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Development of a screening technique for noncovalent complex formation between guanidinium- and phosphonate-functionalized amino acids by electrospray ionization ion trap mass spectrometry: assessing ionization and functional group interaction

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Abstract

These experiments represent work toward the development of an efficient screening protocol for noncovalent complex formation by guanidinium- and phosphonate-containing amino acid molecules (e.g., arginine (Arg) and phospholeucine (pLeu)) with electrospray ionization mass spectrometry (ESI-MS). Mass spectra, acquired with an optimized ESI-MS method, reveal the formation of multiple high order adducts in the positive and negative ionization modes. Relative transmission factors, defined based on the relationship between measured ion intensities and the initial concentration of each component in an equimolar mixture, for all free and bound ion forms are determined for qualitative comparison of the effects of covalent modification of amino acid-type analytes (N-acetylation of Arg and pLeu and C-amidation of Arg) on noncovalent interactions. Correlation of measured mass spectra with solution-phase equilibria are tested through quantitative mass spectrometric titration experiments. Poor correlation with the ascribed model indicates the likelihood that processes other than solution-phase equilibria are responsible for the majority of the observed ionic complexes. For quantitative measurement of binding in the gas phase, collision-activated dissociation (CAD) is used to determine half-dissociation thresholds of parent ions ($E_{1/2}$). These values provide a measure of relative stability of the ionic complexes in the absence of solvent. Results from relative transmission factors and $E_{1/2}$ measurements show a high degree of variation for ionic complex response based on the covalent modifications of the amino acids which form the complex. Overall, the combination of these approaches offers a means for monitoring and selecting (i.e., screening) systems that interact favorably through a combination of ionic and hydrogen-bonding interactions. Favorable cases can then be isolated for further study, preferably by CAD methods.

Keywords: Adduct ion; Noncovalent complex; Electrospray; Arginine; Phosphonic acid

1. Introduction

1.1. Methodological background

The move toward high throughput developmental practices has created an impetus for more efficient analytical methodology to evaluate new materials. In the pharmaceutical and biopharmaceutical fields, the development of highly active, or interactive, compounds remains a goal of many synthetic chemists. Evaluation of these molecules requires a technique sensitive to the formation of specific noncovalent complexes or associates with various substrates. One such technique which has found substantial use in this area is soft ionization mass spectrometry (MS). Soft ionization techniques, such as electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), allow for the formation of gas-phase ions with very little or no fragmentation. In many cases, noncovalent interactions formed in the solution-phase or during the ESI process can be preserved and monitored in the mass spectra (as "adduct ions").

The use of soft ionization techniques for MS analysis of noncovalent complexes has been extensively reviewed [1–8]. These reviews highlight a multitude of diverse systems and different analytical methods. Large biomolecular receptor/ligand systems, involving protein, peptide, oligonucleotide, enzyme and drug interactions [9–17], as well as

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polyether binding and encapsulation studies [18–26], are well represented in the literature. Noncovalent complex formation by small biomolecules in MS has also been investigated, but to a lesser extent. Examples include amino acid and small peptide clustering [27–29], and transition metal-mediated systems for steric and thermodynamic determinations [30–33].

ESI-MS has received the majority of attention in the analysis of noncovalently bound systems through mass spectrometric approaches. Due to its applicability to diverse molecular sizes of polar and ionic compounds, low sample consumption, and facile implementation, ESI-MS, especially when coupled with tandem MS capabilities (ESI-MS/MS), is an ideal choice. A useful and effective toolbox of qualitative and quantitative methods has been developed to characterize structural, functional, and energetic aspects of complexes of interest [2,7]. Still, consistent debates about the mechanism of ion production [34,35], correlation between solution- and gas-phase measurements [3,4,7,13], and validity of assumptions for assessing the latter and the former continue to restrict the ability to make generalizations.

Qualitative and quantitative mass spectrometric methods developed for assessing specificity, functional, and energetic aspects of noncovalent complexes generated during ESI-MS can be separated into solution-phase and gas-phase methods [3,7,36]. Solution-phase methods are designed to probe information about preformed complexes in solution by measuring ion responses in the mass spectra. These include competition [3,7,19,21,37], titration [10,14,15], and temperature dependent methods [38,39]. To establish the relationship between measured gas-phase intensities and solution-phase concentrations, transfer coefficients (or response factors) for each ion form of interest should be evaluated and correlation should be demonstrated [2,7,16,19,24]. However, a transfer coefficient (or response factor; t_X, where t_X is the intensity of adduct ion X divided by its equilibrium concentration in solution, [X]), in its strictest sense, cannot be determined without knowing the equilibrium concentration of solution-phase complex present in a given mixture. Rather, correlation is often established through comparison with other solution-phase analytical techniques or assumptions are made concerning the similar transfer of free and bound species. Gas phase methods for evaluation of noncovalent complexes center on the dissociation of a specific ion form. Thermal dissociation is possible in some systems, but the vast majority of work features tandem MS measurements, following collision-activated dissociation (CAD) [5,11,13,17,18,25]. CAD is inherently useful for studying the stability of the final gas-phase ion forms regardless of the processes responsible for their formation.

To learn about and utilize specific interactions between well-defined functional groups, the interaction types and sites must be isolated and studied. In large molecules, the cooperativity of multiple interaction sites for binding a single ligand precludes unequivocal determination of the role of each functional unit by ESI-MS. Instead, a more direct approach for assessing specific interactions would be to simplify the system and to look at the interaction between small, well-defined functional units (i.e., small molecules). This information can then be applied reliably to more complex systems. However, for small molecules, where the complex formed can be more than twice the size of the unbound molecules, problems related to the correlation of response factors and the presence of multiple interaction equilibria for the different ion forms may exist [2].

1.2. Aim of the study

Guanidinium and phosph[on]ate moieties are functional units studied and employed in numerous biological and synthetic reaction schemes [40–48]. The highly basic guanidinium group is present in free arginine, as well as in a wide array of peptides and proteins containing this amino acid residue. Arginine residues have been shown to mediate numerous biological interactions through a combination of directed hydrogen bonding and non-directed Coulombic interactions [40,49]. Guanidinium-based variations have also been incorporated into synthetic molecules to create selective receptors and ligands [42,46,47]. Complementary units, with which the charged guanidinium unit prefers to interact, are carboxylate, phosph[on]ate, and sulf[on]ate moieties. Phosphorylation and sulfation are important post-translational modifications in biological systems that create phosphorylated and sulfated sites, respectively, which promote interactions with basic groups on neighboring molecules [14,41,50]. Phosphonic and sulfonic acids can be interesting amino acid analogues that have stronger acidity than common carboxyl amino acids. Phosphorylated amino acids may also be used as building blocks for synthesizing phosphorylated peptides [51]. Synthetic ligands and receptors have been developed that feature these groups and their selectivity for guanidinium groups has been demonstrated as well [43–45,48]. The mutual interest in these functional units, both from the standpoint of guanidinium units interacting with anionic molecules and vice versa, makes the study of interactions between these units, and the development of an efficient protocol to do so, a worthwhile undertaking.

In this work, qualitative and quantitative ESI-MS and ESI-MS/MS techniques are used to investigate the interaction and ionization of arginine-derived analytes (focusing on the guanidinium residue) with aminophosphonic acid-based analytes (focusing on the phosphonate residue). Fig. 1 depicts the structures of the arginine (Arg) and phospholeucine (pLeu) derivatives chosen for this study. Covalent modifications of the analytes are used to study the contribution of different ionizable groups to binding and ionization. Relative transmission factors (T_X) are defined and interpreted in lieu of the lack of solution-phase equilibrium concentration data for all ion forms observed with this system. T_X values are calculated as the slope of the correlation between the

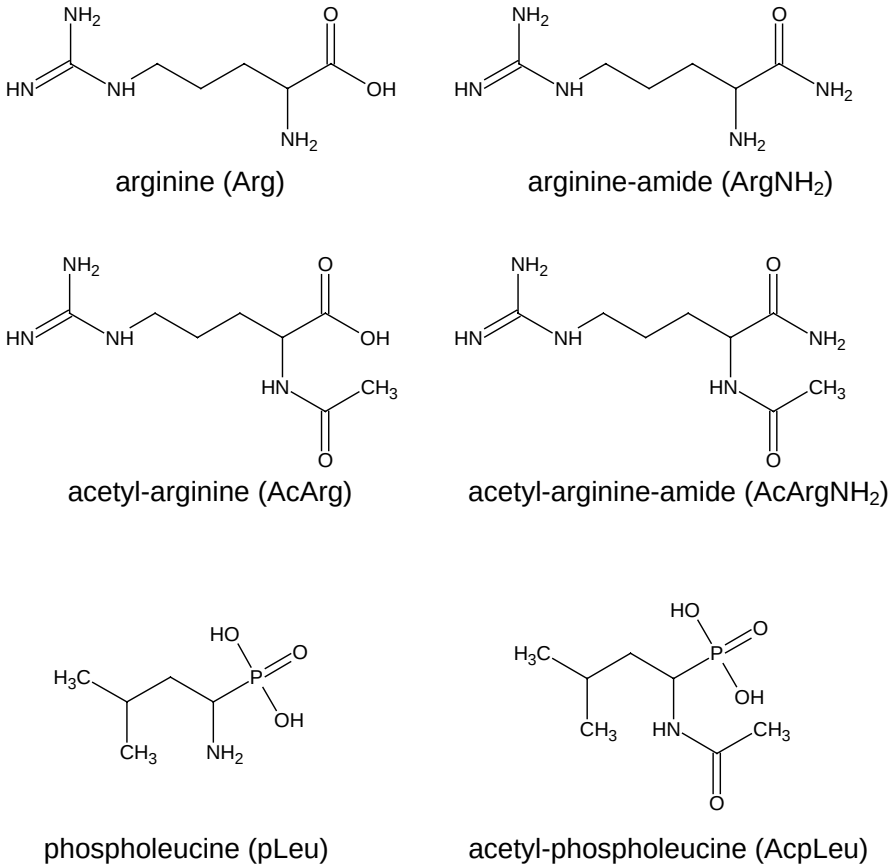

Fig. 1. Small molecule analytes used in this study to investigate noncovalent complex ion formation between guanidinium- and phosphonate-functionalized small molecules.

MS intensity of the various adduct ion forms and the initial concentrations of the components in the mixture. Titration experiments are investigated as a possible means for studying this system in the event of solution-phase correlation. Tandem MS experiments in an ion trap are used to establish quantitative collision thresholds and orders of stability for the major adduct ions observed in the gas phase. A robust ESI-MS analytical method is established that is applicable for these and future studies of noncovalent interactions. The goals of these experiments are threefold: (1) To define the complex-forming nature and ESI behavior of small molecule analytes featuring highly interactive functional units; (2) Investigate the applicability of established qualitative and quantitative techniques for assessing these intermolecular interactions and (3) To develop an efficient gas-phase screening technique for studying small molecule systems by ESI-MS/MS.

2. Experimental

2.1. Instrumentation

Experiments were performed on an Agilent 1100 Series LC/MSD SL ion trap mass spectrometer system (Agilent Technologies, Vienna, Austria) with a pneumatically-assisted electrospray ionization interface. Sample solutions were introduced via a syringe pump operating at 5 μL/min. Mass spectra were collected in both the positive and the negative ionization modes for all sample mixtures with "enhanced scan resolution" (5500 $m/z\,s^{-1}$). Table 1 details the voltages, temperatures, and gas flows employed in the in-

Table 1
Optimized settings of the electrospray ion source and mass spectrometer ion optics for detection of noncovalent ionic complexes

Parameter	Mode setting (+)	Mode setting (−)
ESI ion source		
Spray capillary voltage (V)	5000	−4000
Nebulizer gas pressure (psi)	7.0	7.0
Dry gas flow (L/min)	4.0	4.0
Dry gas temperature (°C)	300	300
Desolvation capillary (V)	100	−105
MS Optics		
Skimmer (V)	25	−35
Oct 1 dc (V)	7.5	−8.5
Oct 2 dc (V)	0.5	−2.4
Lens 1 (V)	−4.5	4.5
Lens 2 (V)	−55	55

Spray capillary voltage in this instrument is applied to the endplate, while the capillary is held at ground. Value indicates the potential difference applied.

strument to optimize the formation of the noncovalent interactions between the guanidinium- and phosphonate-based analytes. Values for these parameters were optimized to maximize adduct ion response (of prominent adduct ions observed) for this analyte system. Although the temperature of the drying gas was set to 300 °C, the low flow rate of the drying gas as well as the voltage settings for the unheated transfer capillary assured maximal and reproducible response for the ions incorporating noncovalent interactions. Except where tandem MS was employed, full scan spectra (100–1500 Th) were collected. Each spectrum collected for evaluation was an average of approximately 75 scans (±3); each scan was an average of five microscans. Values reported for average intensities were the product of triplicate full scan measurements.

2.2. Chemicals

All sample mixtures were prepared from secondary standard sample solutions in 50/50 acetonitrile/water (HPLC-grade acetonitrile from Fisher Chemicals (Schwerte, Germany) and LCMS-grade ultra-pure water from Fluka (Buchs, Switzerland)). The concentration of each component in the final mixtures for analysis was varied (0.005–0.5 mmol/L (mM)) depending on the method being employed (see below). Guandinium-functionalized analytes used in these experiments were H–Arg–OH (unblocked; Arg), Ac-Arg-OH (N-acetylated; Ac-Arg), H-Arg-NH_2 (C-amidated; ArgNH_2), and Ac-Arg-NH_2 (N-acetylated and C-amidated; Ac-Arg-NH_2). Arg (Sigma, Vienna, Austria), Ac-Arg (Bachem, Weil am Rhein, Germany), and Arg-NH_2 (Bachem) were obtained commercially. Ac-Arg-NH_2 was synthesized in-house from Arg-NH_2 and purified by ion-exchange chromatography. Phosphonate-functionalized analytes used were phospholeucine (pLeu) and acetylated phospholeucine (N-blocked; AcpLeu). Phospholeucine was obtained from a previous study [52] and AcpLeu was synthesized in-house from pLeu and purified by ion-exchange chromatography. All analytes investigated were chemically and enantiomerically pure and present in the (*S*) configuration.

2.3. MS adduct ion signals

The adduct ion forms chosen for investigation were those observed in preliminary experiments, under the conditions described above, when an equimolar (0.05 mM each) mixture of Arg and pLeu was analyzed. Fig. 2 shows sample mass spectra for the positive and negative ionization mode analysis of this mixture. Adduct ions in the negative ionization mode composed a higher proportion of the total ion current than was observed in the positive ionization mode. Changes to the positive ionization mode method were investigated with the aim of increasing adduct ion response, however little change was observed. For this reason, most discussion will center on the formation of adduct ions in the negative ionization mode for this system. The major adduct ion forms considered were protonated and deprotonated molecular ions ($[M \pm H]^{\pm}$), homodimeric adduct ions ($[2M \pm H]^{\pm}$), heterodimeric adduct ions ($[M + N \pm H]^{\pm}$), and heterotrimeric adduct ions ($[2M + N \pm H]^{\pm}$ and $[M + 2N \pm H]^{\pm}$). The components M and N represent the complementary analytes in the mixtures.

2.4. Qualitative and quantitative analysis

Since only the mass spectral intensity and initial concentration of each component are known, and not the equilibrium concentration of each complex, we define a relative transmission factor T_X, which relates the observed ion signal to the initial concentration of each component in solution. This is shown in the equation: $I_X = T_X[M_i]$; where I_X is the average intensity of the ion form X and $[M_i]$ is the original concentration of the analyte(s) in the mixture. This approach is analogous to the determination of transfer coefficients t_X based on the equation, $I_X = t_X[X]$ [7], however, it does not distinguish between ionic complexes formed in solution-phase and those formed as a product of ESI or gas phase processes. Relative transmission factors were determined from the concentration range that yielded a linear response in both the positive and the negative ionization modes. Low concentration range values were investigated to ensure specific interactions, and not aggregation, were responsible for the adduct ions observed. The T_X values are useful for qualitative analysis with regards to comparison of ionization efficiencies and complex formation by the various covalently modified Arg- and pLeu-analogues (see also Fig. 1). When T_X values for different ion forms are similar, they provide a means for quantitative comparison [19]. These values were also applied during quantitative titration analysis to investigate the possibility of normalizing or converting the data prior to calculation.

To assess the quantitative correlation between gas-phase measurements and solution-phase equilibria, titration experiments based on a host/guest concept were employed. The role of host and guest, not easily defined for noncovalent complexes formed between analytes of similar size, was varied between the guanidinium- and phosphonate-based analytes. The systems were analyzed by holding one component at constant concentration (0.04 mM) in the linear response range, and varying the complementary component over a concentration range of 0.005 to 0.5 mM. Three mixtures were used as an assessment of the application of this technique: Arg + pLeu; Ac-Arg + pLeu; and Ac-Arg-NH_2 + pLeu. Assuming some simple equilibria between associating molecules were present and dominant over other minor equilibria in solution, a Scatchard-based [53] analysis was applied. The specific model which was applied here is given in a relevant publication by Sannes-Lowery et al. [10]. Variations in this method exist dependent on whether one binding site or multiple binding sites are present. Application, as well as the appropriateness of applying such an approach, to this system will be discussed below.

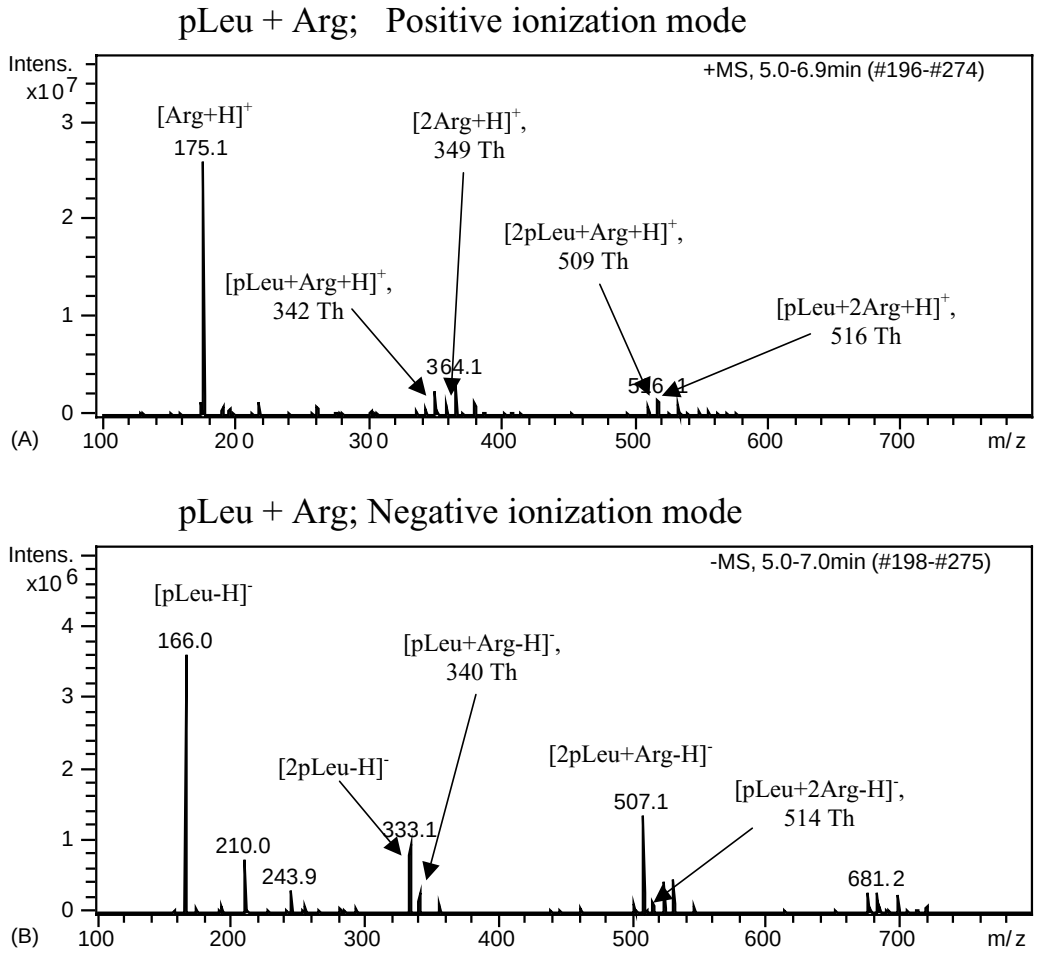

Fig. 2. ESI-MS mass spectrum obtained in (A) the positive and (B) the negative ionization modes for the measurement of an equimolar (0.05 mM each) mixture of pLeu and Arg. Major ion forms observed are labeled.

Since various adduct ion forms for the analyte system are observed in the mass spectrum, an obvious approach to quantitatively assessing the differences between these adduct forms (and the effect of the modified components interacting to create them) is to employ gas-phase tandem MS methods. CAD in the ion trap was applied to determine dissociation thresholds of the ionic complexes in the absence of solvation. "Melting curves" were generated by isolating each adduct ion form and systematically applying an increasing amplitude of excitation voltage (0.1–2.0 V) to monitor the stability of each complex. Stability values are reported as half-dissociation thresholds $E_{1/2}$, or the amplitude necessary to dissociate one-half of the parent ion complex (based on average intensity measured) [13,54].

3. Results

3.1. Relative transmission factor determination

Each free (unbound) and noncovalently-bound adduct ion form which can be observed has an ionization efficiency associated with it for a given ESI-MS method. Relative transmission factors are applied to assess the relationship between MS intensity and the original concentration of the components involved in the complex formation. This allows the effect of small changes to the structure of each component upon transmission (formation and detection) of each ion form during ESI-MS, with a fixed method, to be assessed in a systematic manner. Table 2 shows the

Table 2
Relative transmission factor and linear regression (seven data points; $n = 3$) values determined for monomeric and homodimeric ion form for individual standards (0.005–0.1 mM)

I.D. (M)	T_X (10^5 mM^{-1}) (R^2) values			
	$[M + H]^+$	$[2M + H]^+$	$[M - H]^-$	$[2M - H]^-$
Arg	500 (0.995)	300 (0.973)	5 (0.970)	0
Ac-Arg	200 (0.946)	400 (0.968)	30 (0.881)	100 (0.980)
Arg-NH_2	400 (0.997)	80 (0.844)	2 (0.813)	0
Ac-Arg-NH_2	500 (0.970)	20 (0.909)	0	0
pLeu	0	0	50 (0.981)	30 (0.949)
AcpLeu	0	2 (0.915)	100 (0.985)	6 (0.882)

relative transmission factors (and linear correlations) determined for single standards, without the presence of a second, complementary-functionalized, analyte molecule. In this case, the dominant ion forms are the deprotonated and protonated molecular ions and the homodimeric adduct ions. Ionization efficiency was evaluated over a two orders of magnitude concentration range. T_X values reported in Table 2 are for the linear portion of this response curve (0.005–0.1 mM; seven data points; $n = 3$ for each data point). Above 0.1 mM, saturation and ion suppression create a nonlinear behavior. Values for the ion forms observed with the single standards can be used to assess the effect of covalent modification on ion response. A lowest-value threshold for omission of non-relevant data was set at $t_X = 10^5$ and/or $R^2 = 0.8$. Though a linear correlation coefficient of 0.8 is poor, a positive trend in the data with respect to increasing concentration is still observed in these cases.

Table 3 lists the results of ionization of equimolar mixtures of each guanidinium (Arg)-based (A) with each phosphonate-based (P) analyte molecule. The lowest-value threshold for omission of data protocol is the same as above for the single standard measurements. Again, saturation was observed for concentrations above 0.1 mM, and as such, the values are reported for the linear response region between 0.005 and 0.1 mM. Transmission factors for all ion forms observed with the Arg + pLeu mixture (see Fig. 2) are reported, allowing assessment of the effect of covalent modification of additional Coulombic binding sites (amino and carboxyl functional groups) beyond the dominant guanidinium and phosphonic acid groups on overall ionization and complex formation.

3.2. Titration experiment

Application of the model mentioned previously [10] was made for each adduct (complex) ion form in the positive and the negative ionization mode for each of three representative mixtures. Different models were applied depending on assumptions of whether one or two binding equilibria were dominant in the system. Each ion form was evaluated separately assuming a single dominant equilibrium and using a one binding site model. This was done both by assuming a single molecule precursor (for heterodimeric and heterotrimeric adduct ions) as well as homodimeric and heterodimeric precursors (for heterotrimeric adduct ions) for formation of the product ionic complex of interest. Each heterotrimeric adduct ion was also evaluated along with a homodimeric or a heterodimeric adduct ion in a model capable of evaluating two dominant equilibria. Intensity values for the adduct ions applied in the models were fit to the models directly before and after being normalized/converted with their respective transmission factor, determined previously (i.e., $[X] = I_X/T_X$).

Results for this experiment were, for the most part, poor, indicating a lack of quantitative correlation between gas-phase measurements and solution-phase equilibria in the majority of systems studied with these models. The low correlation of the model equations with the applied data indicated a high degree of uncertainty in these measurements. As such, these values could not be reported with high confidence and were therefore omitted. The quality of the results were unaffected by whether K_D values were calculated directly from measured intensity values or by first converting to concentration units with the appropriate transmission factor.

Table 3
Relative transmission coefficient and linear regression (seven data points; $n = 3$) values determined for dominant homo- and hetero-adduct ion forms for 1:1 mixtures of phospholeucine- (P) and arginine-derivative (A) standards (0.005–0.1 mM)

Adduct I.D.	T_X (10^5 mM^{-1}) (R^2) values							
	Components of mixture: pLeu + ···				Components of mixture: AcpLeu + ···			
	Arg	Ac-Arg	Arg-NH_2	Ac-Arg-NH_2	Arg	Ac-Arg	Arg-NH_2	Ac-Arg-NH_2
Ion mode (−)								
$[P - H]^-$	50 (0.979)	40 (0.972)	50 (0.948)	50 (0.959)	100 (0.942)	100 (0.969)	100 (0.949)	90 (0.926)
$[2P - H]^-$	40 (0.995)	40 (0.976)	30 (0.982)	60 (0.943)	10 (0.980)	9 (0.916)	6 (0.857)	4 (0.847)
$[A - H]^-$	0	20 (0.848)	0	0	2 (0.908)	0	0	0
$[2A - H]^-$	0	30 (0.923)	0	0	1 (0.904)	0	1 (0.929)	0
$[P + A - H]^-$	20 (0.962)	100 (0.993)	3 (0.914)	10 (0.966)	40 (0.991)	100 (0.973)	10 (0.971)	30 (0.972)
$[2P + A - H]^-$	70 (0.980)	20 (0.988)	30 (0.934)	60 (0.976)	10 (0.992)	2 (0.808)	20 (0.949)	10 (0.884)
$[P + 2A - H]^-$	9 (0.973)	200 (0.978)	0	0	10 (0.996)	80 (0.904)	0	0
Ion mode (+)								
$[P + H]^+$	0	0	2 (0.904)	0	0	0	2 (0.897)	0
$[2P + H]^+$	0	0	20 (0.965)	20 (0.936)	0	0	0	0
$[A + H]^+$	300 (0.920)	200 (0.925)	300 (0.964)	500 (0.962)	300 (0.952)	100 (0.887)	300 (0.959)	300 (0.822)
$[2A + H]^+$	60 (0.997)	200 (0.993)	6 (0.976)	8 (0.962)	40 (0.954)	100 (0.983)	3 (0.851)	2 (0.864)
$[P + A + H]^+$	30 (0.828)	0	20 (0.965)	0	20 (0.909)	2 (0.864)	100 (0.990)	0
$[2P + A + H]^+$	20 (0.858)	0	40 (0.935)	4 (0.925)	0	0	5 (0.922)	0
$[P + 2A + H]^+$	100 (0.985)	0	1 (0.868)	0	100 (0.990)	80 (0.994)	0	0

A value of '0' denotes $T_X \leq 10^5$ and/or $R^2 \leq 0.8$.

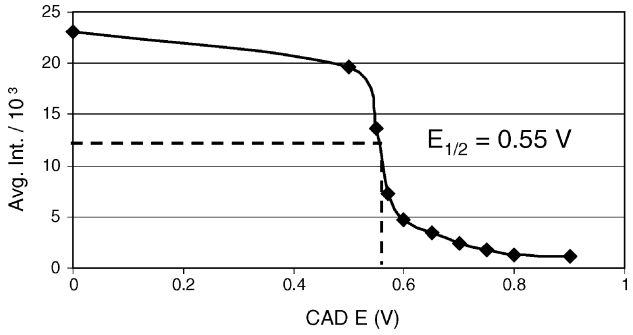

Fig. 3. Example of $E_{1/2}$ determination for [pLeu + Ac-Arg-NH_2-H]$^-$.

3.3. CAD threshold determination

In a similar manner to that described by Wan and coworkers [13], the half-dissociation collision threshold ($E_{1/2}$) was determined for the dominant adduct ions observed. This was done for equimolar mixtures of each combination of P and A analytes. Fig. 3 shows a representative "melting curve" for the heterodimeric adduct ion formed between Ac-Arg-NH_2 and pLeu. The value of CAD energy necessary to dissociate 50% of the parent ion is reported as $E_{1/2}$. Table 4 shows the data set for all of the adduct ion forms. In many cases, the intensity of an adduct ion was insufficient for isolation and fragmentation (denoted as 'ND' in Table 4). Values that are reported are accompanied by the identity of the dominant (most-stable) offspring ion. Where equivalent fragmentation pathways are observed, these results can be used to compare the differences in binding related to the incorporation of the various covalent modifications of Arg and pLeu; in the absence of solvent.

4. Discussion

The formation or transmission of specific noncovalent ionic complexes (or clusters) by small molecules with highly interactive functional units during ESI-MS is evident by the observed signals in the mass spectra. In this study, which features primarily the highly basic guanidinium and the highly acidic phosphonic acid units, heteromeric ionic complexes allow the chance to study the interactions between these groups through various qualitative and quantitative means. These include covalent modification of additional and/or competing chargeable binding sites, determination of relative transmission factors, titration experiments, and determination of gas-phase collision thresholds. Collectively, the results from these experiments offer insight into the potential of studying these sets of interactions by ESI, how these interactions may be formed in this environment, and the capability of applying these techniques in future routine screening applications of similar systems.

Guanidinium and phosphonate groups are known to interact through non-directed Coulombic, and even di-

Table 4
Determination of $E_{1/2}$ values (given in amplitude voltages) and major product ion formed for selected homo- and heterodimeric and trimeric adduct ion forms

Adduct I.D.	$E_{1/2}$ (V) (major product) values							
	Components of mixture: pLeu + ⋯				Components of mixture: AcpLeu + ⋯			
	Arg	Ac-Arg	Arg-NH_2	Ac-Arg-NH_2	Arg	Ac-Arg	Arg-NH_2	Ac-Arg-NH_2
Ion mode (–)								
[2P − H]$^-$	0.95 ([P − H]$^-$)	0.96 ([P − H]$^-$)	0.95 ([P − H]$^-$)	0.95 ([P − H]$^-$)	0.71 ([P − H]$^-$)	0.77 ([P − H]$^-$)	ND	ND
[2A − H]$^-$	ND	ND	ND	ND	ND	ND	ND	ND
[P + A − H]$^-$	1.37 ([P − H]$^-$)	0.65 ([A − H]$^-$)	ND	0.55 ([P − H]$^-$)	0.65 ([P − H]$^-$)	0.7 ([P − H]$^-$)	0.5 ([P − H]$^-$)	0.62 ([P − H]$^-$)
[2P + A − H]$^-$	0.76 ([2P − H]$^-$)	0.84 ([P + A − H]$^-$)	0.71 ([2P − H]$^-$)	0.82 ([2P − H]$^-$)	0.5 ([2P − H]$^-$)	ND	0.5 ([2P − H]$^-$)	0.5 ([2P − H]$^-$)
[P + 2A − H]$^-$	0.8 ([P + A − H]$^-$)	0.55 ([P + A − H]$^-$)	ND	ND	0.76 ([P + A − H]$^-$)	0.55 ([P + A − H]$^-$)	ND	ND
Ion mode (+)								
[2P + H]$^+$	ND	ND	0.96 ([P + H]$^+$)	1.01 ([P + H]$^+$)	ND	ND	ND	ND
[2A + H]$^+$	0.78 ([A + H]$^+$)	0.71 ([A + H]$^+$)	ND	ND	0.86 ([A + H]$^+$)	0.76 ([A + H]$^+$)	ND	ND
[P + A + H]$^+$	1.18 ([A + H]$^+$)	ND	1.25 ([A + H]$^+$)	ND	1.1 ([A + H]$^+$)	ND	0.87 ([A + H]$^+$)	ND
[2P + A + H]$^+$	1.5 ([P + A + H]$^+$)	ND	ND	ND	ND	ND	ND	ND
[P + 2A + H]$^+$	0.69 ([2A + H]$^+$)	ND	ND	ND	ND	ND	ND	ND

Values for ion forms listed as 'ND' were not determined due to inability to isolate the ion form because of low or no abundance in the mass spectra.

rected hydrogen-bonding, interactions in competitive media [45,46]. In the 50/50 acetonitrile/water mixture, the solvent is expected to be an effective competitor for hydrogen-bonding sites. Although this is likely the case, it is possible that the Coulombic interaction between the ionic groups provides a mode for creating proximity between complementary groups which can then arrange into more directed-type interactions. This combination of types of affinity appears to be more pronounced for the highly basic guanidinium interacting with highly anionic functional units, such as phosphonate, compared to alternative arrangements. Correlating this statement, preliminary results (not shown here) have shown a considerable decrease in the propensity of ESI adduct ion formation when carboxylate-based amino acids are used instead of the phosphonate-based ones shown here.

Experiments performed here have been designed to isolate the presumably dominant ionic interaction between guanidinium and phosphonate through systematic covalent modification of other ionic sites in the analyte sets derived from Arg and pLeu. This strategy has been used previously, in a similar fashion, to study binding for larger host/guest systems by MS [55,56]. Covalent modification of the amino and carboxyl groups is also commonly used to block these ionic active sites during synthetic procedures involved in amino acid chemistry.

Differences in the ionization efficiency (relative transmission factors) and dissociation thresholds, measured here for small-molecule analytes by ESI-MS/MS, as a result of the chemical modifications are evident. Several reasons can be offered for explaining the changes. First, the chemical modification of the chargeable groups blocks or transforms the highly interactive ionic site, thus lowering the ability of that site to form intermolecular noncovalent bonds. Through acetylation (N-termini) and amidation (C-termini), amide units which are now hardly capable of ionic interaction, but still can engage in hydrogen-bonding interactions and charge carrying in ESI, are created (see Fig. 1). Such an effect is evident in comparing the $E_{1/2}$ values for dissociation of the homodimeric ionic complex, $[2P\text{-}H]^-$, for pLeu and AcpLeu (see Table 4). In the case of pLeu, this ion form is likely formed through mutual ionic phosphonate–amine interactions. When the amine is blocked, as with AcpLeu, the interaction strength is decreased, indicating a weaker, but possibly hydrogen-bonding-type, interaction. Second, the modification of the ionic sites increases the overall hydrophobicity and, in turn, lowers the hydrophilicity of the free and the complexed ions formed. As it relates to solvation energy in a 50/50 acetonitrile/water solvent system, this will significantly alter ionization efficiency [16]. The two-fold difference between the transfer efficiency of $[P - H]^-$ for pLeu (50×10^5) and AcpLeu (100×10^5), is a fitting example of this expected effect (see Tables 2 and 3). Arginine derivatives, incidentally, show a much lower effect in this respect for covalent modification of the N- and C-termini. This is likely explained by the dominant charge localization ability of the guanidinium group. Along these lines, the third possibility for differences observed between complex formation due to covalent modification is the overall change in the ability of a free or complex ion to become charged (through protonation or deprotonation). This effect is difficult to quantify relative to the other explanations provided. Although it is intuitive that ionic sites more easily form ions, the ESI process certainly allows for charge association by neutral polar sites as well. This, in addition to the ability of phosphonate to lose (2−) and guanidinium to gain (2+) a second hydrogen, makes this point less relevant to further discussion.

Other small differences noted, especially when comparing similar $E_{1/2}$ values across a given ion form, may be due to an incorporation of directed interactions in the complex. Slight differences in the final arrangement of a structure may stabilize or destabilize an ionic complex based on this concept. An example of this is the variation in measured values for all of the heterodimeric ($[P + A - H]^-$) $E_{1/2}$ values reported in Table 4 (Arg + pLeu being an exception). These ion forms are believed to be centered about a dominant guanidinium–phosphonate interaction scenario, based on the measurement of this ion form for Ac-Arg-NH_2 + AcpLeu, where all other additional potential ionic interaction sites are chemically blocked. The forked structure [40] of the guanidinium and the pyramidal structure of the phosphonate group provide various directed arrangements that, when combined with modifications outside of this central interacting unit, could cause slight changes in the stabilization/destabilization of the overall complex and thus, the measured $E_{1/2}$ value. The exception is for the Arg + pLeu mixture which, for $[P + A - H]^-$, has an $E_{1/2}$ value more than twice (1.37) that of the others. This is likely due to a combination of more than one Coulombic binding ligature which act in concert to stabilize this adduct ion form. Overall, the results provided by covalent modification of ionizable sites on the complex-forming partners makes this approach useful for studying binding interactions in systems containing structurally-similar analytes.

The results offered directly from examining the relative transmission factors measured for the free and bound ion forms are informative, even if time-consuming to obtain. Because there is little to no correlation between these values (i.e., the ionization mechanism has a strong influence on formation and/or transmission of the different ion forms), quantitative evaluation of solution-phase binding is difficult to ascertain. This is evidenced by the poor results obtained during titration experiments. In general, large changes in relative transmission factor values provide a good qualitative means for assessing the effect of blocking various ionic groups, regardless of the specific processes responsible for association. A good example is the effect of blocking the C-terminus of Arg on the ionization efficiency of the heterotrimeric adduct, $[P + 2A - H]^-$, shown in Table 3. For Arg or AcArg mixed with pLeu or AcpLeu, this form has a significant T_X value. When the C-terminus is blocked, as with Arg-NH_2 and Ac-Arg-NH_2, the transmission of this

ion form is squelched. Thus, interaction by the C-terminus of Arg (or an Arg derivative) is necessary to the formation of this heterotrimeric ion form. The second heterotrimeric adduct ion investigated, $[2P + A - H]^-$, is possibly a product of adduction of A to $[2P - H]^-$. As discussed earlier, the homodimeric adduct ion for P is diminished when the N-terminus of pLeu is acetylated. This change is similarly reflected in the diminished response of the $[2P + A - H]^-$ ion for all of the arginine derived analytes studied. The offspring ion for dissociation of this form in $E_{1/2}$ measurements also corroborates this hypothesis (with the exception of Ac-Arg + pLeu). In summary, changes in ionization efficiency reflected by large (at least one order of magnitude) changes in the measured transmission factors provides an useful approach for systematically, albeit qualitatively, studying the effect of each group on complex formation through the ESI-MS process, for a given method.

The determination of collisional thresholds by CAD provided a more straightforward and quantitative approach to the study of adduct formation by small molecule analytes in ESI-MS. For small molecule analytes that interact mainly through electrostatic (Coulombic, hydrogen-bonding, and dipolar) interactions, the absence of solvent generally increases interaction strengths. Some notable results have already been described. Table 4 outlines the major adduct ion forms isolated, their $E_{1/2}$ values, and their respective dominant offspring ion. In many cases, the dominant offspring ion was the same for all mixtures tested indicating equivalent fragmentation pathways. Exceptions, such as with the dissociation of $[2P + A - H]^-$ from the Ac-Arg + pLeu mixture may provide insight into unique selectivities provided by dissociation events. With an ion trap instrument, the possibility also exists to use MS^n to isolate and compare the $E_{1/2}$ values for the offspring ions. This was performed (data not shown) for the higher intensity $[2P - H]^-$ offspring ions observed upon CAD of $[2P + A - H]^-$. The values obtained were identical to those recorded for $[2P - H]^-$ in tandem MS mode and reported for the various mixtures in Table 4. In summary, the application of tandem MS methods for determination of dissociation thresholds is reliable and informative. Whether or not these ion forms are created in the solution-phase, gas phase, or the ESI-mediated transmission is irrelevant if useful information can be obtained through gas-phase analysis.

5. Conclusions

The present study was designed to investigate procedures towards the development of an efficient screening protocol to create and elucidate noncovalent interactions between small molecules. Such a protocol can be useful for characterizing the interaction sites between small molecules that contain highly interactive functional units, such as the guanidinium and phosphonate units studied here. An efficient screening technique built around soft ionization mass spectrometry would be analytically useful for assessing the affinity of such molecules towards each other. Relative transmission factors provide a good qualitative assessment of the differences in ionization efficiency and measure of interaction strength (especially where structural modifications on similar analytes are investigated). For studying discrimination, provided by relative binding of analytes (guests, enantiomers, isomers, etc.) to a specific reference (host, substrate, etc.), gas-phase studies, using CAD, can be employed to measure branching ratios from dissociated products. In this case, correlation with solution-phase binding is not necessary and the final adduct ion form is only of interest.

The highly interactive and complementary guanidinium and phosphonate functional units are shown to interact through the formation of Coulombic interactions, with a possible degree of directed hydrogen-bonding character. Additional binding sites can serve to further stabilize an ionic cluster. Reciprocally, removal of these sites can destabilize an adduct form. Relative transmission factors determined for the range of ion forms correlate linearly with solution-phase concentration of the initial components in the mixtures. However, differences in transmission efficiency and the lack of correlation with the applied titration model for the free and bound ions indicate that quantitative solution-phase determination methods are not easily applied. Rather, multiple interaction equilibria in solution and during ESI and gas-phase processes likely account for the majority of ionic complexes observed. As an alternate approach, the formation of intense high order adducts allows the use of CAD to characterize the stability of the adduct ions in the absence of solvents. In this respect, the use of a well-characterized ESI procedure for the promotion of adduct ion formation between certain molecule types, can be beneficial to further study of ionic complex stability in the gas phase. This may lead to more specialized applications with the development of new host/guest (receptor/ligand, reference/analyte, selector/selectand, etc.) systems. In general, the experimental design has provided a robust method for transmission and formation of noncovalent clusters of small molecules. Implementation of this ESI method in a screening scenario should allow for identification of interactive host and guest molecules that can be further exploited for useful and informative analytical determinations.

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