting a ΔpH (pH_o = 7.5, pH_i = 4.0), supporting this conclusion. As in the case of the lysine methyl ester, a drop in the residual pH gradient was also observed, corresponding to protonation of the pentapeptide amino function after traversal of the membrane in the neutral form. The maximal concentrations entrapped were ~35 nmol peptide/μmol phospholipid. This level of internalized peptide was maintained for ≥24 h with no leakage (data not shown). In contrast, uptake in response to ΔΨ revealed slower uptake (Fig. 4 *B*), which was dependent on the internal buffering capacity. Specifically, the uptake was much slower at high internal buffer concentrations (300 mM Hepes, pH 7.5) than for LUVs with lower internal buffer concentrations (20 mM Hepes, pH 7.5). The slower uptake for the system containing 300 mM Hepes may be attributed to a smaller induced ΔpH due to the high internal buffering capacity. Subsequent experiments revealed that the amide form of the pentapeptide exhibited uptake behavior similar to that of the methyl ester form in response to both ΔΨ and ΔpH (results not shown).

Subsequently, experiments were undertaken to verify that the pentapeptide was indeed accumulated in the neutral form, as observed for lysine methyl ester. As outlined previously, the slope of a plot of log *k* versus pH_o should exhibit a slope of one if there is only one basic titratable group present (as is the case for the hydrophobic pentapeptide). It was found that the rate of uptake of the pentapeptide increased significantly as the exterior pH was raised from 6.50 to 7.75. Again, the absolute value of ΔpH was maintained at 3.5 units by varying internal and external pH values in unison. A plot of log *k* versus pH_o revealed a straight line with a slope of 0.85 ± 0.1 (results not shown). This strongly supports a mechanism involving transport of the neutral (deprotonated) form of the pentapeptide.

A final set of experiments was undertaken to determine the activation energy for transport of the pentapeptide. A set of data analogous to that obtained for lysine

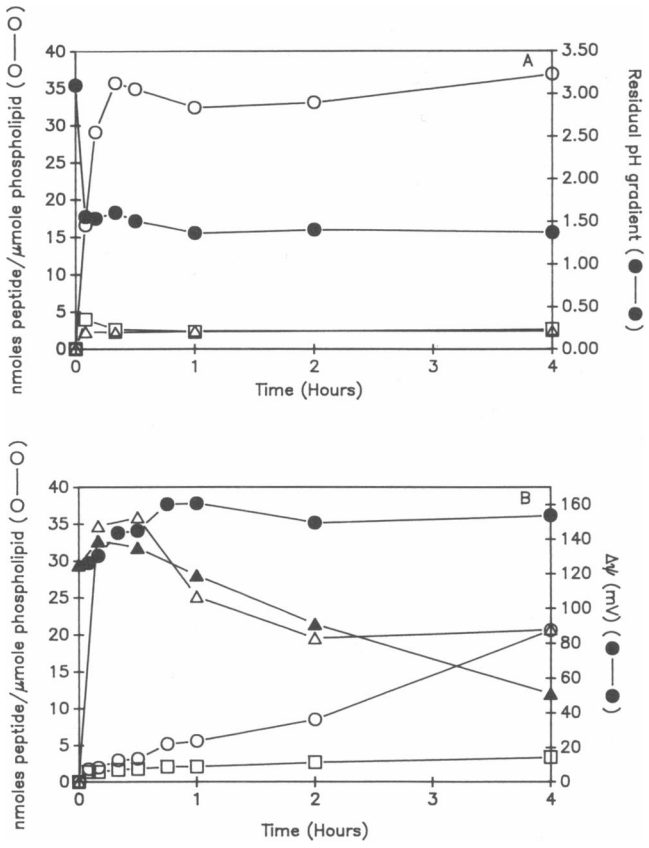

FIGURE 4 (*A*) Time course of uptake (○) of a pentapeptide (ala-met-leu-trp-ala methyl ester) into 100 nm EPC vesicles (6.4 mM) exhibiting a ΔpH (pH_i = 4.0; pH_o = 7.5). Control vesicles with no ΔpH gradient (△, pH_i = pH_o = 7.5; □, pH_i = pH_o = 4.0) were also tested. Uptake was conducted at 20°C, and the initial external peptide concentration was 0.4 mM. (*B*) Time course of uptake of the pentapeptide into 100 nm EPC vesicles (6.6 mM) exhibiting a valinomycin-induced K^+ membrane potential with high (300 mM Hepes) internal buffering capacity (○) and low (20 mM Hepes) internal buffering capacity (△). Control vesicles with no valinomycin present generated background levels of peptide uptake (□). The filled symbols (●, ▲) represent the measured ΔΨ values corresponding to the high and low buffering concentrations used. Uptake was conducted at 20°C, and the initial external peptide concentration was 0.5 mM.

methyl ester (Fig. 3) was generated over the temperature range 23–50°C (data not shown), leading to an activation energy of 30.6 kcal/mol.

DISCUSSION

The results of this report establish that basic amino acids and peptides, in which the C-terminal carboxyl functionalities have been modified to form methyl esters or amides, can be rapidly and efficiently accumulated into LUVs in response to transmembrane ΔpHs (inside acidic). Two points of interest concern the mechanism of uptake and the implications for transbilayer translocation of peptides and proteins in vivo. With regard to mechanism, previous work on a variety of lipophilic amino-containing drugs (Mayer et al. 1986; Madden et al. 1990) yields uptake behavior consistent with transport of the neutral (deprotonated) form of the amine. In the case of the lysine methyl ester investigated here, the dependence of the rate constant k on the external pH, which is consistent with an inverse square relationship between the uptake rates and the external proton concentration, also provides strong evidence for transport of the neutral (deprotonated) form. The rapid uptake of the methyl ester derivative used by de Kroon et al. (1989) in response to ΔpH observed here, the much slower uptake observed in response to ΔΨ at high internal buffer concentrations (which may be attributed to reduced uptake in response to smaller ΔpHs induced by the K^+ diffusion potential), and the linear dependence of the rate constant k on the external proton concentration are also all consistent with accumulation of the neutral form and indicate that uptake in response to ΔΨ per se does not occur.

The rapid uptake of the lysine methyl ester in response to ΔpH at external pH values that are considerably below the pKs of the associated primary amines clearly suggests that the neutral form must be highly membrane permeable. In this regard, the analysis presented under Materials and Methods indicates that the permeability coefficient P of the neutral form obeys the relation $P = kV_0[H^+]_o^2/A_m K_1K_2$. From the data of Fig. 2 *B* at 20°C and pH_o = 7.0, k can be calculated as 1.86×10^{-4}/s. The pK_1 of the NH_2 terminus amino function can be estimated as 8.95, where the pK_2 of the lysine side chain is 10.50. Thus, assuming an area per molecule for EPC of 70 Å^2, it can be calculated that $P = 2.1 \times 10^{-2}$ cm/s, corresponding to extremely rapid transbilayer movement of the neutral form.

The activation energies observed for this uptake of the lysine methyl ester and the pentapeptide are of interest, particularly with respect to those exhibited by PG and PA, which exhibit high activation energies in the range of 30 kcal/mol (Eastman et al., 1991). Such activation energies are comparable with those observed for the pentapeptide (30.6 kcal/mol) but lower than those observed for the lysine methyl ester (36 kcal/mol). Activation energies associated with diffusion across membranes have been rationalized on the basis of the need to break and reform hydrogen bonds (Stein, 1967). Specifically, the activation energies associated with transfer of a molecule into the membrane hydrocarbon can be estimated according to the number (n) of hydrogen bonds (e.g., with water) that must be broken to enter the hydrocarbon, less the number of intramolecular hydro-

gen bonds that reform in the hydrocarbon. Thus, for the lysine methyl ester the number of effective hydrogen bonds to be broken (see Stein, 1967) would be four for the two primary amines and 0.5 for the methyl ester. Assuming a value of 5 kcal/mol for the breaking of each hydrogen bond, this gives an activation energy of ~23 kcal/mol, which is considerably lower than observed here. However, in the case of PG and PA, similar analyses indicate activation energies of 40 and 28 kcal/mol, respectively. It is likely that the value for PG would be reduced by an ability to form intramolecular hydrogen bonds when in the hydrocarbon. Clearly, the net activation energy for the pentapeptide is difficult to estimate, given the considerable potential for forming intramolecular hydrogen bonds on insertion into the hydrocarbon.

This work has implications for mechanisms whereby peptides can insert into and translocate across membranes in vivo. Certain polypeptides can translocate across membranes in the absence of membrane proteins (Rapoport, 1990) by a mechanism that is not yet understood. Furthermore, signal sequences associated with translocated proteins exhibit lipophilic weak base character (Gierasch, 1989); and, again, the mechanisms whereby such sequences move across membranes during protein biosynthesis are not understood (Verner and Schatz, 1988; Gierasch, 1989). It has been suggested that signal sequences may be effectively pulled across bilayers by the electrochemical potential present (Skerjanc, 1990). The work presented here indicates that transmembrane ΔpHs could fulfill such a role by trapping signal sequences in the lumen of the endoplasmic reticulum. Endoplasmic reticulum membranes possess an inwardly directed proton pump that can generate ΔpHs (interior acidic) of up to 2 units, as demonstrated in microsomal systems (Rees-Jones and Al-Awqati, 1984; Thevenod et al., 1989). Net translocation of signal sequences and some peptides in response to such transmembrane ΔpHs in vivo is a clear possibility.

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