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Quantitation of Electrostatic and Hydrophobic Membrane Interactions by Equilibrium Dialysis and Reverse-Phase HPLC

William C. Wimley and Stephen H. White[1]
Department of Physiology and Biophysics, University of California, Irvine, California 92717

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Equilibrium dialysis and reverse-phase HPLC have been used for the sensitive and precise quantitation of both electrostatic and hydrophobic interactions of peptides and small molecules with lipid bilayers. We show that hydrophobic solutes are rapidly and quantitatively released from lipid dispersions when loaded onto a C4 reverse-phase HPLC column equilibrated in water + 0.1% trifluoroacetic acid and that the lipid molecules have no interfering effect on the chromatography. Peptides interacting electrostatically with bilayers are released quantitatively when a higher ionic strength buffer (water + 2% ammonium acetate) is used. As little as 50 ng of solute can be accurately quantitated even in the presence of milligram amounts of lipid. We demonstrate the application of these methods to the hydrophobic interactions between indoles and lipid bilayers and to the electrostatic interaction between defensins, which are cationic antibiotic peptides, and anionic bilayers. The high sensitivity allows nondestructive quantitation of submicrogram amounts of precious solutes and the high precision allows the heat capacity change, an important thermodynamic parameter, to be obtained from the partitioning data.

Knowledge of the forces that drive the interactions of solutes with lipid bilayers and biological membranes is critical for understanding the activity and uptake of many hormones, drugs, insecticides, and toxins, as well as the translocation of polypeptides and the folding of membrane proteins. Bilayer interactions, which are generally expressed quantitatively as partition coefficients, are primarily driven by hydrophobic or electrostatic interactions (1,2). Partition coefficients can be determined either by distinguishing bound from free solute spectroscopically (3) or by separating bound from free solute by centrifugation, filtration, or dialysis (4–6). In the latter case, quantitation of solute concentration is obtained through standard methods such as spectroscopic analysis, chemical derivitization, or through the use of radioactive labels.

These methods often suffer from well-known shortcomings including a limited range of measurable partition coefficients, low sensitivity, spectroscopic interference from lipid dispersions, or the need to use fluorescent or radioactively labeled compounds. In assays involving filtration or centrifugation, precise temperature control is difficult to maintain and loss of solute by adsorption to filters and other surfaces can be a serious source of error. In the work presented here, we use equilibrium dialysis in conjunction with reverse-phase HPLC to measure bilayer partition coefficients. Equilibrium dialysis, although more time-consuming than most other methods, is advantageous because separation of free from bound solute is established under equilibrium conditions, because concentration and temperature can be controlled precisely, and because solute adsorption has no effect on the results.

We are currently investigating the bilayer interactions of a class of very active, broad-spectrum antibiotic peptides known as the defensins (7). The human defensins, designated HNP-1, -2 and -3,[2] are globular, disulfide-stabilized cationic peptides that act by permeabliz-ing cell membranes (7,8). This probably occurs through

[1] To whom correspondence should be addressed.

[2] Abbreviations used: RP HPLC, reverse-phase high-pressure liquid chromatography; IEX, ion exchange; HNP, defensin or human neutrophil peptide; HNP-1, -2, -3: three nearly identical human defensin isoforms; TFA, trifluoroacetic acid; POPC, palmitoyloleoylphosphatdidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; MLV, multilamellar vessicles.

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a two-step process: reversible electrostatic binding of the peptides to the membrane surface followed by a poorly understood irreversible second step that leads to the lethal state (9). In preliminary studies of defensin–bilayer interactions, we found that precise quantitation of microgram amounts of defensins or other peptides in the presence of lipid was not possible using intrinsic absorbance or fluorescence or using standard protein microassay methods such as bicinchoninic acid, orthophlalaldehyde, or fluorescamine (10). In the work presented here we show that reverse-phase HPLC can be used to quantitate as little as 50 ng of peptide even in the presence of milligram amounts of lipid.

In addition to the defensins, we are also investigating the partitioning of the tryptophan side-chain analogs indole, 3-methylindole, and *N*-methylindole into lipid bilayers. These compounds interact with bilayers via the hydrophobic effect, as well as through specific polar interactions with the lipids (17). The high precision of RP HPLC and equilibrium dialysis allows the heat capacity change (ΔC_p) to be obtained from the temperature dependence of the partition coefficients. The heat capacity-induced curvature is a subtle effect not generally obtained from bilayer partitioning experiments because of the lower precision of many detection methods. However, it is now clear that ΔC_p is the key to quantitating hydrophobic processes such as bilayer partitioning (11–13), and therefore determination of ΔC_p from partitioning experiments is of great value (17).

MATERIALS AND METHODS

Materials. 3-Methylindole and *N*-methylindole were obtained from Sigma (St. Louis, MO), and were greater than 99% pure by HPLC. Palmitoyloleoylphosphocholine (POPC) and palmitoyloleoylphosphoglycerol (POPG) were obtained from Avanti Polar Lipids (Birmingham, AL). Lipid purity was confirmed by thin-layer chromatography. The sample buffer used was 10 mM Hepes, 50 mM KCl, 1 mM EDTA, 3 mM NaN_3, pH 7.0. Water was glass distilled. Ammonium acetate and HPLC-grade acetonitrile were from Fisher (Fair Lawn, NJ) and TFA was sequencing grade from Aldrich (Milwaukee, WI). The tripeptide Ac-ala–trp–ala–*O-tert*-butyl was synthesized using standard solution phase methods and was purified by reverse-phase HPLC (4).

Purification of the human defensins HNP-1, -2 and -3. The human defensins HNP-1, -2, and -3 were purified from human polymorphological neutrophil granules. The granules were repetitively extracted with 5% acetic acid at 0°C. The combined extracts, containing approximately 60 mg total HNP-1, -2, and -3 were lyophilized and suspended in 5% acetic acid. The defensins were separated using a 5 × 150-cm Bio-Gel P60 column run in 5% acetic acid at 5°C. The defensins eluted ~0.5 liter after the included volume, due to interactions with the P60 matrix, and contained approximately 70% HNP-1, -2, and -3. Most of the impurities were probably incompletely processed defensin precursors (14). Final purification was obtained with a combination of reverse phase (RP) and ion exchange (IEX) HPLC. RP HPLC was done using 1 × 25-cm C18 column from Rainin Instrument Co. (Woburn, MA) and IEX HPLC was done with a 0.46 × 20-cm polysulfoethyl-aspartamide cation exchange column from PolyLC inc. (Columbia, MD). In both cases water/acetonitrile/NH_4 acetate buffers were used. Purity of the final products was greater than 98% as judged by IEX and RP HPLC, acid/urea gel profiles, and amino acid analysis.

Equilibrium dialysis. Unilamellar lipid vesicles of 0.1 μm diameter were prepared by extrusion (15) and were used in all of the experiments presented here. Equilibrium dialysis was run in a Spectrum equilibrium dialyzer (Spectrum Industries, Los Angeles, CA) using all Teflon cells with half-cell volumes of 1.5 ml. Spectrapor 2 dialysis membranes (Spectrum Industries, 12–14,000 MWCO) were used in all studies. Temperature was controlled to ±0.2°C using a circulating water bath. The lipid containing half-cell contained 1–20 mM lipid. No leakage of lipid across the dialysis membrane was detectable. The dialysis apparatus was rotated at 10 rpm and equilibration was obtained within 5 h for small molecules and within 15–20 h for the defensins. Defensin samples for HPLC were taken directly from the dialysis cells because adsorption to the surfaces of vials and tubes was significant even for siliconized glass and plastic.

Quantitative RP HPLC. Quantitative HPLC was performed with a Rainin HPLC system interfaced to a Macintosh SE computer. Gradient control and data collection and analysis were with Rainin Dynamax software. Between 5 μl and 1 ml of sample was loaded onto a 0.46 × 5-cm guard column handpacked with C4 silica (Western Analytical; 15–20 μm) that was equilibrated with the aqueous buffer. Solutes were eluted with 9- to 12-min gradients ending at 100% of the organic buffer and absorbance detection was with a Hitachi Model 100-40 spectrometer equipped with a Altex flow cell. Gradients were designed such that peaks were always superimposed on a linear baseline, which was extrapolated across the solute elution peak. Baseline noise was ~0.0004 AU.

RESULTS AND DISCUSSION

Typical RP HPLC chromatograms for 0.1 and 1.0 μg of the defension HNP-2 are shown in Fig. 1. Figure 2 demonstrates the linearity of response for HNP-2 detected at either 215 or 280 nm. A peak with an area of 3–5 μV · s is barely detectable above the baseline. This corresponds to approximately 20 and 100 ng of peptide detected at 215 and 280 nm, respectively, so that peptide

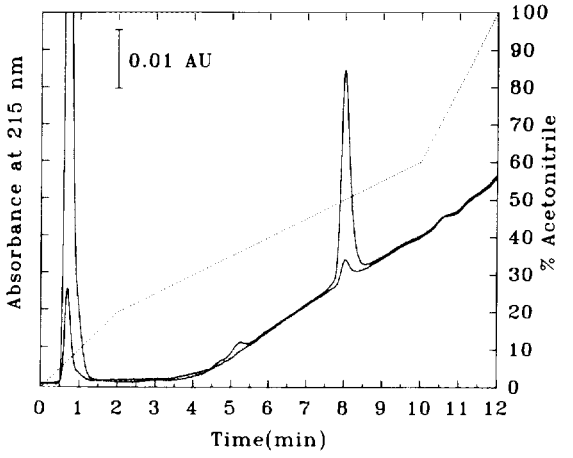

FIG. 1. Reverse-phase HPLC chromatograms of the defensin HNP-2. The column is a 0.46 × 5-cm guard column handpacked with C4 silica (15–20 μm, Western Analytical). Solvents are water/acetonitrile (0.1% TFA). Flow rates were 3 ml/min and the gradient is shown by the dashed line in the figure. Detection is at 215 nm. The peaks at 8 min are 0.1 and 1.0 μg HNP-2.

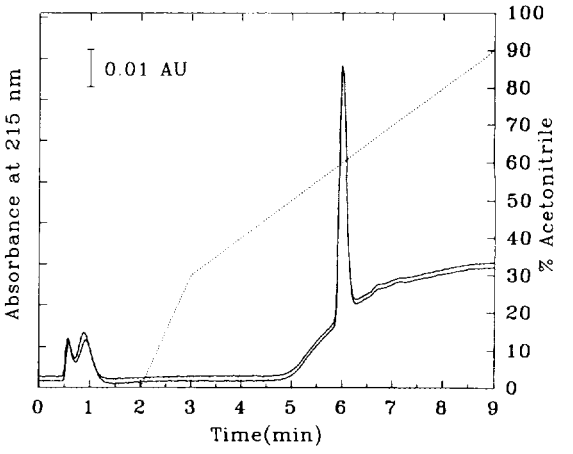

FIG. 3. RP HPLC chromatograms of the hydrophobic tripeptide Ac-ala-trp-ala-*O-tert*-butyl free in solution or entrapped within multilamellar POPC vesicles as described in the text. The HPLC conditions are described in the legend to Fig. 1 and the gradient is shown by the dashed line. Each injection contained 2.3 μg of peptide and 3.2 mg of POPC. The peaks at 6 min are the peptide.

concentrations of less than 1 μg/ml and at least up to 1 mg/ml can be quantitated under these conditions. Similar results were obtained for 3-methylindole and *N*-methylindole where detection was at the aromatic absorbance maximum (280 nm).

Figures 3 and 4 show the effect of lipid on the RP HPLC behavior of systems with bilayer partitioning dominated by hydrophobic or electrostatic interactions. Figure 3 illustrates RP HPLC chromatograms of a hydrophobic tripeptide (Acetyl-ala-trp-ala-*O-tert*-butyl) free in solution and entrapped within large multilamellar vesicles (MLV) of the zwitterionic lipid POPC. MLV are heterogeneous 1- to 10-μm particles that are composed of 100 or more concentric lipid bilayers separated by thin water spaces. The tripeptide molecules were entrapped within the MLV bilayers by repetitive freeze-thawing at high lipid concentration, followed by dilution. Approximately 80% of the total peptide was entrapped within the vesicles, in the interlamellar water space, or bound to the bilayer surfaces (1). The bilayers were found to be impermeant to the peptides over the time course of the experiments. As can be seen in Fig. 3, entrapment within MLV had no effect on the HPLC

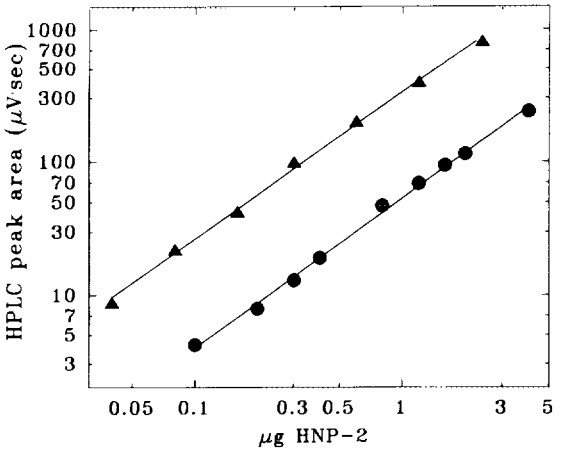

FIG. 2. Double logarithmic plot of HPLC peak area as a function of the amount of HNP-2 loaded. Conditions are as described in the legend to Fig. 1. Detection was at 215 nm (▲) or 280 nm (●). Peak areas are given in μV·s, where 10^6 μV = 1 AU.

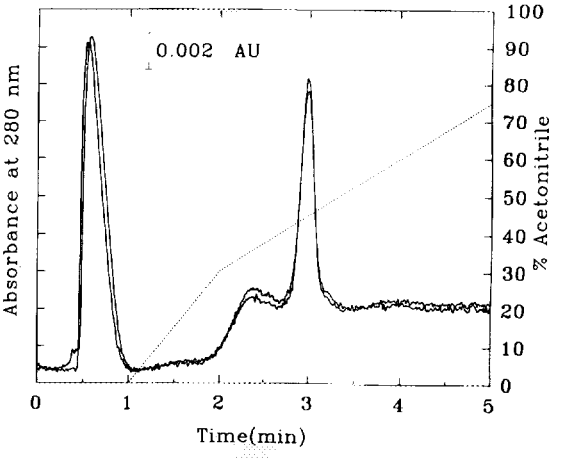

FIG. 4. RP HPLC of HNP-2 free in solution or incorporated into POPG multilamellar vesicles by freeze-thawing. In these experiments solvents were (A) water/2%NH_4 acetate (pH 6.0), and (B) 80% acetonitrile/20% A. The gradient is shown by the dashed line. Detection was at 280 nm. Each sample contained 2.5 μg HNP-2 and 150 μg POPG. The peaks at 3 min are the peptide.

retention time or on the peak area of the peptide. The lipid elutes only after the solvent gradient has reached 100% acetonitrile. These results demonstrate that the entrapped peptides are rapidly and quantitatively released from the lipid dispersions when they are loaded onto the reverse-phase column and that there is no interference from the lipid molecules bound to the column, which, in this case, are present in a 1400-fold weight excess. Similar results were obtained using 3-methylindole, which is dissolved in the bilayers under these conditions. Therefore, the butyl-silica surfaces of the RP column matrix appear to act as a very effective "detergent" that rapidly solubilizes lipid dispersions to release bound and entrapped solutes.

Figure 4 shows the results of a similar experiment in which strong electrostatic interactions are present. The MLV used in this experiment are composed of the anionic lipid POPG into which the cationic human defensin HNP-2 has been incorporated by repetitive freeze-thawing. There are strong electrostatic interactions between HNP-2 and POPG bilayers. For instance, HNP-2, which does not bind to uncharged POPC vesicles, causes rapid aggregation of 0.1-μm unilamellar POPG vesicles into large (>10 μm) insoluble particles. In experiments using water/acetonitrile/TFA solvents, such as in Fig. 3, no peptide elution peak was detectable, indicating that the electrostatic peptide–lipid complexes are not readily dissociated under these conditions. The results in Fig. 4 were obtained by using higher ionic strength buffers (A: water/2% (0.25 M) NH_4 acetate, pH 6.0; B: 80% acetonitrile/20% A). Under these conditions, the electrostatic complexes are "solubilized" on the RP column. It is clear from the identical retention times that there are no residual electrostatic interactions between the defensins and the POPG molecules bound to the RP column.

The lipid-containing half-cell in an equilibrium dialysis experiment is separated from the buffer-containing half-cell by a dialysis membrane, and the compound of interest is allowed to equilibrate across the membrane. The mole fraction partition coefficient (K_x) is given by

$$K_x = \frac{[S]_{bil}/[L]}{[S]_{water}/[W]} = \left(1 - \frac{[S]^{dial}_{lipid}}{[S]^{dial}_{buffer}}\right)\frac{[W]}{[L]}, \qquad [1]$$

where $[S]_{bil}$ and $[S]_{water}$ are the bilayer and water concentrations of solute, respectively. $[L]$ and $[W]$ are the molar concentrations of the lipid and water. $[S]^{dial}_{bil}$ and $[S]^{dial}_{buffer}$ are the measured concentrations of solute in the buffer and lipid dialysis halfcells and $[S]^{dial}_{lipid} = [S]_{bil} + [S]_{water}$. HPLC peak areas alone are sufficient for partition coefficient determination because the experimentally determined concentrations are expressed as a ratio. Also, because K_x is independent of total solute concentration, adsorption will have no effect if only the remaining soluble peptide is assayed. This is an important consideration for solutes such as defensins and hydrophobic peptides where adsorption onto dialysis membranes can be a serious problem.[3]

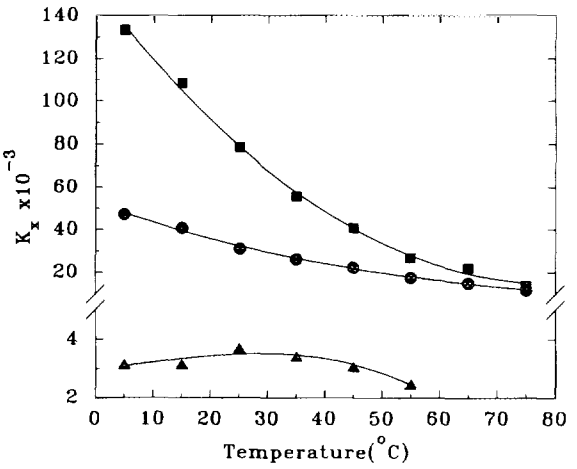

FIG. 5. Examples of mole-fraction partition coefficients determined using equilibrium dialysis and RP HPLC. The three compounds shown here are 3-methylindole (■), *N*-methylindole (●) and Ac-ala-trp-ala-*O*-*tert*-butyl (▲). Mole fraction partition coefficients were determined as described in the text.

Figure 5 shows examples of indole partition coefficients measured using equilibrium dialysis and HPLC. We have also used fluorescence spectroscopy to measure K_x for all three of these compounds (not shown) and the measured partition coefficients were very similar. Mole-fraction partition coefficients ranging from 10^2 to 10^7 can be measured using equilibrium dialysis and HPLC. This accessible range is larger than those for many other methods of K_x determination. Partition coefficients for small molecules such as 3MI or NMI can be determined with a precision[4] of ±2–3%, which is at least as good as the precision of experiments using radiolabeled compounds and is significantly better than most other methods that typically have precisions of 5–10%. For example, we found uncertainties of 6–9% for 3MI/bilayer partition coefficients assayed using fluorescence intensities. The increased precision with which K_x can be determined is crucial in thermodynamic analysis of partitioning because it allows the heat capacity change, ΔC_p, to be determined from the curvature of the temperature dependence of the free energy of partitioning. The heat capacity change, which is usually not obtained from bilayer partitioning data, is the key to un-

[3] For example, approximately 20% of HNP-2 binds irreversibly to 47-mm-diameter Spectrapor 2 dialysis membranes in an equilibrium dialysis experiment begun with 50 μg of peptide in 1 ml of buffer.

[4] RMS deviation of individual measurements from the mean for separate determinations of partition coefficients for distinct, identical samples run simultaneously.

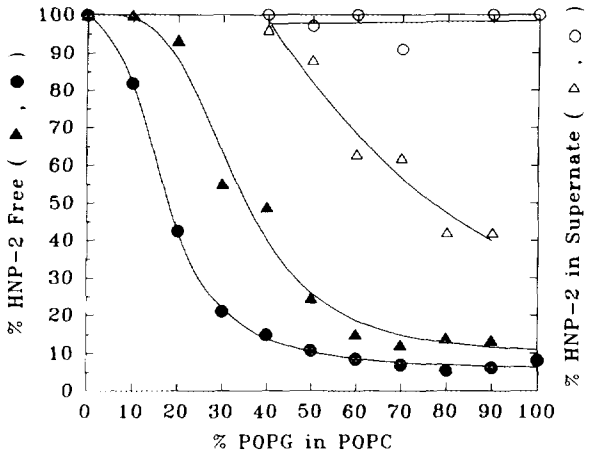

FIG. 6. Examples of electrostatic interactions measured using equilibrium dialysis and RP HPLC. Binding of HNP-2 to unilamellar vesicles of mixtures of POPC and POPG at 1 mM (▲) and 10 mM (●) total lipid. % free is the percentage of total peptide which is not bound to the vesicles. The solid lines are sigmoidal functions fit to the data. % soluble is the percentage of HNP-2 in the HNP-2 vesicle solutions, at 1 mM (△) or 10 mM (○) lipid, which is not removed by centrifugation at 1000*g* for 10 min.

derstanding the thermodynamics of bilayer partitioning (17) as it is for other hydrophobic processes (11–13).

Figure 6 shows an example of electrostatic interactions assayed by equilibrium dialysis and HPLC. In this experiment, unilamellar vesicles composed of mixtures of zwitterionic lipid POPC and the anionic lipid POPG were equilibrated with the defensin HNP-2 at 25 μg/ml which has a net charge of +3 (7). Lipid concentrations of 1 and 10 mM were used. After 24 h equilibration, peptide concentrations were assayed by HPLC using approximately 1 μg of peptide for each determination. In this case detection was at 280 nm because of the high background absorbance of 2% NH_4 acetate at 215 nm. The sigmoidal binding curves are typical of polycation interactions with anionic bilayers (16). The amount of peptide-induced aggregation of the vesicles was also assayed in these experiments by HPLC. HNP-2 concentration was measured in aliquots of HNP-2/vesicle solutions before and after centrifugation at 1000*g* for 10 min (see Fig. 4). These mild centrifugation conditions will only remove large aggregates (>1 μm). These results indicate that 25 μg/ml (7 μM) HNP-2 causes significant aggregation of anionic vesicles at 1 mM lipid, but not at higher lipid concentrations. Both the binding and the aggregation results will be discussed in detail in a future publication.

We have described the application of equilibrium dialysis and RP HPLC to the quantitation of both hydrophobic and electrostatic interactions with lipid bilayers. Other methods frequently used are fluorescence spectroscopy, radioactive labels, chemical derivitization, absorbance spectroscopy, and zeta potential measurements (16). Quantitative HPLC in combination with equilibrium dialysis is, in many cases, preferable to all of these methods because of its high sensitivity and nondestructive nature, because derivitization or labeling is not required, and because precise temperature and concentration control are possible. The high sensitivity is important when working with precious samples such as the defensins we are studying. Peptide bilayer partition coefficients can be determined with less than 1 μg of peptide, and all peptide samples can be recovered from the peptide–lipid mixtures for repurification. In addition, the precision of the measurement is significantly higher using equilibrium dialysis and RP HPLC than for many other methods and is free from errors resulting from solute adsorption. This allows the extraction of ΔC_p from partitioning data. These methods will have broad applicability both in studies of peptides and proteins in membranes and in studies of membrane partitioning of small molecules.

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