A Novel Method to Measure Self-association of Small Amphipathic Molecules

TEMPERATURE PROFILING IN REVERSED-PHASE CHROMATOGRAPHY*

Biophysical techniques such as size-exclusion chromatography, sedimentation equilibrium analytical ultracentrifugation, and non-denaturing gel electrophoresis are the classical methods for determining the self-association of molecules into dimers, trimers, or other higher order species. However, these techniques usually require high (mg/ml) loading concentrations to detect self-association and also possess a lower size limit that is dependent on the ability of the technique to resolve monomeric from higher order species. Here we describe a novel, sensitive method with no upper or lower molecular size limits that indicates self-association of molecules driven together by the hydrophobic effect under aqueous conditions. “Temperature profiling in reversed-phase chromatography” analyzes the retention behavior of a sample over the temperature range of 5–80 °C during gradient elution reversed-phase high-performance liquid chromatography. Because this technique greatly increases the effective concentration of analyte upon adsorption to the column, it is extremely sensitive, requiring very small sample quantities (microgram or less). In contrast, the classical techniques mentioned above decrease the effective analyte concentration during analysis, decreasing sensitivity by requiring larger amounts of analyte to detect molecular self-association. We demonstrate the utility of this technique with 14-residue cyclic and linear cationic peptides (<2000 Da) based on the sequence of the de novo-designed cytolytic peptide, GS14. The only requirements for the analyte molecule when using this technique are its ability to be retained on the reversed-phase column and to be subsequently removed from the column during gradient elution.

The detection of molecular self-association and aggregation is an important concern for molecules intended for biological applications, such as proteins, peptides, and small organic drug molecules. A plethora of biophysical techniques already exist for the detection of molecular self-association in aqueous solutions, including spectroscopic (NMR, CD, Fourier-transform infrared spectroscopy (FTIR), fluorescence, light scattering), chromatographic (affinity, size-exclusion (SEC)/gel filtration), and other techniques (matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), non-denaturing PAGE, sedimentation equilibrium analytical ultracentrifugation). However, some of the classical techniques commonly used for measuring self-association (sedimentation equilibrium analytical ultracentrifugation, non-denaturing PAGE, and size-exclusion chromatography) possess a lower limit to the molecular size that can be clearly resolved, such that fewer methods exist for detecting self-association in smaller molecules. With a view to solving this problem, we describe here a novel method for measuring self-association, referred to as “temperature profiling in reversed-phase chromatography,” based on observation of the conformation-dependent response of peptides to RP-HPLC under changing temperature.

Much of the efficacy of RP-HPLC as a probe of polypeptide stability, folding, and conformation lies in the extensive range of stationary phases and/or mobile phase conditions available to the researcher when relating polypeptide elution behavior with structural features (e.g. the amphipathicity of α-helices or β-sheets; destabilization of conformation) (1–11) and/or biological activity (e.g. antimicrobial potency, receptor binding) (12–14). Such studies are based on the premise that the hydrophobic interactions between polypeptides and the nonpolar stationary phase characteristic of RP-HPLC (15–17) mimic the hydrophobicity and interactions between nonpolar residues, which are the major driving forces for protein folding and stability, including those dictating the level and stability of polypeptide oligomerization. We believe temperature adds another dimension to such applications, with physicochemical studies of RP-HPLC of polypeptide solutes under conditions of varying temperature (hence, “temperature profiling”) allowing even more insight into conformational stability and self-association of peptide solutes.

The present study demonstrates the utility of temperature profiling in RP-HPLC to monitor self-association of small cyclic peptides based on the de novo-designed amphipathic cytolytic peptide, GS14, through the interpretation of peptide elution behavior over a temperature range of 5–80 °C. This approach is shown to be simple to operate, highly sensitive, requires low sample quantities (nanograms to low micrograms) and is capable of analyzing small molecules (<2000 Da). In addition, the applicability of this novel approach for other small molecules is also discussed.

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‡ The abbreviations used are: SEC, size exclusion chromatography; RP-HPLC, reversed-phase high performance liquid chromatography; lin, linear.
**Materials and Methods**

**Peptide Synthesis, Purification, and Cyclization**—Linear and cyclic peptides were synthesized using standard Fmoc/t-butoxycarbonyl chemistry, as described previously (17). N-terminal and C-terminal groups of linear peptides were left as free amino and carboxyl groups, respectively. Peptides were cleaved using standard HF protocol and purified by reversed-phase chromatography. Peptides intended for cyclization were cyclized, deformylated, and purified using the method of Kondejewski et al. (17).

**Analytical Reversed-Phase Analysis of Peptides**—Peptides were analyzed on an Agilent 1100 Series liquid chromatograph (Little Falls, DE). Runs were performed on a Zorbax 300SB-C8 column (2.1 mm inner diameter) × 150 mm, 300-A pore size, 5-μm particle size) from Agilent Technologies using a linear AB gradient (0.5% B/min) and a flow rate of 0.35 ml/min, where solvent A is 0.05% aqueous trifluoroacetic acid, pH 2 and solvent B is 0.05% trifluoroacetic acid in acetonitrile. Analyses were performed in 5 °C increments, from 5 to 80 °C.

**CD Spectroscopy**—CD measurements were obtained on a JASCO J-810 Spectropolarimeter at 20 °C. Temperature was controlled in a Lauda K-810 Spectropolarimeter at 20 °C. Concentrations were calculated based on average isotopic masses. In the GS14 X4 series, GS14 A4 denotes D-alanine substituted at position 4 in GS14. In the GS14K4 hydrophobicity series, GS14K4 L3/A3 denotes substitution of three leucine residues by three alanine residues in GS14K4.

**Results**

**Peptide Design and RP-HPLC Retention Behavior**

Parent Peptide (GS14)—GS14 is a 14-residue cytolytic amphipathic peptide designed de novo in our laboratory (Fig. 1). The sequence of GS14 was originally developed as an analog of the cationic antimicrobial peptide gramicidin S (17), whose activity is thought to be a result of interaction with lipid membranes (9, 17–25). GS14 has been previously studied by a number of techniques, including RP-HPLC (9, 26), CD spectroscopy (9, 17, 24–26), NMR spectroscopy (9, 27), UV absorption, and fluorescence spectroscopy (26). It is a cyclic β-sheet molecule that contains six aliphatic residues (3 Val and 3 Leu) on the nonpolar face and four basic (Lys) residues on the polar face; the segregation of nonpolar and charged residues on opposite sides of the molecule makes it an extremely amphipathic peptide (Fig. 2A) (27). GS14 is also a suitable choice for de novo peptide design and structure-activity studies because the cyclic nature of the peptide prevents amino acid substitutions from significantly altering secondary structure, i.e. switching the β-sheet to an α-helix or random coil. Previous studies indicated that GS14 aggregated above 50 μM peptide concentration in aqueous solution (26). Unfortunately, attempts to determine the oligomerization state failed by sedimentation equilibrium analytical ultracentrifugation (because of the low molecular weight) and by size-exclusion chromatography (because of high sample load requirements and low peptide solubility), thus emphasizing the need for an alternative technique to examine self-association of this molecule. GS14 is biologically active against human red blood cells (hemolytic); however, it shows little or no antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and yeast (17).

**RP-HPLC of Amphipathic Peptides**—It is well documented that the formation of a hydrophobic binding domain due to peptide secondary structure can affect peptide interactions with reversed-phase matrices, this effect having been observed both for amphipathic α-helical peptides (4, 6, 7, 11–14, 28, 29) and amphipathic, cyclic β-sheet peptides (8, 9). Thus, peptides containing such preferred binding domains will exhibit significantly greater retention times compared with analogs with the same amino acid composition (i.e. the same intrinsic hydrophobicity) but lacking such a domain (8, 28). Indeed, the chromatography conditions characteristic of RP-HPLC (hydrophobic stationary phase, nonpolar eluting solvent) are able to induce and stabilize secondary structure in both potentially α-helical (28, 30–33) and cyclic, β-sheet (33) peptides; concomitantly, tertiary and quaternary structure is disrupted by such conditions (1, 12, 28, 34–37). Due to the intimate interaction of the nonpolar faces of amphipathic cyclic analogs with a reversed-phase matrix, any differences in effective hydrophobicity of these preferred binding domains via amino acid substitutions in this domain will be readily monitored through subsequent differences in RP-HPLC retention time behavior.

**Peptide Diastereomer (GS14K4)—**Because GS14 has such low specificity toward microbial membranes, analogs of GS14 have been studied in an attempt to reverse this activity profile and to create an antimicrobial peptide with minimal toxicity to human cells and high activity toward pathogens. One such analog is GS14K4 (Fig. 2B), a diastereomer of GS14 with the D-enantiomer of L-Lys substituted at position 4 (9, 24). While
the positively charged l-Lys 4 in GS14 is normally on the polar face, the d-Lys 4 in GS14K4 is positioned on the nonpolar face and disrupts the β-sheet structure in benign medium. GS14K4 exhibits an activity profile opposite to that of GS14 with a 10^3- to 10^4-fold improvement in therapeutic index (a measure of peptide specificity for microbial cells over human cells). Since the highly hydrophilic d-Lys is positioned on the nonpolar face of the peptide, the amphipathicity of the molecule is significantly decreased, thus decreasing its retention time in RP-HPLC relative to GS14 by over 23 min at 80 °C (GS14, 85.6 min; GS14K4, 62.0 min, Table I). A comparison of the effect of temperature on the elution behavior of GS14 and GS14K4 is shown in Fig. 3. While GS14 aggregates at concentrations above 50 μM (84 μg/ml) in solution due to the high hydrophobicity of its nonpolar face, no aggregation was observed in GS14K4 up to the highest concentration studied, 175 μM (292 μg/ml) (26). Because GS14 and GS14K4 possess the ability to be analyzed in an RP-HPLC system and exhibit marked differences in activity, structure, and aggregation phenomena, these de novo-designed cyclic peptides are ideal candidates for the investigation of relationships between molecular self-association and biological activity using the RP-HPLC technique of temperature profiling.

**GS14 X₄₄ Analogs**—The GS14 X₄₄ analogs replaced the d-Lys in GS14K4 with a series of other d-amino acids at position 4 (Fig. 1). The substituted amino acids ranged in hydrophobicity from D-Asn and D-Tyr (least hydrophobic) to D-Leu and D-Phe (most hydrophobic) (12, 38) and altered both the overall hydrophobicity and amphipathicity of the peptide caused by the substituted amino acid being positioned on the nonpolar face. The analogs also had a lower net charge versus GS14K4 (+3 instead of +4 from the Lys residues in GS14, Fig. 2). The differences in the peptide composition influenced peptide retention time in RP-HPLC, with the most hydrophobic amino acid substitution (d-Leu in GS14L₄₄) producing the largest retention time, compared with GS14K4, which had the least hydrophobic amino acid (d-Lys) at position 4 and the lowest retention time within the series. Retention times at 80 °C ranged from 62 to 100 min for d-Lys and d-Leu peptides, respectively (Table I).

**GS14K4 Hydrophobicity Analogs**—To investigate the role of peptide hydrophobicity in microbial specificity, analogs of GS14K4 were previously created with variations of the aliphatic residues in the nonpolar face (24). A subset of these hydrophobicity analogs was now assessed for self-association in the present study (Fig. 1). The selected analogs had lower nonpolar face hydrophobicity relative to GS14K4, with valines and/or leucines in GS14K4 substituted with the less hydrophobic alanine residue (12, 38), e.g. peptide GS14K4 V₃/A₃ had three valines substituted with three alanines, producing a peptide with one lysine, three leucines, and three alanine residues in the nonpolar face. GS14K4 and GS14K4 V₃/A₃ showed antimicrobial activity with minimal toxicity to human red blood cells; the other analogs selected for this study possessed insufficient hydrophobicity to exert cytolytic effects on either microbial or human cells. This series of analogs varied substantially in hydrophobicity (retention times ranged from 25 to 62 min at 80 °C, Table I).

**Linear Analogs**—The peptide analogs GS14 lin, GS14K4 lin, and GS14K4 A₆ lin served as linear controls versus cyclic peptides, possessing the same intrinsic hydrophobicity owing to the identical amino acid sequences but having different effective hydrophobicity due to their noncyclic nature. For example, at 80 °C, GS14 lin had a retention time of 55 min whereas its cyclic analog, GS14, had a retention time of 85 min (Table I), clearly demonstrating the enhanced hydrophobicity in forming the preferred binding domain by cyclization. GS14K4 A₆ lin served as the control peptide in the normalized temperature profiles for all peptides studied. This peptide lacked any defined structure and had insufficient hydrophobicity to dimerize.

**Proposed Mechanism of Action**—The proposed mechanism of action for temperature profiling in RP-HPLC is shown in Fig. 4. The mechanism is based on two assumptions, with the first based on the fact that loading the peptide onto the top of the column in aqueous conditions concentrates the peptide locally on the hydrophobic matrix bound by its hydrophobic preferred binding domain. As the acetonitrile concentration increases during gradient elution, the acetonitrile content becomes high enough to allow peptide partitioning between the matrix and the mobile phase. Thus, in the early stages of partitioning, our assumption is that the effective peptide concentration in solution is extremely high as a result of the concentration of peptide achieved on binding to the matrix during sample loading. The second assumption is that because of this concentrating effect in solution, peptides in this study that are capable of self-association during gradient elution are in equilibrium between a monomeric peptide bound to the matrix (Fig. 4A, top left), an unbound monomeric form in the mobile phase (Fig. 4A, top center), and an unbound dimeric form in the mobile phase (Fig. 4A, top right). As a result, peptides that dimerize in solution are eluted faster since the on-rate for the bound state is decreased, i.e. retention time is lower than what would be observed if the molecule was unable to dimerize and was only in the monomeric state in the mobile phase. The dimerization is temperature-dependent, with more dimerization occurring in solution at low temperatures. As the temperature is increased, equilibrium is shifted toward the monomeric form in solution.
due to disruption of the dimer. The higher solution concentration of monomer during partitioning increases the on-rate for the bound state, and retention time thus increases, as clearly shown for GS14 in Fig. 3. It should be noted that the increase in temperature also introduces other general effects on retention time because of lower mobile phase viscosity and a significant increase in mass transfer between the stationary and mobile phases. These effects decrease retention time with increasing temperature in a linear fashion. Thus, in the case of peptides that do not undergo a significant conformational change over the temperature range profiles, one would expect to see a linear decrease in retention time with increasing temperature because of the lower mobile phase viscosity and increase in mass transfer. This would be the case for a monomeric peptide with either a random coil or an undefined structure (Fig. 4B) or for a very stable peptide with a specific structure that does not change over the entire temperature range; for example, a stable amphipathic monomeric α-helix.

There are thus two opposing effects on peptide retention time

### Table I

| Peptide        | $t_R(80^\circ C)$ | $t_R(5^\circ C)$ | $T_P$ | $t_R(T_P)$ | $t_R(T_P) - t_R(5^\circ C)$ | $t_R(T_P) - t_R(80^\circ C)$ |
|----------------|------------------|------------------|-------|------------|-----------------------------|-------------------------------|
| GS14           |                  |                  |       |            |                             |                               |
| GS14X_4 analogs |                  |                  |       |            |                             |                               |
| GS14 LD_4      | 100.4            | 96.8             | 55    | 101.8      | 5.0                         | 1.4                           |
| GS14 FD_4      | 94.1             | 90.6             | 55    | 95.3       | 4.7                         | 1.2                           |
| GS14 ND_4      | 83.0             | 83.2             | 40    | 85.1       | 1.9                         | 2.1                           |
| GS14 AD_4      | 73.3             | 73.5             | 40    | 75.3       | 1.8                         | 2.0                           |
| GS14 KD_4      | 62.0             | 63.5             | 20    | 65.3       | 0.3                         | 3.3                           |
| GS14K4 hydrophobic analogs | 62.0 | 65.0 | 20 | 65.3 | 0.3 | 3.3 |
| GS14K4 V3/A3  | 47.4             | 52.5             | 5     | 52.5       | 0                           | 5.1                           |
| GS14K4 L3/A3  | 39.8             | 46.4             | 5     | 46.4       | 0                           | 6.6                           |
| GS14K4 A6     | 25.8             | 33.3             | 5     | 33.3       | 0                           | 7.5                           |
| Linear analogs |                  |                  |       |            |                             |                               |
| GS14 lin       | 55.6             | 62.8             | 15    | 63.1       | 0.3                         | 7.2                           |
| GS14K4 lin    | 46.2             | 51.6             | 5     | 51.6       | 0                           | 5.4                           |
| GS14K4 A6 lin | 21.5             | 30.7             | 5     | 30.7       | 0                           | 9.4                           |

*a* Peptide retention time at 80 °C as determined by reversed-phase HPLC (see "Materials and Methods").

*b* Peptide retention time at 5 °C as determined by reversed-phase HPLC (see "Materials and Methods").

*c* Temperature at which the maximum retention time is observed over the temperature range 5–80 °C.

*d* Peptide retention time at temperature $T_P$.

*e* Difference in retention time between $t_R$ at the $T_P$ and $t_R$ at 5 °C.

*f* Difference in retention time between $t_R$ at the $T_P$ and $t_R$ at 80 °C.

*g* GS14KD_4 and GS14K4 denote the same peptide with D-Lys at position 4.

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**FIG. 3.** Effect of temperature on RP-HPLC elution profiles of GS14 and GS14K4. The runs were performed on a Zorbax 300-SB C8 column (2.1 (inner diameter) × 150 mm) at 0.5% B/min, a flow-rate of 0.35 ml/min, and temperatures of 5, 20, 55, and 80 °C, where solvent A was 0.05% aqueous trifluoroacetic acid and solvent B was 0.05% trifluoroacetic acid in acetonitrile. The sequences of GS14 and GS14K4 are shown in Fig. 1.

**FIG. 4.** Proposed mechanisms of temperature profiling by RP-HPLC. Panel A, at lower temperatures, peptides capable of self-associating in aqueous solution via the hydrophobic effect establish equilibrium between monomer and dimer in the mobile phase. Only the monomeric form of the peptide in solution can partition with the alkyl ligands on the reversed-phase column, and retention time is decreased due to the large population of dimers in solution. At higher temperatures, the population of dimers in solution during partitioning decreases, increasing the concentration of monomeric peptide in solution and increasing retention time. At some temperature no dimer would exist in solution. Panel B, with a peptide that does not form an amphipathic structure and cannot dimerize, the peptide binds to the stationary phase and partitions in the mobile phase as a monomer at both low and high temperatures. Thus, the effect of temperature on retention time is linear and decreases with increasing temperature.
as the temperature is increased: increasing retention time as dimerization is disrupted and decreasing retention time from these general temperature effects. For molecules that dimerize, the effect of dimerization is dominant at low temperature over the general temperature effects. Thus, at low temperatures, the dimerization effect dominates and retention time increases with increasing temperature until a certain temperature is reached, which we designate $T_p$ (the temperature at the transition point, equivalent to the temperature at which maximum peptide retention time is achieved). At this temperature, the temperature effects that increase retention time (disruption of the dimer) are equivalent to those that decrease retention time. Above the temperature, $T_p$, the net result is a decreased retention time as lower viscosity and higher mass transfer effects predominate.

**Peptide Structure**

**CD Spectroscopy**—The structure of peptides in this study was characterized by CD spectroscopy in aqueous buffer and in a mixture of buffer and trifluoroethanol (Fig. 5 and Table II), an organic solvent known to mimic the nonpolar environment characteristic of RP-HPLC (28). Cyclic peptides do not exhibit CD spectra of typical $\beta$-sheet character (the classic $\beta$-sheet spectrum possesses a single minimum at 215–218 nm). However, the solution structure of GS14 has previously been determined by NMR techniques and is known to be a $\beta$-sheet (Fig. 2) (27); thus, the CD spectrum for GS14 shown in Fig. 5A, resembling that of an $\alpha$-helix with minima near 208 and 222 nm, is due to the dominating effects of the type II $\beta$-turns in these small cyclic peptides. GS14K4 was known to exhibit disrupted $\beta$-sheet structure by NMR (Fig. 2) (27); its CD spectrum is shown in Fig. 5A with a significantly less negative mean residue molar ellipticity at 222 nm ([$\theta$]$_{222}^\circ$) than GS14. GS14 $\chi_1$,4 peptides all exhibited CD spectra similar to GS14K4 in benign and 50% trifluoroethanol conditions (data not shown). GS14K4 and its hydrophobicity analogs GS14K4 L3/A3, GS14K4 V3/A3, and GS14K4 A6 all exhibited a lesser degree of $\beta$-sheet structure than GS14 in benign buffer as shown by the smaller molar ellipticity values at 222 nm, but were induced to form $\beta$-sheet structure in trifluoroethanol (Table II). The linear control peptides GS14 lin and GS14K4 A6 lin both exhibited spectra typical of a random coil in aqueous conditions with a minimum below 200 nm and a maximum at 220 nm (Fig. 5A). But in 50% trifluoroethanol, GS14 lin appeared to be induced into substantially more $\beta$-sheet structure than GS14K4 A6 lin (Fig. 5B). Based on GS14K4 A6 lin behavior in 50% trifluoroethanol, we assume that it behaves similarly in the hydrophobic environment of the column matrix and also binds mainly in an undefined structure.

**Temperature Profiles**

**RP-HPLC Analysis**—The absolute retention times of GS14 and other cyclic peptides differed greatly because of the differences in one or more of the following properties: structure, amphibipathy, and/or intrinsic hydrophobicity (Table I). To examine graphically the effect of temperature on retention time, peptide retention times were normalized relative to the values obtained at 5 or 80 °C, the lowest and highest temperature used in this study (Fig. 6). Normalizing at 5 °C allowed determination of $T_p$, the transition temperature (Fig 6A), while normalizing at 80 °C corrected the retention time to a temperature where the effects of structure and self-association are minimal (Fig 6B).

**GS14 and GS14K4**—The cyclic $\beta$-sheet peptides GS14 and GS14K4 showed a deviation from linearity in the normalized temperature profiles (Fig. 6, A and B), indicating a change in structure over the 5–80 °C temperature range. In the temperature profile normalized to retention times at 5 °C, GS14 had a $T_p$ of 55 °C, significantly higher than the $T_p$ of GS14K4, which was 20 °C (Fig. 6A). Thus, from Fig. 3, the illustrated elution profiles for GS14 and GS14K4 at the $T_p$ values of 55 and 20 °C, respectively, represent the maximum retention times for these peptides. Note that the clear sharpening of the GS14K4 peak above 20 °C (increased peak height and decreased peak width) is due to the shift of the monomer–dimer equilibrium to favor the monomeric species in solution during partitioning. Below the $T_p$ value of 20 °C for GS14K4, the peak width is broad due to the increased presence of the dimeric form in solution. The decrease in retention time above 20 °C for GS14K4 is consistent with the aforementioned general effects of temperature.
specifically concerning the increase in mass transfer between stationary and mobile phases as the temperature is increased. In contrast, the peak width of GS14 is increasing as the temperature is raised from 5 °C up to its \( T_p \) value of 55 °C and remains broad even at 80 °C since the temperature is still not high enough to eliminate the monomer-dimer equilibrium (Fig. 6). It is the shifts in the monomer-dimer equilibrium with varying temperature, in conjunction with general temperature effects, that affect the overall peak elution behavior (retention time and peak shape). These effects are much more dramatic for GS14K4 since the proportion of monomer in solution is high at a much lower temperature compared with GS14.

In contrast to the results obtained from cyclic GS14 and GS14K4, the linear peptides generally exhibited a negative relationship between RP-HPLC retention time and temperature, e.g. GS14K4 A6 lin (Fig. 6A). Interestingly, GS14 lin had normalized changes in retention time above 0 at temperatures of 10, 15, and 20 °C (\( T_p = 15 \) °C), suggesting that self-association may have occurred at these low temperatures (Fig. 6A). Similarly, Fig. 6B shows the temperature profiles normalized to retention times at 80 °C. The profiles of GS14 lin and GS14K4 A6 lin were almost superimposable except at temperatures below 20 °C. This indicates that additional structural effects (e.g. changes in \( \beta \)-sheet content or degree of self-association) did not occur from 20 to 80 °C.

The temperature profiles in Fig. 6C for GS14 and GS14K4 cyclic and linear peptides were zeroed at 80 °C and also normalized for general temperature effects using the control peptide, GS14K4 A6 lin. Thus, these profiles showed the effects of temperature only on self-association, without the negative linear retention time decrease from general effects of temperature. GS14 exhibits the most self-association, with a change in retention time of 12.5 min at 5 °C compared with 80 °C, significantly larger than that of GS14K4 (6.5 min). It is interesting that the linear peptides GS14 lin and GS14K4 lin also show the ability to change structure/self-associate at low temperatures. Fig. 6C also illustrated our hypothesis that by further increasing temperature beyond 80 °C, equilibrium is completely shifted to the monomeric state in the mobile phase, and the temperature profiles of all the peptides would fall along the normalized baseline at sufficiently high temperature.

**GS14 X\(_{D}A\) Analogs**—All peptides with \( D \)-amino acid substitutions had temperature profiles that deviated from linearity (Fig. 7). The change in retention time at \( T_p \) for all peptides in the series was proportional to the hydrophobicity of the \( D \)-amino acid substituted in the peptide, as determined by previous amino acid hydrophobicity scales (12, 38). The \( D \)-peptides GS14 F\(_{D}A\), GS14 L\(_{D}A\), and GS14 A\(_{D}A\) had \( T_p \) values identical to GS14 (\( T_p \) of 55 °C, Table I). Notably, an increase in hydrophobicity of the nonpolar face (e.g. with \( D \)-Phe, \( D \)-Leu, or \( D \)-Ala in
addition to the three Leu residues and three Val residues of GS14) increased the magnitude of the change in retention time only slightly (5.2, 5.0, and 4.7 min, respectively, compared with GS14 with a value of 4.2 min, Table I). This result showed that, above a certain nonpolar face hydrophobicity, there was no further increase in $T_p$ for these cyclic peptides, suggesting that 55 °C may represent a maximum limit for $T_p$ in this cyclic $\beta$-sheet structural framework. Decreasing the hydrophobicity of the $\phi$-substitution at position 4 from $\phi$-Phe, $\phi$-Leu or $\phi$-Ala to $\phi$-Tyr or $\phi$-Asn dramatically decreased the $T_p$ value to 40 °C and the magnitude of the change in retention time to 1.9 and 1.8 min for $\phi$-Tyr and $\phi$-Asn, respectively, compared with GS14 (Fig. 7A and Table I). $\phi$-Lys at position 4 had the lowest intrinsic hydrophobicity, the lowest $T_p$ (20 °C) and the lowest difference in retention time between $T_p$ and 5 °C (0.3 min, Fig. 7A, and Table I). Fig. 7B shows the temperature profiles normalized to retention times at 80 °C and illustrates that, despite the large change in hydrophobicity of these cyclic peptides, they all show large deviations from the linear behavior of control peptide GS14K4 A6 lin. Following normalization for general temperature effects using the GS14K4 A6 lin peptide, all these cyclic peptides show the ability to dimerize (Fig. 7C).

**GS14K4 Hydrophobicity Analogs**—The peptides with temperature profiles that were closest to the control GS14K4 A6 lin profile, GS14K4 L3/A3 and GS14K4 A6, had the lowest intrinsic hydrophobicity (Fig. 8A). The analogs also showed that as nonpolar face hydrophobicity decreased, $\beta$-sheet structure was diminished both in benign medium and in the presence of 50% trifluoroethanol (compare $\theta_{222}$ in Table II) and the temperature profile was closer to linearity (Fig. 8B). Note also that the normalized profiles all fell below that of GS14K4, showing that GS14K4, possessing the highest hydrophobicity of the series, also had the highest degree of self-association among the hydrophobicity analogs (Fig. 8B). Except for GS14K4 V3/A3, the peptides that showed the smallest deviations from linearity also showed no antimicrobial or hemolytic activity (Table II).

In this study, we used the differences in retention time at 80 °C as a measure of the differences in apparent hydrophobicity of the various analogs. Fig. 9, A and B show the relative hydrophobicity of the analogs using a 1% acetonitrile/min linear gradient at 80 °C. The RP-HPLC retention times shown in Table I were determined at a gradient rate of 0.5% acetonitrile/min. This shallower gradient was used to amplify the differences between peptide analogs. At 80 °C, the effects of dimerization are essentially eliminated, with all amphipathic molecules being in their monomeric form during partitioning in RP-HPLC. For example, compared with GS14K4 A6, the addition of 3 Leu residues (i.e. GS14K4 V3/A3) increases retention time at 80 °C by 21.6 min (47.4–25.8 min, Table I), with each leucine residue contributing 7.2 min. In comparison, the addition of 3 Val residues (i.e. GS14K4 L3/A3) increases retention time at 80 °C by 14 min (39.8–25.8 min, Table I), with each valine residue contributing 4.7 min. Thus, the sum of 3 Val and 3 Leu residues added to GS14K4 A6 should increase retention time by 14 + 21.6 = 35.6 min, which is close to the observed value difference of 62.0–21.6 = 40.4 min (i.e. the difference between GS14K4 and GS14K4 A6 retention times at 80 °C).

With the GS14K4,4 analogs the effects of substitution were in the identical order of the hydrophobicities of the substituted residues as determined by RP-HPLC from a previous study. Thus, Sereda et al. (12) determined the relative hydrophobicities of amino acid side chains in the center of the nonpolar face of an amphipathic $\alpha$-helix containing 6 Leu residues as 1.99, 0.96, 0, −3.94, −8.85, and −12.25 min for Leu, Phe, Ala, Tyr, Asn, and Lys, respectively. Interestingly, the relative side chain RP-HPLC-derived hydrophobicities determined in the present study from substitutions in the nonpolar surface of the amphipathic cyclic $\beta$-sheet peptide containing 3 Val and 3 Leu residues were similar to those obtained from amphipathic $\alpha$-helices when compared at the same gradient rate (3.1, 2.1, 0, −5.5, −10.4, and −16.0 min for Leu, Phe, Ala, Tyr, Asn, and Lys, respectively).
Finally, it is worth noting that, from Table I, the minimum number of hydrophobic residues required for substantial self-association of the cyclic peptide analogs appears to be six residues, specifically in this case, 3 Leu and 3 Val residues making up the hydrophobic face of GS14 ($T_p = 55$ °C). Replacement of the 3 Leu residues (GS14K4 L3/A3) or the 3 Val residues (GS14 V3/A3) with the considerably less hydrophobic Ala residues decreased self-association to almost negligible levels ($T_p = 5$ °C for the latter two analogs). This observation reflects in an interesting manner extensive work carried out in this laboratory on the role of hydrophobic residues in stabilizing amphipathic $\alpha$-helical coiled-coils (10, 39–44). Thus, it was determined that a minimum of six hydrophobic residues, capable of strong affinity interactions, were required for $\alpha$-helices to dimerize in solution, i.e. two hydrophobic residues in each of the three heptads making up the seven-residue sequence repeat characteristic of $\alpha$-helical coiled-coils (39).

**DISCUSSION**

**Reversed-Phase Chromatography and Mechanism of Elution**—The time that a molecule is retained by a reversed-phase chromatography column is based on analyte partitioning between the aqueous mobile phase and the hydrophobic stationary phase. During linear gradient elution, the composition of the mobile phase changes with an increased percentage of organic solvent as a function of time. As noted previously, amphipathic molecules are generally retained longer since the hydrophobic region is localized to one side of the molecule, forming a preferred binding domain (4, 6–9, 11–14, 28, 29); also, the tertiary and quaternary structures of globular proteins are generally disrupted upon binding to the stationary phase in RP-HPLC, since hydrophobic interactions responsible for the higher order molecular structures are disrupted (1, 12, 28, 34–37). If the monomeric structure of a protein is unfolded in the hydrophobic environment of the matrix and in the organic solvents used in RP-HPLC, then specific self-association cannot occur. However, both organic solvent in the mobile phase and contact with the hydrophobic matrix can stabilize the secondary structure of amphipathic molecules ($\alpha$-helix and $\beta$-sheet) (28, 30–33). Though oligomerization of amphipathic molecules will be disrupted on binding to the hydrophobic matrix, the stabilization of the secondary structure by the organic solvent in the mobile phase may facilitate the potential for association of secondary structural elements. The association of amphipathic molecules in the mobile phase during partitioning will decrease the retention time of the molecule compared with the retention time of a molecule where no self-association takes place. This decrease would presumably be proportional to the molecular binding affinity of the secondary structure element in the mobile phase. Thus, this technique will work for any molecule whose structure is stabilized by the hydrophobic matrix and hydrophobic solvents in the mobile phase.

**Size-exclusion Chromatography versus Reversed-Phase Chromatography**—Unlike RP-HPLC, in which hydrophobic interactions between the analyte molecule and the column matrix primarily determine retention time, SEC retention time is determined by the hydrodynamic radius of the molecule in solution. Smaller molecules are retained by the SEC column and thus have longer retention times; high affinity self-association in solution increases hydrodynamic radius and decreases retention time, so that it is possible to observe multiple peaks in the case of a molecule adopting several oligomeric species if they are in slow exchange. An excellent example of SEC monitoring of the effect of amphipathic $\alpha$-helical peptide concentration on oligomerization state was reported by Wagschal et al. (45). These authors observed an equimolar mixture of trimeric and monomeric species at a peptide concentration of 500 $\mu$M, which, upon dilution to 2.5 $\mu$M, was converted predominantly to monomer. Due to a slow exchange rate for interconversion of these oligomerization states relative to overall run time, two distinct peaks representing the trimeric and monomeric states were observed at high peptide concentrations. SEC has also been used to demonstrate the oligomerization state of small amphipathic $\alpha$-helices that form dimeric or trimeric $\alpha$-helical coiled-coils (34, 35, 37). There is an inherent separation range in all SEC columns, with the lower limit typically ~0.5–1 $k_D$ for peptide columns; however, in RP-HPLC, there are no such size limitations. In SEC, the analyte concentration is diluted on the column during elution, whereas, during RP-HPLC, the analyte is eluted as a single narrow concentrated peak, regardless of initial loading concentrations. Because of the dilution effect in SEC, it is also less sensitive to detecting lower affinity self-association than RP-HPLC. Finally, since salt is typically included in the elution buffer for SEC (100–300 mM) to prevent nonspecific electrostatic interactions with the matrix (46), molecules may have different solubility limits in SEC buffer, as was the case for GS14 (insoluble in 100 mM NaCl).

**Nonlinear Behavior of Linear Controls**—The temperature profiles of both GS14 lin and GS14K4 lin were not completely linear compared with the profile of GS14K4 A6 lin. The cause of the deviation from a linear temperature dependence in GS14 lin and GS14K4 lin can best be explained by peptide self-association in aqueous solvent. GS14 lin showed deviation from linearity only at lower temperatures, suggesting that it exhibited self-association between 5–15 °C. Previous molecular modeling studies of linear GS14 showed that the N and C termini are oriented close to one another and that the adoption of $\beta$-sheet structure was possible (47). By adopting a $\beta$-sheet structure, linear GS14 would become more amphipathic and increase its likelihood of dimerizing in solution. Since secondary structure is disrupted at higher temperatures, the adoption of $\beta$-sheet structure and reduction in retention time occurred only at temperatures below 15 °C for GS14 lin. This observation is not surprising considering that GS14 lin was also shown to adopt a $\beta$-sheet structure in 50% trifluorethanol (Fig. 5), a solvent, as noted previously, known to mimic the hydrophobic environment of the reversed-phase matrix (28).

**Role of Self-association in Antimicrobial Peptide Activity**—Based on RP-HPLC results in the present study, as well as previous biological activity data (24), we observed that the GS14K4 peptides that showed the least amount of self-association in solution had little or no activity against microbial or human cells. However, some peptides that showed a large extent of self-association in solution (GS14 and GS14 X$_{D4}$ analogs GS14 F$_{D4}$, GS14 L$_{D4}$, GS14 A$_{D4}$, Fig. 7) showed strong hemolytic activity with weak antimicrobial activity. It is known that antimicrobial peptides with high peptide hydrophobicity lose microbial specificity and become very hemolytic, i.e. toxic to red blood cells (24). It is plausible that the high peptide hydrophobicity also results in greater peptide self-association in solution, as observed with GS14 and other GS14 X$_{D4}$ peptides. The most efficacious peptide studied to date, GS14K4, had a lower degree of self-association, with a $T_p$ of 20 °C, a hemolytic activity of 200 $\mu$g/ml and an average antimicrobial activity of 4 $\mu$g/ml (larger values correspond to poor activity). This suggests that high degrees of self-association may not be desirable for optimal microbial specificity. Conversely, the complete absence of self-association also negates the desired anti-
microbial activity and is also undesirable for high microbial specificity.

Thus, the loss of microbial specificity in the cyclic peptides of this study is observed in conjunction with two distinct and opposing behaviors in molecular self-association. On the one hand (compare GS14 to GS14K4), increased dimerization of GS14 relative to the lesser degree of self-association of GS14K4 reduces antimicrobial activity (Table II); on the other hand (compare GS14K4 to GS14K4 V3/A3, GS14K4 L3/A3 and GS14K4 A6), the absence of dimerization of the GS14K4 hydrophobicity analogs, compared with the observed self-association of GS14K4, has also significantly reduced antimicrobial activity (Table II).

The enhancement or reduction of biological activity depending on the degree of peptide dimerization can be explained by the dimerization effect on interactions between the peptide and the lipid membrane. In the case of GS14, the rigid β-sheet structure and high hydrophobicity of the nonpolar face enhance dimerization in aqueous benign conditions. This structure also stabilizes the positively charged (+4) polar face, which in turn results in the high affinity of GS14 for anionic LPS (24, 27). These effects, along with the shielding of the nonpolar face of GS14 via strong hydrophobic interactions between two GS14 molecules, prevent the peptide from penetrating through (traversing) the lipid bilayer. GS14K4, in contrast, has a sufficiently nonpolar face to allow a degree of dimerization but not to the extent that the dissociation of two GS14K4 molecules cannot readily occur, thus allowing migration of GS14K4 into the polar-apolar interfacial regions of the lipid layer where the nonpolar face interacts with the acyl chains (48, 49). Concerning the absence of self-association of the GS14K4 hydrophobicity analogs, this lack of dimerization is a reflection of these analogs not possessing sufficient inherent hydrophobicity to interact with the nonpolar region of the lipid membrane. To summarize, dimerization can inhibit the ability of the peptide to penetrate the lipid bilayer; however, even if dimerization (or too strong a self-association) is prevented, the inherent hydrophobicity of the peptide may be insufficient to penetrate the bilayer. Thus, an optimum hydrophobicity is required to control dimerization and maintain antimicrobial activity and microbial specificity.

The role of aqueous self-association in peptide cytolytic activity has not been well studied, due to the relatively small size of peptides and the lack of suitable analytical methods to date. Temperature profiling in RP-HPLC may be a useful technique to examine other antimicrobial peptides to see if a relationship exists between the degree of self-association in solution, antimicrobial activity, and hemolytic activity.

**Temperature Profiling with Other Types of Molecules**—The temperature profiling behavior described in the present study is not unique to just cyclic and β-sheet peptides. Thus, temperature profiling in RP-HPLC in our laboratory has also been used to study the unfolding of monomeric α-helices and α-helices that dimerize (50), as well as α-helices that dimerize to form coiled-coils (51). In all cases, we have observed similar nonlinear profiles among the two classes of structures able to dimerize (β-sheet and α-helix) and linear profiles with unstructured peptides. Interestingly, the observation that substituting a Lys residue onto the center of the nonpolar face of GS14 (to produce GS14K4) significantly reduced dimerization of these cyclic peptides mirrored a similar result with α-helical peptide analogs. Thus, replacing a Leu residue in the center of the nonpolar face (made up of seven Leu residues) of an amphipathic α-helix with a Lys residue also effectively blocked/reduced dimerization of these molecules (50).

The β-sheet peptides in the present study do not have an easily identifiable parameter that changes during the transition between higher order self-association and monomer. Admittedly, the ratios of the wavelengths of the local minima appeared to shift with higher GS14 concentration, suggesting aggregation, and this hypothesis was supported by additional fluorescence studies (26). Unlike α-helical coiled-coils, which lose both quaternary and secondary structure upon denaturation, we believe that only the quaternary structure is lost when the cyclic β-sheet peptides in our study no longer self-associate, i.e. one observes essentially the same β-sheet secondary structure over the entire denaturation range. In all cases, the control peptides with undefined structure were used to normalize profile data to account for general temperature effects associated with changes in solvent viscosity and mass transfer rates, so that we could examine the effect of temperature specifically on peptide structure.

**Additional Considerations**—While temperature profiling in reversed-phase chromatography is a useful technique for evaluating self-association in small amphipathic molecules, there are aspects of the technique that merit further discussion if one considers extending the methodology to analyze other kinds of molecules. Since the technique denatures proteins by exposing hydrophobic side chains upon binding to the stationary phase and contacting organic solvent, this technique is unsuitable for assessing the self-association of native folded globular proteins stabilized by hydrophobic interactions, since organic solvent and the hydrophobic matrix stabilize and unfold most tertiary structures.

On the positive side, the sensitivity of the temperature profiling described in this work could be further amplified by using narrower diameter (micro-, capillary, or nano-) reversed-phase columns, which would require less sample for analyses than the narrow bore (2.1-mm diameter) column used in the present study. Also, a complete temperature profile only requires time spent on sample preparation, since the Agilent 1100 chromatograph can be programmed to perform multiple runs, automatically regulate temperature, inject and monitor samples, and store data after each elution. Thus, although a complete set of runs may take sixteen hours of real time, the actual time the experimenter requires to set up the system is minimal. Also of significance is the point that, because the peptide oligomerization being measured is due to hydrophobic interactions and is, thus, pH-independent, this approach may be employed at any pH value suitable for chromatography, generally pH 2–8 for silica-based packing materials, although more alkaline conditions are possible with non-silica-based supports (16). Hence, this temperature profiling approach is not restricted to just the pH 2 conditions employed in the present study.

**CONCLUSIONS**

Temperature profiling in RP-HPLC can be used to detect and measure self-association of small molecules driven together by the hydrophobic effect, caused by the technique’s extremely high sensitivity. By performing a series of reversed-phase elutions at a range of temperatures, we have demonstrated that self-association of small molecules in solution can be detected using extremely small (nanogram) quantities. One can easily identify self-association based on the nonlinear relationship between analyte retention time and temperature. The technique is applicable to association in both α-helical and β-sheet structures, cyclic and linear, and does not have either an upper or a lower size limit for detection. We envision that this technique can also be used for nonpeptide analytes as well as larger biomolecules, as long as the domain responsible for self-association is hydrophobic and is stabilized by the organic modifier in the aqueous/organic solvent used in RP-HPLC.
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A Novel Method to Measure Self-association of Small Amphipathic Molecules: TEMPERATURE PROFILING IN REVERSED-PHASE CHROMATOGRAPHY
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