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Using electrospray ionization-mass spectrometry/tandem mass spectrometry and small molecules to study guanidinium–anion interactions

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Abstract

A previous study highlighting the interaction between guanidinium- and phosphonate-functionalized molecules and the development of a screening protocol for noncovalent interactions using ESI-MS and MS/MS methodologies is extended here to incorporate sulfonate- and carboxylate-functionalized binding partners for guanidinium. Multiple high order homomeric and heteromeric adduct ions are observed in the mass spectra when mixtures of complementary analytes are ionized. Comparison of relative binding and ionization efficiencies are made using the solution-phase competition methods and gas-phase collision threshold dissociation ($E_{1/2}$) measurements. Transmission factors are determined to compare the effect of structural variation of the analytes on their relative ionization efficiencies. Results indicate that while phosphonate- and sulfonate-functionalized analytes form more and higher order adduct ion complexes with guanidinium-containing molecules (represented here by free and modified arginines) as a result of the ESI process, when solvent is removed and collisional dissociation is employed, the trend is reversed, and the carboxylate group yields a stronger interaction with guanidinium, relative to the other oxoanions. Ionization differences reflected in the mass spectra are attributed to pH effects present in the condensed phase, whereas differences in stability measured in the gas-phase are attributed to the gas-phase acidities of the oxoanions and their geometric complementarity when forming noncovalent interactions with guanidinium. This work highlights the interaction of guanidinium with oxoanion binding partners using various ESI-MS and MS/MS methods, but also addresses explicitly the advantages and disadvantages of using small molecule analytes for routine analysis of noncovalent interactions.

Keywords: Guanidinium; Oxoanion; Small molecule; Noncovalent interaction; Adduct ion; Electrospray

1. Introduction

The propensity of noncovalent interactions in biological systems and the interest in studying novel structures and functions of molecules in this setting has created an impetus for the development of efficient, effective, and information-rich methods and techniques of analysis. Of the more common techniques used for studying these interactions, soft ionization mass spectrometry, specifically electrospray ionization-mass spectrometry (ESI-MS), has shown the greatest development in the last several years. Several comprehensive reviews have been published which cover this topic [1–8]. These reviews detail a plethora of methodologies which have become commonplace in the analysis of noncovalent complexes between a large variety of different molecule types. Still, in light of the wealth of information which exists, the study of small molecule interactions, specifically designed to highlight complementarity between different functional groups, has been investigated to a lesser extent. Analysis of amino acid and peptide clustering [9–11] and the use of transition-metal mediated systems for conformational and configurational determinations [12–16] are the most

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prevalent small molecule investigations of noncovalent interactions encountered in the literature. What has become apparent is that through careful consideration of the electrospray ionization process and the choice of suitable interaction systems of interest, useful information can be extracted. Current work in our laboratory is focused upon understanding the interactions between the basic guanidinium functional unit and complementary acidic functional groups and development of the methodology to do so.

The proton-loving guanidinium group is present in a vast number of naturally occurring and synthetic biologically and pharmacologically relevant interaction systems [17–27]. This functional unit, composed of a forked, Y-shaped, planar geometry is known to be capable of both directed hydrogen-bonding, as well as nondirected Coulombic interactions with complementary groups [17,20]. In biological environments, referring to amino acids, peptides, and proteins as the most dominant species, guanidinium is most commonly encountered in the side chain of arginine and arginine residues. Here, interacting partners are composed mainly of acidic carboxylate, phosphate and sulfate groups. These anionic groups can be present as the side chains of aspartic and glutamic acid residues (carboxylate) or as a result of post-translational modification (phosphorylation and sulfation). Together, interactions between these units in biological systems are important for processes such as protein stabilization, RNA messaging, membrane transport of small and large biomolecules, and enzymatic catalysis, to name a few [20,24,25]. In synthetic systems, other variations of both guanidinium (free or cyclized) and anionic interacting partners (phosphonate, sulfonate, acid esters, etc.) may be encountered. These systems are designed for a variety of purposes, including pharmaceutical and bio-pharmaceutical (drugs, synthetic peptides, etc.) utility [22], as well as selective recognition (receptor–ligand, host–guest, etc.) and sensing [26,27], often mimicking biological schemes. Overall, studies of the interaction between guanidinium-based units and complementary anionic groups, particularly those resulting from phosphorylation events, currently comprise a relevant and analytically interesting topic in biochemical, pharmaceutical, and other related fields.

In this regard, we have recently published a study of the interaction between guanidinium and the phosphonate group using amino acids and various ESI-MS and tandem mass spectrometry (MS/MS) techniques [28]. The use of small molecules (previously, free and blocked arginine and aminophosphonic acid analytes) and mass spectrometry to assess noncovalent interactions between specific functional units has several advantages and disadvantages. In contrast to large molecules, where the cooperativity of multiple interaction sites precludes the determination of the role of each specific functional unit by ESI-MS, small molecules allow a more simplified and direct approach to isolating connectivity between two interacting partners. Analytes, such as amino acids, are useful because ionizable sites can be easily modified (e.g., C- and N-terminal blocking) to: (a) study that group's effect on the binding of a partner analyte; and (b) isolate functional units (in our previous work, the guanidinium and phosphonate groups [28]), making them the dominant interaction sites in a system under study. By systematically varying the analytes of interest, MS-based analysis techniques can be applied to screen specific and nonspecific interactions in a large number of complementary systems for comparison of different functional group interactions. In addition, the established mass spectrometric techniques are widely varied in their approach and the information which they provide; offering versatility to the experimentalist focused upon new systems of interest. Although this is appealing, disadvantages to performing experiments based upon small molecule interaction analysis by ESI-MS do exist. Inherently, the structure of each analyte greatly affects the efficiency by which it can be transferred from solution to the gas-phase during the electrospray process [29]. These changes in ionization efficiency are also apparent when comparing ionic complexes formed ("adduct ions") which incorporate different analytes. To minimize this effect, analytes with similar structure must be used and careful consideration of the affect of each of their ionization efficiencies must be made. Also, in cases where several ionizable sites on small molecules exist, the multiple interaction equilibria present, both in solution and during ESI and gas-phase processes, can result in complex mass spectra. This can hamper interpretation of the spectra as well as application of simple models or assumptions, useful when applying many of the established MS-based techniques for analysis. For example, the application of the equilibrium partition model [30] for predicting ionization response in a simple host–guest scheme, such as reported by Sherman and Brodbelt, becomes extremely difficult [31]. Still, concepts based on assessing the molecular and complex activities and relative partition factors inside the droplet are valid, even if they cannot be quantitatively elucidated. In general, useful information can be extracted from such systems through careful choice of experimental procedures, as well as explicit consideration of the effect of the ionization process on what is observed in the mass spectra.

The common methods for qualitative and quantitative analysis by ESI-MS and MS/MS can be separated into solution and gas-phase methods [7]. Solution-phase methods are designed for probing information about preformed complexes in solution by measuring ion abundances observed in the mass spectra. These include competition [3,7,32–34], titration [35–37], and temperature-dependent methods [38,39]. In these approaches, where specific information about interaction equilibria in solution is not known, assumptions must be made which state that the solution-to gas-phase transfer of a bound ionic complex is equal to that of the free, unbound host. This is often valid for large molecules, but is problematic for small molecules where the host–guest complex is often twice the size of either the host or the guest by itself. In such cases, gas-phase methods may offer a better approach for quantitatively evaluating interaction

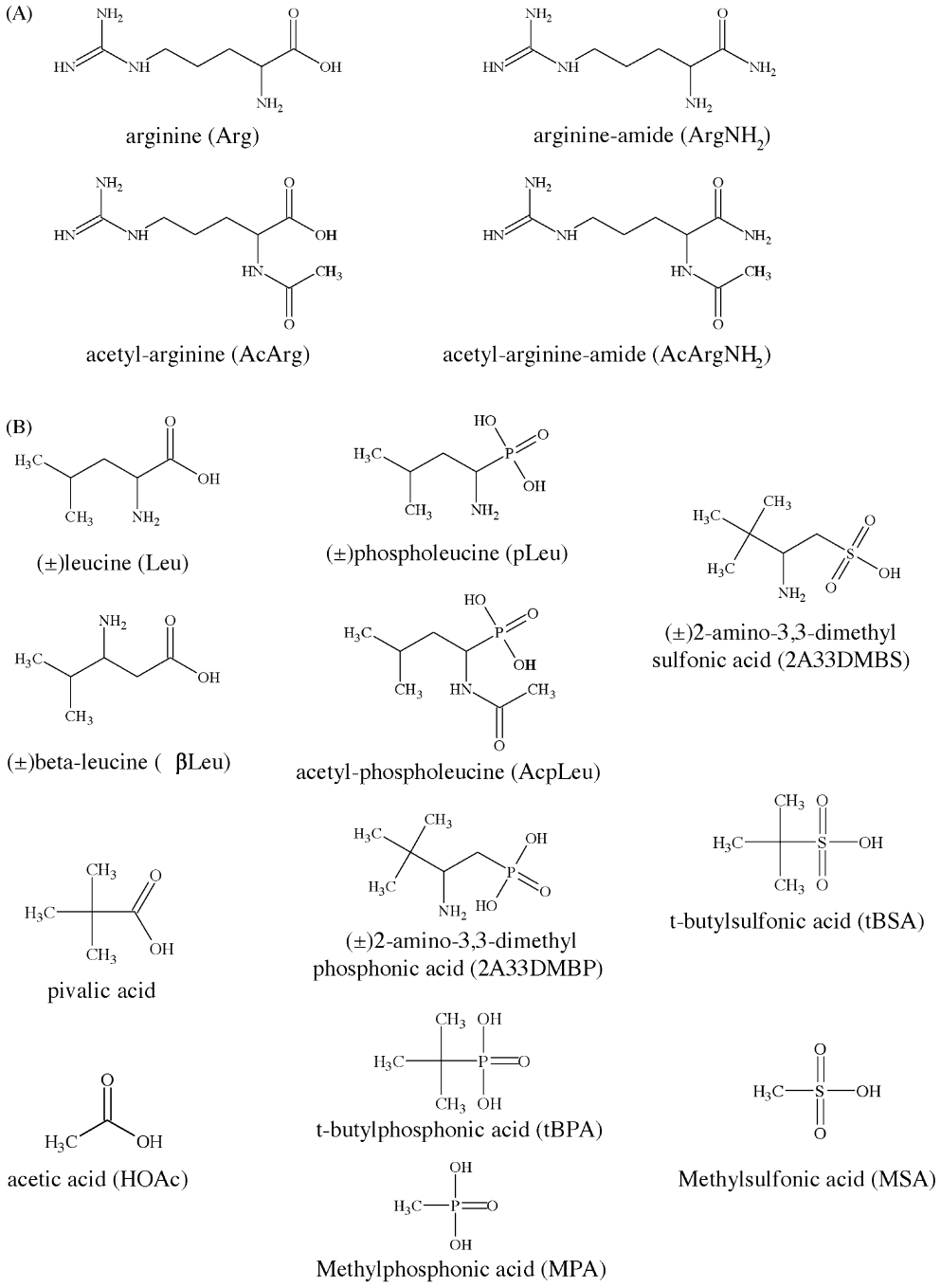

Fig. 1. Guanidinium (A)- and anionic (B)-based analytes used in this study to investigate noncovalent complex formation by ESI-MS and MS/MS.

energies. Gas-phase methods are used to make measurements independent of the solution-phase equilibria and the ESI process. This, in turn, may make interpretation and analysis, in the absence of solvent, simpler. However, by the same token, these methods offer neither specific information about condensed-phase equilibria nor the ESI process. Also, the interaction behavior of many ionic (acidic/basic) molecules is decidedly different in the solution and gas-phase due to large changes in solvation and the dielectric of the medium. These changes can be expected to effect quantities measured between the two states. The most common gas-phase techniques are thermal dissociation (in a heated transfer line) [7] and tandem MS measurements, following collision-activated dissociation (CAD) [5,40–43].

In this work, we seek to extend both the variety of interaction systems studied in conjunction with the highly basic guanidinium functional unit, as well as the methodologies employed to do so. To compare with binding to the guanidinium by phosphonate groups (aminophosphonic acids and methylphosphonate) studied previously [28], we

have added a larger array of phosphonic acid analytes, as well as carboxylate- (leucines and alkylcarboxylic acids) and sulfonate-based (aminosulfonic acid and alkylsulfonic acids) analytes. Fig. 1 depicts the guanidinium (A)- and anionic (B)-based analytes employed in this experiment. CAD threshold experiments are used to compare relative binding strengths between these three different oxoanions for the set of blocked (N-acetylated and/or C-amidated) and unblocked arginine analytes in the gas-phase. Also, variations on competitive equilibrium methods are used to establish orders of binding between the analytes in the condensed phase (in solution or during the ESI desolvation process, as related to the observed ionic complexes). The competition methods are used to study the effect of different guest (oxoanion) structure on relative responses of the adduct ions during simultaneous measurements. Introduced in our previous work [28], transmission factors are used again here to assess the affect of structural changes on the ionization efficiency of the analytes under scrutiny. The term transmission factor described here should not be confused with the transmission of an ion through the ion optics of a mass spectrometer only. Rather, this refers to an all-encompassing ionization factor which measures the relationship between the observed ion abundance of a given ion form and the initial concentration of a molecule contributing to that ion form. Overall, this work, in conjunction with our previous work, allows for a qualitative and quantitative comparison of the interactions of different oxoanions with guanidinium using small molecule analytes in conjunction with new and established MS-based techniques. The experiments are designed such that future extension of this work to peptides and other more complex interacting systems should be more straightforward.

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. Samples were introduced via a syringe pump operating at 5 μL/min. Because adduct ion formation was previously determined to be a much more significant portion of the total ion current in the negative ionization mode, compared to the positive ionization mode, the former was used here for all experiments [28]. Adduct ion formation in the positive ionization mode with these analyte systems is characterized by low abundances and the inability to isolate and dissociate the ion forms of interest with acceptable reproducibility. In the negative ionization mode, the following parameters were optimized for maximum adduct ion response in the investigated analyte systems: Spray capillary voltage (potential applied to the endplate in this model) = −4000 V; nebulizer gas pressure = 7.0 psi; dry gas flow = 4.0 L/min; dry gas temperature = 300 °C; desolvation capillary voltage (unheated transfer line to the high vacuum region) = −105 V; skimmer = −35 V; octapole 1 DC = −8.5 V; octapole 2 DC = −2.4 V; lens 1 = 4.5 V; and lens 2 = 55 V. Mass spectra were collected with "enhanced scan resolution" (5500 s^{-1} *m/z*). Except where tandem MS was employed, full scan mass spectra (50–1000 Th) were collected. Each spectrum collected for evaluation was an average of 75 ± 3 scans and each scan was an average of five microscans. Values used for statistical evaluation and reported as average intensities were the product of triplicate measurement.

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 concentrations of each component in the final equimolar mixtures used for analysis were 0.06 mmol/L (mM) for CAD threshold determinations and 0.04 mM for competition experiments. These concentrations were chosen for the purpose of operating in a linear response region and to avoid the onset of saturation of response which is observed when each component is present at greater than 0.1 mM. Measurements of transmission factors were made from solutions ranging in concentration between 0.005 and 0.1 mM. This linear operating range was determined previously [28]. Guanidinium-functionalized analytes used in these experiments were H-Arg-OH (unblocked arginine; Arg), Ac-Arg-OH (N-acetylated arginine; AcArg), H-Arg-NH_2 (C-amidated arginine; ArgNH_2), and Ac-Arg-NH_2 (N-acetylated and C-amidated arginine; AcArgNH_2). Arg (Sigma, Vienna, Austria), AcArg (Bachem, Weil am Rhein, Germany) and ArgNH_2 (Bachem) were obtained commercially. AcArgNH_2 was synthesized in-house from ArgNH_2 and purified by ion-exchange chromatography. All guanidinium-based analytes were chemically and enantiomerically pure and present in the (*S*) configuration. Anionic analytes employed in these experiments were leucine (Leu) (Fluka), beta-leucine (βLeu) (Acros, Geel, Belgium), pivalic acid (Sigma), acetic acid (HOAc) (Fluka), phospholeucine (pLeu) (obtained in-house from a previous study [44]), N-acetylated pLeu (AcpLeu) (synthesized from pLeu and purified by ion-exchange chromatography in-house), 2-amino-3,3-dimethylphosphonic acid (2A33DMBP) (obtained in-house from a previous study [45]), *tert*.-butylphosphonic acid (*t*BPA) (Acros), methylphosphonic acid (MPA) (Sigma), 2-amino-3,3-dimethylsulfonic acid (2A33DMBS) (obtained in-house from a previous study [45]), *tert*.-butylsulfonic acid sodium salt (*t*BSA) (Acros), and methylsulfonic acid (MSA) (Merck, Darmstadt, Germany). All anionic analytes were chemically pure and, if chiral, present as racemic mixtures.

3. Results and discussion

Electrospray ionization of equimolar mixtures of the above described analytes (A + B) with the given method, optimized for adduct ion formation, leads to mass spectra containing multiple ionic noncovalent complexes with varying degrees of average intensity and stoichiometry. These adduct ion responses are the result of numerous equilibria present in solution and during the ESI process. Previously, titration methods, using a simple theoretical solution-phase equilibrium model [35], were investigated for a guanidinium- and phosphonate-based system [28]. Results showed poor correlation with the ascribed model and indicated that: (a) the model used was two simplistic for the system to which it was applied; and/or (b) the observed adduct ion responses were due to formation during ESI and gas-phase processes. Instead, qualitative information gathered using the calculation and comparison of relative transmission factors, and relative quantitative data using CAD threshold determination methods, were shown to be superior in providing useful information regarding the interaction of the guanidinium- and phosphonate-based small molecule analytes. In the current

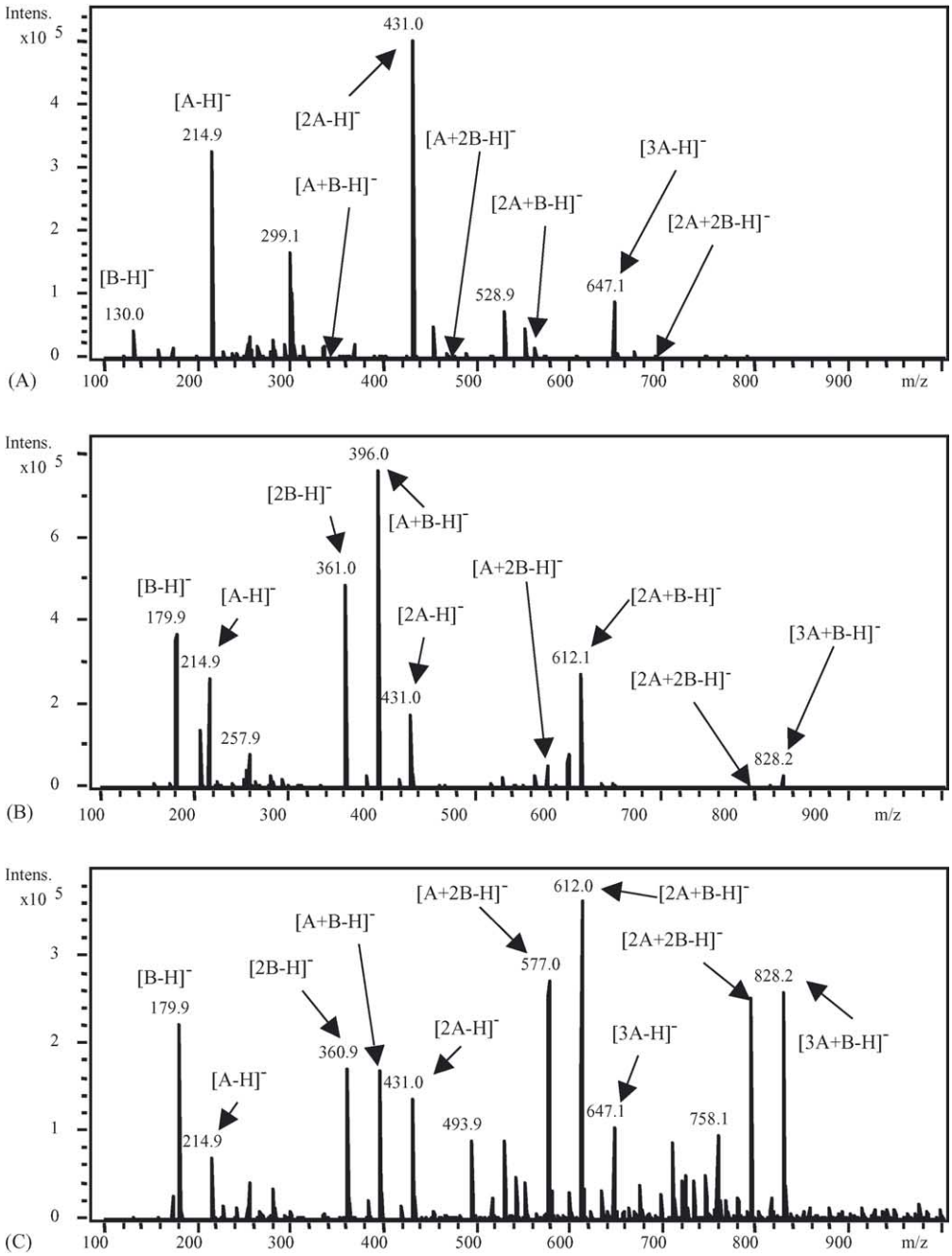

Fig. 2. Sample full scan mass spectra recorded for equimolar mixtures of AcArg with βLeu (A), 2A33DMBP (B), and 2A33DMBS (C).

study, we seek to compare adduct ion formation and relative strength of interaction of adduct ions based on the systematic variation of the anionic interaction partner (carboxylate-, sulfonate-, and phosphonate-based analytes) with the guanidinium group. To accomplish this, full scan mass spectra were collected to visually compare the degree of adduct ion formation under a fixed ESI method. Following this, solution-phase competitive host–guest experiments, with some variation (see below), were employed to rank the order of relative adduct ion formation probability for each of the different oxoanions. Transmission factors were determined for the various anionic guest analytes to assess ionization efficiencies and the ability to use structurally similar small molecule probes to compare oxoanion binding with a guanidinium group, as found in arginine and arginine-containing peptides. Finally, the relative strength of binding in the absence of solvent was measured using CAD threshold determination.

3.1. Adduct ion formation

The prominent adduct ion forms observed when performing ESI-MS of low-concentration (0.04 mM) equimolar mixtures of one analyte from each group, A and B, comprise various intensities and stoichiometries depending on the specific components employed. The dominant ion forms observed in the negative ionization mode were $[B-H]^-$, $[A-H]^-$, $[2B-H]^-$, $[2A-H]^-$, $[A+B-H]^-$, $[A+2B-H]^-$, $[2A+B-H]^-$, and $[2A+2B-H]^-$. Fig. 2 shows typical mass spectra observed for mixtures incorporating each of the different oxoanions. Fig. 2A shows AcArg mixed with βLeu; 2B shows AcArg mixed with 2A33DMBP (a β-amino phosphonic acid); and 2C shows AcArg mixed with 2A33DMBS (a β-amino sulfonic acid). All of the anionic components are similar in structure (see Fig. 1). Each has an amino group in the beta-position relative to the acid group. The only difference is the lack of one methyl group on the side chain of βLeu which is present for the other analytes. Although this group conceivably increases the ionization efficiency of the phosphonate- and sulfonate-based free and adduct ions (due to a slightly higher hydrophobicity [29]), the difference is expected to be small.

What is observed is a dramatic difference in the degree of adduct ion formation, depending on the anionic component in the mixture. The spectra for βLeu is dominated by free ($[A-H]^-$ and $[B-H]^-$) and homomeric ($[2A-H]^-$ and $[3A-H]^-$) adduct ions. Possessing a carboxylate anionic group, βLeu does not appear to interact strongly with AcArg to form heteromeric or mixed adducts. This trend is observed with all sample mixtures of Arg derivatives and carboxylate-based guest analytes used in this study under the prescribed ESI method. In contrast, the propensity for adduct ion formation by the phosphonate- and sulfonate-based analytes is much increased. Multiple heteromeric adduct ions are observed indicating an increased interaction between these oxoanions and AcArg. For the mixture of AcArg with 2A33DMBP, the base peak is a 1:1 adduct ion ($[A+B-H]^-$), whereas for the AcArg + 2A33DMBS mixture, a 2:1 ($[2A+B-H]^-$) ion form is the most dominant. This trend of greater heteromeric adduct ion formation (as well as higher intensity adduct ions) for the phosphonate- and sulfonate-based analytes relative to that for the carboxylate-based counterparts is also observed for the other similar sample mixtures tested. The full scan mass spectra indicate that the systems incorporating sulfonate and phosphonate groups should provide sufficient ion signals for further study. They also indicate that it is unlikely that native solution-phase interactions are reflected in the gaseous ions observed. These observations, however, are speculative and should be evaluated more rigorously with methods which take into account (or remove, if possible) the effects of ionization or transfer efficiency of the different ion forms.

3.2. Transmission factors

To assess the ionization efficiencies of the analytes of interest, transmission factors T_X have been calculated based on the relationship $I_X = T_X[M_i]$, where I_X is the intensity of the ion of interest and M_i is the original solution concentration of the analyte component in the ion form being measured. The use of T_X is analogous to the determination of transfer coefficients t_X, based on the equation $I_X = t_X[X]$ [7], but instead, since the equilibrium solution concentration of an ionic complex of interest X is unknown, T_X does not distinguish between ionic complexes formed in the solution-phase or as a result of ESI or gas-phase processes. Instead, transmission factors represent a qualitative way to compare the ionization efficiency (including detection by the mass spectrometer) of structurally similar or dissimilar analytes under fixed ionization conditions. When T_X values for different ions are similar, so then is their ionization efficiency. In these cases, a more quantitative comparison, especially when utilizing competition methods for assessing relative binding, can be made.

Table 1 lists the transmission factors for the anionic analytes (see Fig. 1B) measured from sample solutions in the absence of guanidinium-based components. Focusing first on the values recorded within each anionic group, the effect of analyte structure is apparent. For carboxylate-based analytes, T_X for Leu, βLeu, and pivalic acid are similar. A marked decrease in ionization efficiency is apparent for HOAc, as expected, due to the molecule's much lower hydrophobicity (and hence, more favorable solvation energy in the solution media used here) [29]. A parallel comparison is apparent in each of the other two anionic analyte groups. In all cases, the *tert*.-butyl analyte components (pivalic acid, *t*BPA, and *t*BSA) possess similar ionization efficiency to the other analytes in their groups. If only the $[B-H]^-$ T_X values are considered, it is apparent that the solvation energy contribution provided by additional ionizable functional units in the majority of the analytes (increasing analyte affinity for the condensed phase) is offset by the larger alkyl chains so that, overall, ionization efficiency of these analytes is closer

Table 1
Transmission factors (and linear correlation coefficient, R^2) for anionic interactions components in the absence of guanidinium-functionalized analytes

Anionic group	Analyte (B)	T_X (10^5 mM^{-1}) (R^2)			
		$[B-H]^-$	$[2B-H]^-$	$[3B-H]^-$	Sum all
COOH	Leu	6 (0.988)	0	0	6 (0.987)
	βLeu	3 (0.967)	0	0	3 (0.960)
	Pivalic acid	4 (0.984)	0	0	4 (0.984)
	HOAc	0.04 (0.919)	0	0	0.05 (0.945)
PO_3H_2	pLeu	30 (0.984)	40 (0.887)	30 (0.943)	100 (0.963)
	Acpleu	60 (0.985)	3 (0.954)	0	60 (0.989)
	2A33DMBP	40 (0.954)	50 (0.907)	0	100(0.911)
	*t*BPA	20 (0.987)	6 (0.867)	0	20 (0.989)
	MPA	8 (0.987)	0	0	8 (0.986)
SO_3H	2A33DMBS	40 (0.976)	60 (0.986)	20 (0.936)	100 (0.989)
	*t*BSA	30 (0.960)	0	0	30 (0.960)
	MSA	7(0.981)	0	0	7(0.981)

Measurements for $[B-H]^-$ and $[2B-H]^-$ were made using seven data points (0.005–0.1 mM; $n=3$ for each data point), whereas those for $[3B-H]^-$ were made using five data points (0.02–0.1 mM; $n=3$ for each data point). Data with correlation coefficients below $R^2=0.85$ were omitted.

to the value of a singly functionalized analyte with a shorter alkyl unit. This in itself may prove to be a convenient means for comparison; however, the presence of higher functionality also contributes additional associative equilibria. Shown in Table 1 by T_X values for the higher homomeric adduct ion forms ($[2B-H]^-$ and $[3B-H]^-$), the phosphonate and sulfonate analytes with free amine groups are particularly amenable to higher order adduct formation. Therefore, it seems more practical to use the transmission factor determined from the sum of all ion signals when comparing the ionization efficiency of small molecules. The effects of these additional equilibria (i.e., higher order adduct ion formation) will be considered in more detail with the application of competitive binding experiments discussed below.

The main reason for generating transmission factors in these experiments is to assess the ionization efficiency between the analytes possessing different anionic functional units. In this sense, it is apparent from the higher values of T_X, that the phosphonylated and sulfonylated analytes are much more amenable to creating high intensity negative ions with the ESI method used here. Again, the presence of additional functional units outside of the anionic groups of interest makes it difficult to account for ionization equilibria in an analyte which is transferred into the gas-phase as a singly negative charged ion. Consideration of the series of *tert*.-butyl analytes, however, makes it clear that under the ionization conditions employed, the phosphonate and sulfonate groups contribute much more significantly to ionization than does the carboxylate group. The calculated pK_a values for pivalic acid (4.94 ± 0.10), *t*BPA (2.48 ± 0.42; pK_{a1}), and *t*BSA (1.99 ± 0.50) (values calculated used ACD/pK_a DB pK_a calculator) indicate that all analytes should be completely ionized to a -1 charge in a solution mixture of 50/50 acetonitrile/water. This is assumed for the sake of comparison, even though: (a) it is difficult to assess the local pH effects in a small evaporating droplet; and (b) it has been previously reported that the presence of organic modifier can increase the pK_a of an acidic buffer in a mixed aqueous/organic solution system [46–48]. In general, the T_X values for the phosphonylated and the sulfonylated analytes are similar, offering a good comparison of the different binding effects with guanidinium for these groups in the subsequent competition experiments below. In contrast, the carboxylate analytes, even though they possess similar backbone structures to their corresponding phosphonate- and sulfonate-based counterparts, are ionized approximately an order of magnitude less efficiently.

3.3. Competition experiments

Competition experiments are solution-phase based measurements useful for comparing the relative binding of two guests for one host (or vice versa) in a single experiment. As such, an obvious advantage of this approach is the removal of run-to-run variability. Still, in a system where the ionization efficiency of the free and bound hosts and guests are not constant, it is imperative to account for or normalize the different responses due to structural variation of analytes being compared. Also, it is important to consider the degree of adduct ion formation that is present (i.e., how many homo- and heteromeric ion forms are present for a given system?). The presence of multiple equilibria (and thus, adduct ion forms) complicates data interpretation and decreases the feasibility of accounting for all observed responses due to a given analyte.

With these considerations in mind, a traditional approach to evaluating competition between two guests for one host by simply taking the ratio of adduct ion abundances seems impractical. For this reason, we have chosen to evaluate multiple approaches for assessing the data. The result of each approach must be interpreted differently, but collectively a more complete picture of the important processes can be gathered. The basis of each approach lies in the calculation of a fundamental "selectivity factor" which differentiates the response due to each anionic guest for a given host [3].

Table 2
Competitive binding selectivity factors calculated by three methods for varied competition systems

Anionic group	Competitor (B_b)	Method 1 $\left(S_1 = \frac{\text{Int}([A+B_b-H]^-)}{\text{Int}([A+B_a-H]^-)}\right)$		Method 2 $\left(S_2 = S_1\left(\frac{\text{Int}([B_a-H]^-)}{\text{Int}([B_b-H]^-)}\right)\right)$		Method 3 $\left(S_3 = \frac{\text{all(A:B}_b)/\text{non(A:B}_b)}{\text{all(A:B}_a)/\text{non(A:B}_a)}\right)$	
		$AcArgNH_2$ + AcpLeu + ...	Arg + pLeu + ...	$AcArgNH_2$ + AcpLeu + ...	Arg + pLeu + ...	$AcArgNH_2$ + AcpLeu + ...	Arg + pleu + ...
COOH	Leu	0.16	0.01	1.69	0.07	3.23	0.82
	βLeu	0.14	0.01	2.38	0.10	3.55	0.81
	Pivalic acid	0.02	0.01	2.37	0.04	39.77	0.57
	HOAc	0.03	0.01	9.76	4.53	22.21	22.38
PO_3H_2	pLeu	0.94	–	2.37	–	4.72	–
	AcpLeu	–	1.23	–	0.55	–	1.28
	2A33DMBP	1.00	1.82	2.29	1.33	5.06	1.40
	*t*BPA	0.33	0.72	0.66	0.69	1.71	1.43
	MPA	0.10	0.28	6.77	10.74	17.81	7.31
SO_3H	2A33DMBS	0.53	0.56	0.81	0.26	6.43	0.97
	*t*BSA	0.32	0.17	0.44	0.08	1.09	0.47
	MSA	0.03	0.17	1.42	5.39	5.19	3.79

Table 2 lists selectivity factors using two base competition systems and three separate methods for calculating selectivity factors. The base systems were chosen to cover the range of adduct ion formation complexity in the host–guest systems evaluated. The first base system, $AcArgNH_2$ + AcpLeu + competitor, is simple. All ionizable groups except for the guanidinium ($AcArgNH_2$) and phosphonate (AcpLeu) groups have been chemically modified. As such, the dominant ion-pair driven adduct ion form observed for this system is the 1:1 adduct ion. The second base system, Arg + pLeu + competitor, possesses the highest degree of complexity. Because there are many ionizable groups (none are chemically modified in this system), there are many more association equilibria which can complicate the mass spectra. Most of the homomeric and heteromeric adduct ion forms of interest, and listed previously, are observed with this system. Therefore, selectivity factors calculated for a single ion form, for example, will be largely "diluted" by equilibria for formation of other adduct ion forms and difficult to interpret.

The three methods chosen to evaluate these systems were conceived based on previously established methods [3] and with consideration of the difficulties associated with small molecule noncovalent complex analysis by ESI-MS. Method 1 focuses on evaluation of the 1:1 ($[A + B - H]^-$) adduct ion and is reported simply as the ratio of responses for this ion form between the competitor and the base system. Method 1 is akin to a traditional competition method used to evaluate systems where large hosts bind small guests. The selectivity factors calculated with this method will be greatly affected by the different structures of the anionic guests. Method 2 focuses also on the 1:1 adduct ion form and is designed to remedy the difference in ionization due to structure by normalizing the data to the free ion responses. Method 3 incorporates all of the adduct ion forms observed. As such, this method likely fails to explicitly isolate the guanidinium–anion interaction, but should be more comprehensive in accounting for complex formation. What can be gauged is the appropriateness of treating the 1:1 adduct ion form by itself, as in Methods 1 and 2, while neglecting the other responses (and therefore, equilibria) present in the mass spectra. Selectivity factors for Method 3 are calculated as the ratio of the responses for all of the observed heteromeric ion forms of the competitor normalized to the summed responses of all of the free and homomeric ion forms with that of the base system.

What is immediately evident upon examination of the competition experiment data (Table 2) for the different calculation methods utilized is that there exist large differences due to both the base system selected and the structural properties of each analyte employed. To reiterate, this is expected with this type of analysis using small molecules. Beginning with Method 1, and considering the first base system, $AcArgNH_2$ + AcpLeu + competitor, all of the values are less than or equal to one. This indicates a greater 1:1 adduct ion response for AcpLeu + $AcArgNH_2$, compared to that for nearly all of the other competitors tested. Values of 1.00 and 0.94 are recorded for 2A33DMBP and pLeu, respectively. Both are phosphonate-based analytes with similar structures to AcpLeu, indicating that: (a) the statement regarding similar transfer/ionization for similarly structured analytes is valid; and (b) that the amino group (blocked in AcpLeu, α to the phosphonate in pLeu, and β in 2A33DMBP) is not likely involved in the interaction with $AcArgNH_2$. This confirms that these three molecules comprise suitable controls for assessing the isolation of the guanidinium–phosphonate interaction in this system. However, staying within the PO_3H_2 interaction groups in Table 2, and assessing the competition by MPA in this base system, it is apparent that the 1:1 adduct ion formed from the much smaller MPA is not ionized as efficiently as that for AcpLeu. Assuming that the interaction strength between guanidinium and phosphonate is equivalent for MPA and AcpLeu with $AcArgNH_2$, such a result is a prime example of why direct quantitative comparison

of adduct ions is largely affected by the ionization process when small molecules are involved. This finding echoes that described above in the determination of transmission factors. The S_1 value associated with *t*BPA is slightly larger than that for MPA, also indicating the effect of increased hydrophobicity.

Next, those analytes with similar structure to AcpLeu, but possessing different anionic functionalities can be compared. The interaction by the sulfonate group in the 1:1 adduct ion, shown by 2A33DMBS, is approximately half as strong as that for AcpLeu. S_1 for *t*BSA is between that of MSA and 2A33DMBS, mimicking the results of the phosphonate competitors. The carboxylate equivalents, Leu and βLeu, show responses of approximately 15% of that of AcpLeu, whereas the values for pivalic acid and HOAc are even smaller. Thus, according to Method 1, a relative order is established for binding in the condensed phase with the guanidinium group: phosphonate > sulfonate > carboxylate. Though binding appears to be controlled by condensed (solution) phase acid/base equilibria, previous results [28], hint that it is likely ESI processes (droplet shrinking and local concentration effects) and not native solution equilibria which are responsible for the observed ion forms.

Results from the second base system in Method 1, Arg + pLeu + competitor, are less informative and difficult to assess, because it is likely that the presence of many other adduct ion forms, detracts from the ability to effectively isolate and look at just the 1:1 adduct ion form. Still, the relative order of response is preserved as the phosphonate-based analytes show a higher selectivity factor than the sulfonate-based analytes, which again is greater than the carboxylate-based analyte equivalents. The COOH analytes, especially, are unable to effectively compete in this system.

Method 2, where each of the 1:1 adduct ion responses is normalized to the response of the free ion form, was expected to be a more information-rich approach to the competition experiments where small molecules are employed. By normalizing to the free ion response, we expected to remove the difference due to ionization caused by the different structures. However, even more so than with Method 1, the effect of structural variations convolutes the calculated values. It is easiest to consider the effect mathematically. The selectivity factor S_2 in Method 2 is calculated as $S_2 = ([B_b]_{1:1}/[B_b]_{free})/([B_a]_{1:1}/[B_a]_{free})$, where $[B_b]_{1:1}$ and $[B_b]_{free}$ are the adduct and free ion responses of the competitor, respectively, and $[B_a]_{1:1}$ and $[B_a]_{free}$ are those, respectively, for the base system studied. AcpLeu, because it is the most hydrophobic of the molecules investigated, also has the highest free ion ($[B-H]^-$) response. When present in the base system, this raises $[B_a]_{free}$ and causes S_2 to be greater than one for all of the competitors tested. The values of S_2 are much greater than one for HOAc and MPA in this case, because both of these analytes have very low free ion responses ($[B_b]_{free}$). Also, comparison of values from the SO_3H analytes shows results which are not mirrored by the other structural counterparts. Since it is not true that $[B_b]_{free}$ is similar for MSA and Leu, we believe that there is interference in the measured value caused by the propensity of the sulfonate group to induce more and varied interactions (higher order adduct ions) in this system. The same reasoning can be used to explain the S_2 value calculated for *t*BSA. This tendency can also be inferred qualitatively from Fig. 2.

In the base system Arg + pLeu + competitor, evaluated by Method 2, again a combination of effects, noted with the first base system, is present. Compared to the carboxylate-based analytes (with the exception of HOAc) pLeu binds in a 1:1 adduct ion form much more readily. The order determined previously concerning the sulfonate-based 2A33DMBS also holds, with a response greater than the carboxylate-based but less than the phosphonate-based analytes. Again, the S_2 values calculated for the very small (methylated) anionic molecules are greater than one, due to their poor ionization efficiency as free ions. Differences between pLeu and the other phosphonate-based analytes can also be reconciled. The S_2 value with respect to AcpLeu is less than one ($S_2 = 0.55$) due to the higher free ion formation by the more hydrophobic structure. Such an explanation also holds for *t*BSA and *t*BPA. The response of 2A33DMBP is greater than one ($S_2 = 1.33$), but does not differ very significantly from one. This change may be due to different overall adduct ion formation (outside of the 1:1 adduct ion form focused upon with Method 2) as a result of α- versus β-amino analyte forms. In general, Method 2 appears to be less quantitative than Method 1, due to the added effect of small changes in response for free versus adducted ions which are enhanced in a normalization-type procedure.

Method 3 allows the evaluation of the effect of additional adduct ion formation (presence of additional equilibria) on the values calculated for Methods 1 and 2, which focused only on the 1:1 adduct ion form. By accounting for the additional response by other heteromeric adduct ($[2B+A-H]^-$, $[B+2A-H]^-$, $[B_a+B_b+A-H]^-$, and $[2B+2A-H]^-$) and free or homomeric adduct ($[2B-H]^-$ and $[B_a+B_b-H]^-$) ion forms, outside of those used for Method 2, some attenuation of the S_3 values are observed, especially in the first base system; with a few exceptions. An overall increase of the S_3 values in the first base system leads to two valid interpretations: (1) the overall adduct ion forming propensity of the competition systems remains the same, with an exception for the sulfonate-based competitors; and (2) the ratio of the bound to free ion forms in this calculation for the base system (based on AcpLeu) has decreased. The reduction of the bound to free ion form ratio for AcpLeu means that there is little additional contribution from other adduct ion forms for this analyte, and that there is an increased contribution from the other homomeric adduct ion forms for the competitors included in the calculation. Both the former, an important premise behind our methodology, and the latter, an expected result, are acceptable and reinforce the statements made previously. The main difference is the increase of the sulfonate analyte S_3 values by changing calculation from Method 2 to Method 3. This also indicates

Table 3
CAD threshold values ($E_{1/2}$) and most abundant ion fragment observed for isolable adduct ion forms

Analyte mixes (A + B)		Adduct ion forms and $E_{1/2}$ (V) (most abundant fragment ion)				
A	B	$[2B - H]^-$	$[A + B - H]^-$	$[A + 2B - H]^-$	$[2A + B - H]^-$	$[2A + 2B - H]^-$
Arg	Leu	1.32 ($[B - H]^-$)	ND	0.78 ($2B - H]^-$)	1.06 ($[2A - H]^-$)	0.69 ($[A + 2B - H]^-$)
	βLeu	1.39 ($[B - H]^-$)	ND	1.13 ($[2B - H]^-$)	ND	0.71 ($[A + 2B - H]^-$)
	pLeu	1.19 ($[B - H]^-$)	1.21 ($[B - H]^-$)	0.86 ($[2B - H]^-$)	0.94 ($[A + B - H]^-$)	0.67 ($[2A + B - H]^-$)
	2A33DMBP	0.81 ($[B - H]^-$)	1.14 ($[B - H]^-$)	0.92 ($[2B - H]^-$)	0.95 ($[A + B - H]^-$)	0.65 ($[2A + B - H]^-$)
	2A33DMBS	0.76 ($[B - H]^-$)	ND	1.00 ($[2B - H]^-$)	0.94 ($[A + B - H]^-$)	0.69 ($[A + 2B - H]^-$)
AcArg	Leu	ND	ND	0.98 ($[2B - H]^-$)	ND	0.92 ($[A + 2B - H]^-$)
	βLeu	ND	ND	1.03 ($[2B - H]^-$)	ND	0.83 ($[A + 2B - H]^-$)
	pLeu	1.21 ($[B - H]^-$)	0.82 ($[A - H]^-$)	0.95 ($[A + B - H]^-$)	0.67 ($[A + B - H]^-$)	0.77 ($[2A + B - H]^-$)
	2A33DMBP	0.80 ($[B - H]^-$)	0.80 ($[A - H]^-$)	0.61 ($[A + B - H]^-$)	0.65 ($[A + B - H]^-$)	ND
	2A33DMBS	0.76 ($[B - H]^-$)	0.77 ($[B - H]^-$)	0.68 ($[A + B - H]^-$)	0.68 ($[A + B - H]^-$)	0.89 ($[A + 2B - H]^-$)
$ArgNH_2$	Leu		1.12 ($[B - H]^-$)	1.20 ($[B - H]^-$)	ND	ND
	βLeu		1.21 ($[B - H]^-$)	1.20 ($[B - H]^-$)	ND	ND
	pLeu		1.89 ($[B - H]^-$)	0.90 ($[2B - H]^-$)	ND	ND
	2A33DMBP		1.81 ($[B - H]^-$)	0.89 ($[2B - H]^-$)	ND	ND
	2A33DMBS		2.01 ($[B - H]^-$)	1.03 ($[2B - H]^-$)	ND	ND
$AcArgNH_2$	Leu		1.17 ($[B - H]^-$)	1.07 ($[2B - H]^-$)	ND	ND
	βLeu		1.18 ($[B - H]^-$)	0.88 ($[2B - H]^-$)	ND	ND
	pLeu		0.77 ($[B - H]^-$)	0.90 ($[2B - H]^-$)	ND	ND
	2A33DMBP		0.81 ($[B - H]^-$)	0.54 ($[2B - H]^-$)	ND	ND
	2A33DMBS		0.68 ($[B - H]^-$)	0.69 ($[2B - H]^-$)	ND	ND

'ND' denotes the inability to isolate this ion form due to insufficient ion signal.

that sulfonate-based analytes have a higher propensity for forming higher order adduct ions (those other than the 1:1 adduct ion form measured in Method 2), reinforcing previous hypotheses.

The base system Arg + pLeu + competitor was chosen because of the much increased high order adduct ion response relative to the other base systems. The competitors in this system remain in the overall trend for adduct ion formation established previously: phosphonate > sulfonate > carboxylate. Also, the similar S_3 values measured for the similar analyte structures in each respective anionic group (0.82 and 0.81 for Leu and βLeu, respectively, and 1.28 and 1.40 for AcpLeu and 2A33DMBP, respectively) emphasizes the ability to compare these systems if there is little or no variation in structure outside of the primary interaction group. These molecules, by virtue of their similar functionality and arrangement, have a similar propensity for forming various adduct ions with the Arg derivatives studied. The increase in S_3 values, relative to Method 2, with the exception of MPA and MSA, are due to the incorporation of the homomeric ion forms in the normalization factors for the calculation (pLeu has a high tendency for dimer ion formation). By evaluating these different approaches to calculating competitive selectivity values for small molecule systems we see that the presence of multiple equilibria makes it difficult to make quantitative comparisons. Instead, the solution-phase based method really only provides qualitative information which can be used to assess trends in the data. When the ionization efficiency of two analytes being compared is similar (as can be evidenced from their respective transmission factors), a more reliable comparison can be made. In this system, the data shows that the propensity to form adduct ions in the solution-phase, especially the 1:1 adduct ion form of interest, when the guanidinium–anion interaction is isolated, is given by the following: phosphonate > sulfonate > carboxylate. Method 1 and Method 2 provide means for focusing directly on a single interaction type, however both are highly effected by any differences in ionization efficiency between the competing analytes. For overall assessment of binding and consideration of all equilibria present, Method 3 seems to be the better choice. The changes in calculated selectivity factors with respect to evaluating a system that exhibits a high degree of adduct formation (Arg + pLeu) versus that which adducts to a lesser degree ($AcArgNH_2$ + AcpLeu) are apparent with Method 3. Care must be taken with uncovering and selecting all relevant ion forms in this case.

3.4. CAD threshold determination

Determination of the threshold for dissociation of a non-covalent complex by using tandem MS and CAD, following isolation of an ion in an ion trap, is a convenient method for assessing the interaction between species in the absence of solvent. To evaluate the relative strength of interaction for prominent adduct ion signals, "melting curves" (collision energy titration curves) were generated by measuring the intensity of the parent ion through a step-wise increase in collisional excitation [41,43]. The value of collision energy where one-half of the parent ion is dissociated is reported as the half-dissociation threshold value, or $E_{1/2}$. Table 3 shows the $E_{1/2}$ values (and the major fragment ion observed) for all of the isolable adduct ion forms with analytes used in this

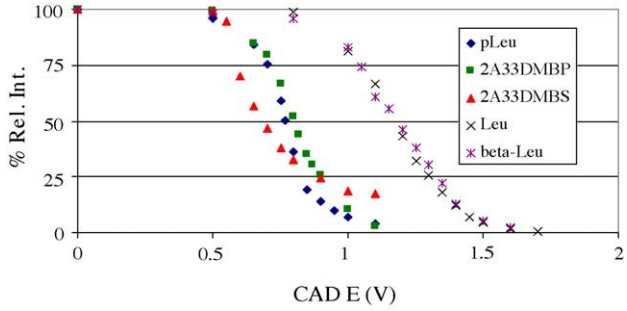

Fig. 3. Experimental data for determination of $E_{1/2}$ values for $AcArgNH_2$ mixed with carboxylate (Leu, βLeu)-, phosphonate (pLeu, 2A33DMBP)-, and sulfonate (2A33DMBS)-based interaction partners. The increased interaction strength of the carboxylate group with guanidinium is apparent from the shift in data points to higher CAD energy.

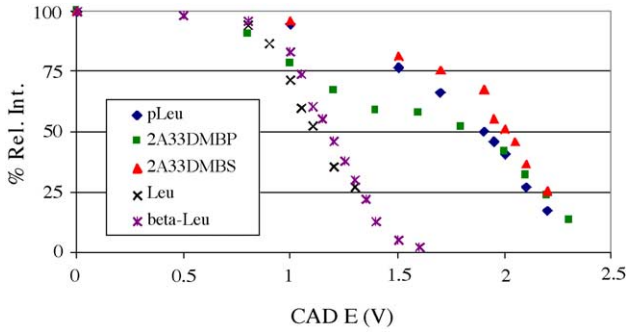

Fig. 4. CAD threshold determination data for 1:1 adduct ions formed between $ArgNH_2$ (N-terminal Arg peptide mimic) and five different amino acid interaction partners. The shift in interaction strength and the irregular shape of the titration curve for the phosphonate- and sulfonate-based systems likely indicates additional interaction by these groups with the free, unblocked amine on $ArgNH_2$.

study. It is important to note that quantitative comparison of collision thresholds between two dissociated complexes requires identical dissociation pathways (i.e., the same fragment ions) for the systems being compared.

Each isolable adduct ion form for the amino acid guest molecules were evaluated for their half-dissociation collision threshold. The main goal of these experiments was to isolate and study the guanidinium–oxoanion interaction. This is exhibited by the values in Table 3 for $AcArgNH_2$ + anionic substituent. Looking at the 1:1 ($[A + B - H]^-$) adduct ion form, what is immediately apparent is a reversal in the order of relative binding strength for that established with the solution-phase competition methods. Here, the order of binding is: carboxylate > phosphonate ≥ sulfonate. This trend, as well as the excellent agreement for the α- and β-amino carboxylate and phosphonate analyte variants studied, may be more easily observed in Fig. 3.

The difference between the trend observed in solution-phase and the relative quantitative values established with CAD is the absence of solvent. In solvent, the combination of an electrostatic and hydrogen-bonding environment allows the phosphonate and sulfonate, stronger acids than carboxylate, to bind with guanidinium more strongly than carboxylate, resulting in more and greater intensity adduct ions. There are two possibilities for reasoning the observed trend in the gas-phase measurements. The first is the difference in gas-phase acidity associated with the different anion groups. In the gas-phase, the relative order of acidity of the sulfur-, phosphorous-, and carbon-based oxoanions reverses from that which is observed in solution. Measurements of gas-phase acidity (free energy for deprotonation) of the specific analytes used here are not available, however, the order can be established by examining literature values of similar oxoanion species. The gas-phase acidity of acetate (1428.8 kJ/mol) is greater than that for dimethylphosphate (1359.8 kJ/mol), which is in turn greater than that for methylsulfonate (1318.0 kJ/mol) [49]. Magnitude of gas-phase acidity (or basicity) has been previously attributed to the observed stability of gas-phase complexes [50,51] and, for example, the greater gas-phase reactivity of phosphate over carboxylate [52]. The second possible contributor to the observed trend in stability is the geometric arrangement of the interacting functional units. When the solvent is removed, the hydrogen-bonding contribution to binding, and thus, the directionality of the interaction, between the analytes of interest becomes more important. The geometrical complementarity of the assembled guanidinium–carboxylate (fork–fork) interaction could therefore conceivably create a stronger interaction than the guanidinium–phosphonate/sulfonate (fork–pyramid) interaction. Overall, though these results are informative, they emphasize the importance of: (a) considering the role of solvent in solution-phase interactions; and (b) considering the differences in attractive forces measured between two groups in solution- versus gas-phase. Thus, it is sometimes difficult (and possibly, erroneous) to report information about the behavior of a solution-phase system through gas-phase measurements.

Other interesting information is also present in the measured $E_{1/2}$ values. In general, the measurement of the dissociation of the high order adducts must be observed carefully. Especially with analytes having multiple strong interaction sites (e.g., the unblocked Arg analytes), the measured values may pertain to the loss of guest molecules outside of the interaction sphere (guanidinium–oxoanion) of interest. Computational evaluation may be useful in the future to address these arrangements and losses. However, by blocking one or the other ionizable groups remote to the guanidinium group on Arg, thus mimicking the Arg moiety within a peptide chain, the interaction of the anionic molecules with the N- (using $ArgNH_2$) or C- (using AcArg) terminus of a peptide can be modeled. Little can be compared with the AcArg results since the carboxylate-based amino acid analytes do not form an isolable high intensity 1:1 adduct with AcArg. The 1:1 interactions by the anionic analytes with $ArgNH_2$, in contrast, are very interesting. Fig. 4 shows the half-dissociation collision threshold determinations for the 1:1 adduct ions between $ArgNH_2$ and the five amino acid analytes. Compared

to the interactions of these analytes with $AcArgNH_2$, the response of the carboxylate-based analytes remains essentially unchanged. However, the phosphonate- and sulfonate-based analytes show both a shift to higher interaction energy, as well as what appears to be a bimodal dissociation profile. This can be explained by the presence of the free amino group in $ArgNH_2$; similar to what would be encountered through interaction with a peptide containing an N-terminal arginine residue. While the carboxylate group can be locked into a fork–fork interaction with the guanidinium, the sulfonate and phosphonate groups are larger and possess the ability to form additional interactions with their remaining oxygen. Phosphonate possesses the ability to lose an additional hydrogen, however, this would change the *m/z* of the observed ion, and therefore probably does not contribute to this phenomenon. It is also of interest to note that these results do not follow the trend in gas-phase acidity, indicating the possibility for multiple interactions which attenuate the measured relative binding strength for the phosphonate- and sulfonate-based interactions in this case. Dissociation in these systems is assumed to follow the same pathway as identical 'most abundant fragment ions' are observed. These phenomena will be interesting to study and confirm in future experiments with diverse peptide molecules.

4. Conclusions

These experiments were designed and carried out with the aim of: (a) studying the interaction between guanidinium and complementary anionic functional units; and (b) evaluating the applicability of well-established MS-based approaches for studying noncovalent interactions between small molecules. Both solution-phase competition and gas-phase dissociation mass spectrometric methods for determination of noncovalent interactions have been employed. As with previous experiments [28], the solution-phase method provided only qualitative information due to the lack of correlation of mass spectrometric signals to solution-phase equilibria and the presence of multiple interactions equilibria (likely a result of the ESI process) which complicate interpretation. Still, a reliable trend using a competitive binding procedure for adduct ion formation with the given ESI-MS method by the oxoanionic analytes studied was revealed: phosphonate > sulfonate > carboxylate. This was in contrast with that found for the order of binding using CAD threshold determination in the gas-phase. Due to the lack of solvent and the higher gas-phase acidity and better hydrogen-bonding complementarity of carboxylate in the absence of solvent, analytes with the COOH oxoanion interacted more strongly with the guanidinium unit than those with sulfonate or phosphonate functional units. Overall, these results indicate that screening of small molecule noncovalent interactions by ESI-MS: (a) can be performed using chemical modification to reduce complexity and directly isolate functional group interactions; (b) provides more quantitative informative through gas-phase measurements where the effects of the ESI process on response can be neglected; (c) requires careful consideration when comparing solution-phase and gas-phase data, not only due to differences in ionization from structure, but also due to changes in functional group interaction strength; and (d) is an effective way for directly screening and comparing the interactions between complementary functional units. The calculation of transmission factors as a qualitative tool to identify analytes with similar (and dissimilar) ionization efficiencies is also useful when attempting to assess interactions between small molecule analytes by ESI-MS methodologies. Future experiments will serve to extend these concepts to more complex and more biologically or pharmaceutically relevant analytes.

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