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# Mass spectrometry evaluation of the solution and gas-phase binding properties of noncovalent protein complexes

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**Abstract**

Recent study on the gas-phase binding of noncovalent protein complexes formed between bovine pancreatic trypsin inhibitor (BPTI) and its target enzymes by ESI-MS/MS revealed that interactions existing in the gas-phase are not reflective of their solution behavior [J. Mass Spectrom. 36 (2001) 950]. In the work described here, the question whether mass spectrometry evaluation of the solution binding properties of these noncovalent protein complexes are reflective of their solution-phase interactions was addressed. Assessment of the titration and competitive binding experiments from the abundance of gas-phase complex ions produced by an ESI source showed correlation with known solution binding order in relative, rather than in absolute, terms; suggesting disruption of the previously existing solution equilibrium. In addition, new data on the dissociation of the gas-phase ions of noncovalent protein complexes produced by nanoESI source were also acquired. These data were compared with previously published observations for dissociation of gas-phase ions of these noncovalent complexes produced by ESI source. Gas-phase binding of noncovalent protein complexes was assessed from the ease of dissociation of gas-phase ions using a collision model for an ion activation. It is shown that gas-phase ions of noncovalent protein complexes produced by both nano- and ESI sources have similar binding energies, however, different from that of the solution complexes, implying that the solution structure of these noncovalent protein complexes was not preserved in the gas-phase. Conclusions on the influence of the method chosen to study solution interactions on the outcome of investigation and comparison with previously reported observations were made. (Int J Mass Spectrom 221 (2002) 147–161)


*Keywords:* Noncovalent protein complexes; Solution and gas-phase binding; NanoESI; MS/MS

## 1. Introduction

It is well known that the biological function of a protein can directly depend on noncovalent interactions with other components existing in the living system. If the nature of the interactions existing in solution can be predicted based on the gas-phase measurements it would provide rapid screening of potential therapeutic targets, which would be of a considerable interest to the pharmaceutical industry.

Application of electrospray ionization mass spectrometry (ESI-MS) to study noncovalent protein complexes allowed detection of conformational changes [1,2], measurement of absolute, and relative dissociation constants [3,4] and has provided accurate measurements of mass and stoichiometry of noncovalent complexes [5–7]. The introduction of the nanoelectrospray ionization (nanoESI) source allowed additional

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flexibility during MS analysis [8]. Tandem mass spectrometry (MS/MS) experiments can provide additional information on the composition and gas-phase binding of observed complexes.

However, the question on the correlation of the solution and gas-phase properties of noncovalent complexes still produces vigorous scientific discussion. Both qualitative and quantitative evaluation of the solution affinity data from the abundance of gas-phase complex ions was demonstrated [9–11]. Correlation of solution binding constants measured by ESI-MS with the known literature data suggested that the solution characteristics of the noncovalent complexes might be retained in vacuo [3,9,12]. Even so, this conclusion was challenged by a number of reports probing gas-phase binding of noncovalent protein complexes using collision-activated dissociation (CAD) MS/MS. Use of the low-energy CAD in triple quadrupole mass spectrometer [13], frond-end dissociation [14,15], sustained off-resonance irradiation (SORI) [16], and resonant RF excitation [17] suggested that solution interactions were not preserved in the gas-phase. These studies have been principally concerned with the binding of relatively small molecules to enzymes [18–20], or with protein–protein interactions in natural oligomers with a low spread in their affinities [21–23]. To date application of MS to study the energetics of solution and gas-phase interactions of noncovalent complexes formed between different proteins has been limited [24]. In particular, there have been few MS studies examining the solution and gas-phase binding properties of the same noncovalent protein complexes [25,26]. Further, the measurement of solution binding properties of noncovalent protein complexes by titration and competitive binding experiments have been seldom accompanied by the evaluation of gas-phase binding properties of the same complexes using CAD-MS/MS. To address these concerns this paper examines the solution and gas-phase binding properties of the same noncovalent protein complexes formed between bovine pancreatic trypsin inhibitor (BPTI) and its target enzymes: trypsin, chymotrypsin, and trypsinogen. The interaction of BPTI and its target enzymes has been studied extensively [27–29]. Trypsin, chymotrypsin, and trypsinogen have similar molecular weights but different affinities for BPTI in solution, and, therefore, present an attractive model for studying protein–protein interactions (Fig. 1).

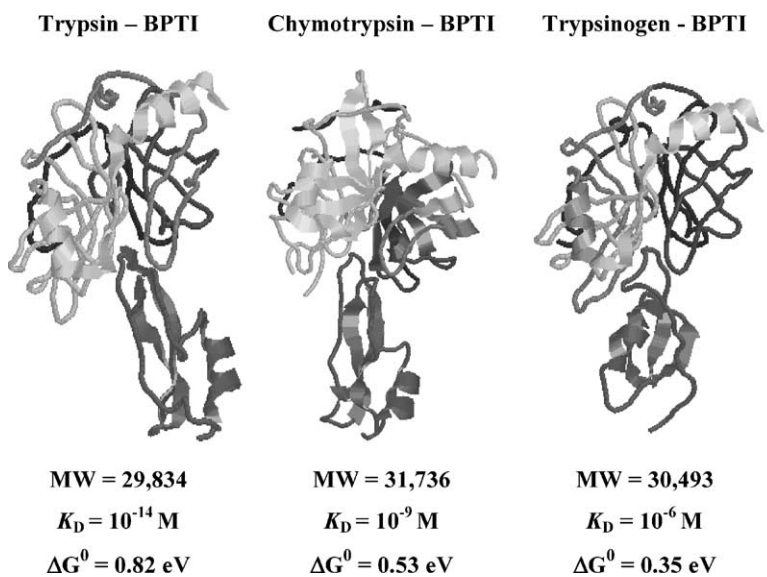


Fig. 1. Noncovalent complexes between BPTI and its target enzymes (trypsin, chymotrypsin, and trypsinogen). The drawings are made from coordinates in the Protein Data Bank files 1TPA, 1CBW, and 2TGP, respectively.

## 2. Experimental

### 2.1. Materials and methods

Trypsin, trypsinogen, and chymotrypsin were from Worthington Biochemical Corporation (Lakewood, NJ, USA), bovine pancreas trypsin inhibitor (cat # 236 624) from Boehringer-Mannheim (Indianapolis, IN, USA), and reserpine (cat # R-0875) was obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Poly(propylene glycol) was from Aldrich-Chemie (Steinheim, Germany). These materials were used without further purification. Gases were Praxair (Mississauga, Ont., Canada), Ne UHP grade (99.996% manufacturer's stated purity), Kr research grade (99.995% manufacturer's stated purity), and $N_2$ UHP grade (99.999% manufacturer's stated purity). The pressure in the collision cell was measured with a precision capacitance manometer (MKS model 120, manufacturer's stated precision 0.12% of reading).

### 2.2. Formation of noncovalent protein complexes

BPTI and its target enzymes were mixed to a final concentrations of 500 and 100 μM, respectively, and incubated overnight at 4 °C to form a complex. Mass spectra were obtained immediately after the solutions has been diluted 10-fold with 10 μM ammonium acetate (pH 7).

### 2.3. ESI- and nanoESI-MS

ESI-MS was performed with a triple quadrupole mass spectrometer as described previously [24]. NanoESI-MS was performed using Protana (Odense, Denmark) source. No nebulizer gas or sheath flow was employed. A low flow was achieved by an introduction of gold-coated capillaries drawn with a short taper to a fine tip of ∼1 μm inner diameter. Clean (99.99999%) nitrogen was used as the curtain gas at a flow rate of 0.2–0.6 L/min. The needle spraying voltage was 1600–1700 V, the skimmer voltage was typically 110 V and the orifice potential was 120 V. A new capillary was used for each experiment.

### 2.4. Tandem mass spectrometry and collision cross-section measurements

Tandem mass spectrometry experiments were performed on a triple quadrupole mass spectrometer as described previously [24]. Details of an energy loss method used to measure collision cross-sections of noncovalent protein complexes are described elsewhere [24,30]. Experiments were done with samples at room temperature (21±2 °C). The averages of six separate cross-section experiments done on different days were calculated along with respective standard errors.

### 2.5. Binding studies

For titration experiments a 10 μM solution of tryptic proteins was mixed with increasing concentrations of BPTI (2, 5, 10, 20, 30, 40, 50, 60, 70, and 80 μM). The extent of BPTI binding to tryptic proteins was determined using the ligand occupancy percentage defined by the number of bound BPTI ligand molecules ($\nu$), divided by the potential number of ligand bound ($n$). It was assumed that the potential occupancy meant binding of one BPTI molecule to one molecule of tryptic proteins. The ligand occupancy was calculated as a function of the BPTI concentration by the summation of integrated signal intensities for the various multiply charged ions for the free tryptic proteins and noncovalent protein complexes. Ligand occupancy $= \Sigma C/(\Sigma C + \Sigma P_{\mathrm{un}})$ where $C$ is the integrated peak intensity of the complex ion and $P_{\mathrm{un}}$ is the integrated peak intensity of the unbound tryptic protein ion. In the competitive binding experiments, a solution of BPTI was added to a solution containing trypsin and tryspinogen, trypsin and chymotrypsin, and trypsinogen and chymotrypsin. The prepared solutions were allowed to incubate overnight and were infused directly into the ESI source.

### 2.6. Calculations

Analysis of the tandem mass spectrometry results was done using Kaleidagraph® program (Synergy Software, Reading, PA, USA) as was described

previously [24]. Assessment of the gas-phase binding of noncovalent protein complexes was done using a collision model for ion activation [24,30]. Calculations of the changes in internal energy were performed using a program written in Matlab® software (The MATH WORKS Inc., Natick, MA, USA). Nonlinear regression analysis was used to draw the best fit curves for data presented in the figures.

## 3. Results

### 3.1. Evaluation of the gas-phase binding properties by nanoESI-MS/MS

#### 3.1.1. Detection of noncovalent protein complexes by nanoESI-MS

NanoESI mass spectra of mixtures of BPTI with trypsin, chymotrypsin, and trypsinogen are shown in Fig. 2. The gas-phase ions of noncovalent complexes formed by nanoESI source produced charge states from +13 up to +15 and in the case of trypsin up to +16 with the maximum at + 14 charge state. Interestingly, ESI mass spectra reported in the previous work [24] showed charge states ranging from +12 to +14 for noncovalent complexes, while the individual tryptic proteins produced charge states ranging from +9 to +12. Thus, mass spectra of noncovalent complexes formed by nanoESI source showed a shift towards higher charge states in the charge state distribution and also in the position of the major peaks in comparison to mass spectra of noncovalent complexes produced by ESI.

A minimal difference between the orifice and skimmer potentials of 10 V was used to maintain specific interactions of noncovalent protein complexes during nanoESI-MS. Other experimental parameters were also optimized to get a maximum signal from the complexes. Variation in solution composition and modifications of the interface conditions were used to verify the specificity of observed noncovalent interactions, which was also suggested by the observed 1:1 stoichiometry of noncovalent complexes and absence of nonspecific forms of aggregation.

#### 3.1.2. NanoESI-MS/MS

In the previous work +12 and +13 charge states were used for analysis of noncovalent complexes by tandem mass spectrometry using an ESI source [24]. In this work only the +13 charge state was chosen for analysis of gas-phase noncovalent complexes produced by nanospray ionization source as the intensity of the signal from the +12 charge was too low to conduct any experiments (Fig. 2). In all three cases the difference between +10 charge state signals corresponding to individual proteins and +13 charge state of their complex was ranging from 82 to 38 *m*/*z* which was sufficient enough to perform further data analysis. Dissociation of the +13 charge states of the noncovalent complexes produced corresponding pairs of product ions, which were formed as a result of the fragmentation of the precursor ion (Fig. 3). Product ions corresponding to BPTI produced charge states ranging from +3 to +6 with the maximum intensity at the +5 charge state, while fragment ions corresponding to tryptic proteins produced charge states ranging from +7 to +12 with prevailing +9 and +10 charge states. Examination of the dissociation pathways of the +13 charge states of noncovalent complexes produced by nanospray ionization source revealed a pattern similar to that previously obtained with the ESI source [24].

Fig. 4 shows dissociation curves of the gas-phase noncovalent complexes of BPTI and its target enzymes: trypsin, chymotrypsin, and trypsinogen formed by nanospray ionization source. The halfway dissociation voltage ($D_{50}$), i.e., the voltage difference between $Q_0$ and $Q_2$, corresponding to 50% dissociation of the precursor ion, was calculated from dissociation curves and used in later computations. Experiments were repeated three times. No tests of statistical significance were performed. Dissociation curves of the +13 charge state of the noncovalent protein complexes formed by the nanoESI source showed that the chymotrypsin-BPTI complex had the highest halfway dissociation voltage. This result was in accordance with an earlier observation obtained with ESI source [24]. However, a shift in the position of the dissociation curves corresponding to

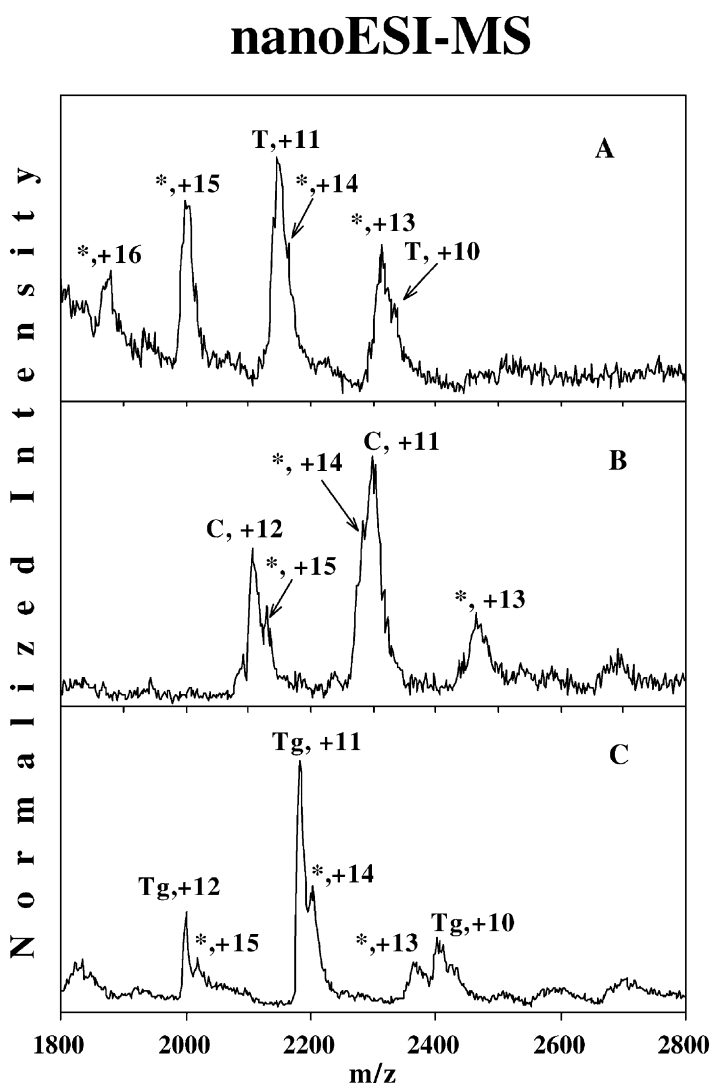


Fig. 2. NanoESI mass spectra of noncovalent protein complexes. (A) Trypsin-BPTI, (B) chymotrypsin-BPTI, and (C) trypsinogen-BPTI. BPTI and its target enzymes were mixed to a final concentrations of 500 and 100 μM, respectively, and incubated overnight at 4 °C to form a complex. Mass spectra were obtained immediately after the solutions were diluted 10-fold with 10 μM ammonium acetate (pH 7). T: trypsin, C: chymotrypsin, Tg: trypsinogen, ∗: complex. Numbers indicate the charge state.

trypsin- and trypsinogen-BPTI complexes was observed. Dissociation curves of trypsin- and trypsinogen-BPTI complexes produced by nanospray ionization source revealed a comparable halfway dissociation voltages (Fig. 4), which was bigger for gas-phase ions of trypsinogen-BPTI complexes produced by ESI source [24].

### *3.1.3. Collision cross-sections and relative internal energies*

In order to assess gas-phase binding energies of noncovalent complexes using collision model for an ion activation it is necessary to account for the different cross-sections of these complexes resulting in different number of collisions [30]. The averages of six

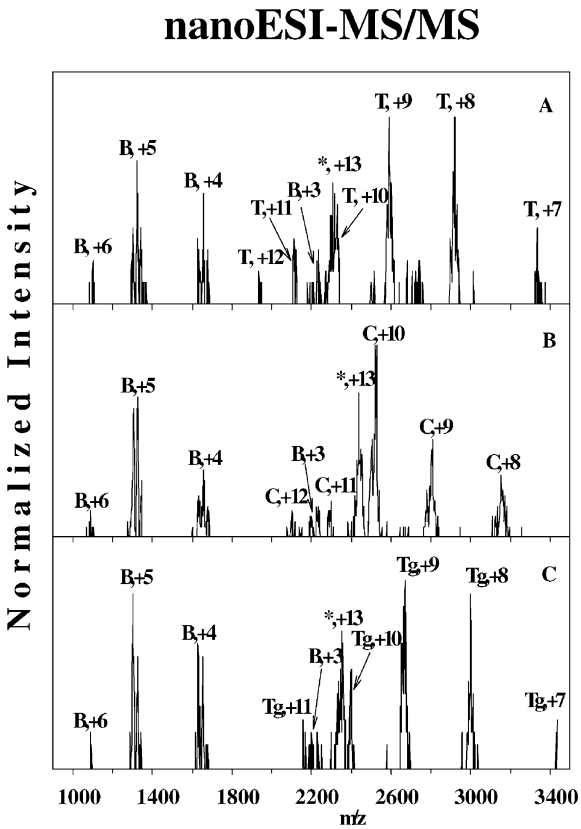


Fig. 3. Dissociation pathways of the +13 charge states for noncovalent protein complexes formed by nanoESI source. (A) Trypsin-BPTI, (B) chymotrypsin-BPTI, and (C) trypsinogen-BPTI. B: BPTI, T: trypsin, C: chymotrypsin, Tg: trypsinogen. Numbers indicate the charge states of fragment/precursor ions. The collision gas was krypton at the pressure of 0.3 mTorr at acceleration potential of 80 V.

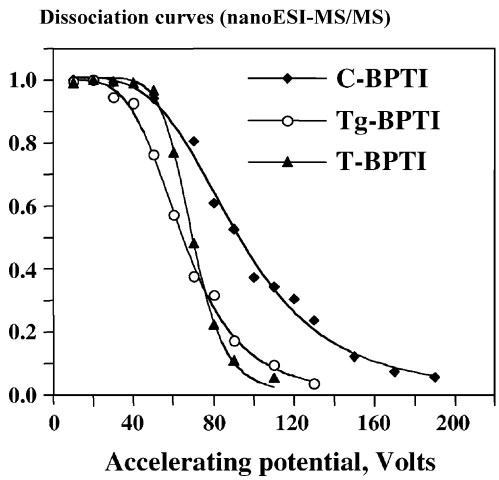


Fig. 4. Dissociation curves of the noncovalent protein complexes formed by nanoESI source. +13 charge state, collision gas krypton, $P = 0.4$ mTorr.

separate measurements along with respective standard errors are shown in Table 1. Previous data showed that for a given charge state of a gas-phase ions produced by ESI source, the collision cross-sections were almost identical among different noncovalent complexes [24]. The measurement of the collision cross-sections for gas-phase ions of noncovalent complexes formed by the nanospray ionization source showed greater differences in values among the complexes and these values were generally of lower magnitude in comparison to data obtained previously by ESI (Table 1).

The change in the internal energy needed to induce 50% loss of the gas-phase ions of noncovalent com-

Table 1
Collision cross-sections (CCS) and relative internal energies ($\Delta E_{int}$) of gas-phase ions of noncovalent protein complexes measured by nano- and ESI sources

| | ESI | | | | NanoESI | |
|---|---|---|---|---|---|---|
| | CCS (Å$^2$) | | $\Delta E_{int}$ (eV) | | CCS (Å$^2$) | $\Delta E_{int}$ (eV) |
| | +12 | +13 | +12 | +13 | +13 | +13 |
| Trypsin-BPTI | 1866 ± 26 | 1928 ± 21 | 108.6 ± 0.7 | 93.4 ± 0.9 | 1812 ± 10 | 106.2 ± 1.1 |
| Chymotrypsin-BPTI | 1887 ± 18 | 1912 ± 17 | 155.9 ± 1.1 | 125.7 ± 1.5 | 1643 ± 25 | 122.3 ± 4.3 |
| Trypsinogen-BPTI | 1899 ± 14 | 1896 ± 18 | 135.5 ± 6.0 | 108.1 ± 4.4 | 1760 ± 20 | 98.7 ± 1.9 |

plexes formed by nanospray ionization source was used as a measure of the relative binding energies. The average values of relative internal energy were calculated for every complex at five different pressures of collision gas and are shown in the Table 1 with their respective standard errors. Calculations for gas-phase ions of noncovalent protein complexes formed by nanospray ionization source showed that the chymotrypsin-BPTI complex had the most binding energy in the gas-phase and it took approximately the same amount of energy to dissociate gas-phase ions of trypsin- and trypsinogen-BPTI noncovalent complexes. These findings are similar to previous data on the relative internal energy required to dissociate gas-phase ions of noncovalent protein complexes formed by ESI source [24]. It was shown that for both +12 and +13 charge states, the chymotrypsin-BPTI complex had the greatest binding energy in the gas-phase followed by the trypsinogen-BPTI complex, while the trypsin-BPTI complex had the lowest binding energy (Table 1). Observation on gas-phase binding of noncovalent complexes formed by both nano- and ESI are in contrast with the known solution behavior, where the most strongly bound trypsin-BPTI complex is followed by chymotrypsin- and trypsinogen-BPTI complexes.

### 3.2. Evaluation of the solution binding properties by ESI-MS

#### 3.2.1. Titration

In ESI-MS titration experiments the relative abundance of noncovalent protein complexes was monitored with increasing concentrations of BPTI. The results of these experiments are plotted in Fig. 5A. To produce this figure, integrated ion abundance from the various charge states have been summed for noncovalent complexes proteins and tryptic proteins as a function of concentration of added BPTI. Due to inability to resolve +14 and +11 charge state signals corresponding to complexed and uncomplexed proteins, their integrated ion abundances were omitted during analysis of titration experiments. The average number of BPTI bound was calculated as described previously. Fig. 5A suggests that the binding of BPTI to its target proteins can be differentiated by ESI-MS as revealed by the change of the slope observed in the titration curves. Difference in the slope of the titration curves indicates that, in accordance with solution behavior, binding of BPTI to trypsinogen has a lower affinity than binding of BPTI to chymotryspin or trypsin. Nonlinear regression analysis was used to extract information from the titration

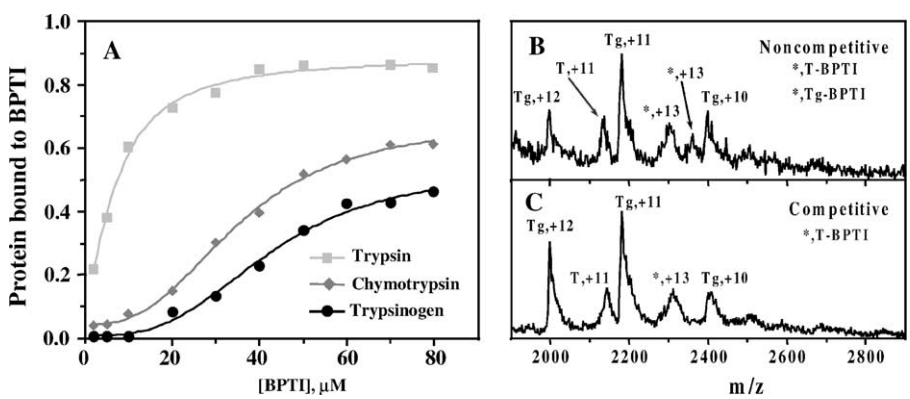


Fig. 5. Evaluation of solution binding properties of noncovalent protein complexes by titration and competitive binding experiments. (A) ESI-MS titration plot, monitoring the amount of bound noncovalent protein complexes with increasing concentrations of BPTI. The concentration of tryptic proteins was 10 μM in 10 μM ammonium acetate buffer, pH 7. (B and C) ESI-MS competitive binding experiments between trypsin and trypsinogen with BPTI. The relative concentration of trypsin:trypsinogen:BPTI in 10 μM ammonium acetate buffer, pH 7, was 1:1:6 (noncompetitive binding) and 1:1:1 (competitive binding conditions).

curves about binding constants. A plot of measured ligand occupancy vs. BPTI concentration has been fit to a Michaelis–Menten equation. Dissociation constants of 7.8, 56.9, and 108.3 μM have been calculated respectively for trypsin-, chymotrypsin-, and trypsinogen-BPTI complexes. Fit to other equations did not produced significant difference in absolute numbers and preserved their relative order.

#### *3.2.2. Competitive binding experiments*

Determination of solution-phase binding constants is also possible based on the relative abundance of the bound and unbound species in the single ESI mass spectrum. To test whether relative ion abundance in ESI mass spectra matches BPTI solution affinities, a study under competitive and noncompetitive binding conditions was carried out. In each competition experiment, solutions of two proteins were mixed with a solution of BPTI. All three mixtures were interrogated by ESI-MS by using the same spray solvent under identical ESI-MS interface conditions. Unfortunately, the resolving limits of instrumentation allowed analysis of only trypsin and trypsinogen mixture with BPTI. In addition, strong overlap existing between +14 and +11 charge state signals corresponding to complexed and uncomplexed proteins forced to consider only the +13 charge state for analysis of competitive binding experiments.

An experiment under noncompetitive condition between BPTI (60 μM) and equimolar amounts of trypsin and trypsinogen (10 μM) clearly showed the appearance of +13 charge states for both noncovalent complexes (Fig. 5B). The dominant ion signals observed in the mass spectra correspond to protonated trypsin and trypsinogen ions. Identification of additional charge states of noncovalent protein complexes was limited by their overlap with charge states corresponding to free protein. Inspection of the mass spectra revealed that the signal corresponding to +13 charge state of the trypsin-BPTI complex was comparable in magnitude to that observed for the trypsinogen-BPTI complex.

In competitive binding experiments equimolar concentrations of trypsin and trypsinogen (10 μM) are mixed with 10 μM BPTI. In common with known solution behavior, signal for the +13 charge state of trypsin-BPTI complex is considerably more abundant than that of the corresponding trypsinogen-BPTI complex (Fig. 5C). This result is consistent with the reported difference of more than eight orders of magnitude in BPTI binding affinity between trypsin and trypsinogen [28], and serves as further proof that ESI-MS spectra obtained under applied experimental conditions reflect specific solution interactions. The difference in binding affinities of BPTI to trypsin and trypsinogen remained unchanged with the increase in buffer concentrations from 10 to 50 μM. Unfortunately, differences in response factors between trypsin and trypsinogen precluded direct quantification of the obtained results.

## 4. Discussion

### *4.1. MS evaluation of solution binding properties of noncovalent protein complexes*

The results derived from the ESI-MS assessment of titration experiments and presented in Fig. 6 are pointing toward a reasonable agreement in *relative* binding order between calculated dissociation constants and the known solution values of the noncovalent complexes investigated. However, obvious differences in the absolute values clearly indicate that the correlation is far from perfect. These results are echoed by the data obtained from competitive binding experiments. Very good agreement with solution binding values was noted under competitive binding conditions where concentrations of BPTI, trypsin, and trypsinogen were equimolar. Only the ions of trypsin associated with BPTI in a 1:1 complex were observed. No peaks corresponding to the 1:1 complex ion of trypsinogen with BPTI were evident. Under noncompetitive conditions both trypsin and trypsinogen were observed to bind BPTI. However, the known affinity difference of eight orders of magnitude is hardly evident from the relative abundance of the trypsin and trypsinogen-BPTI complexes. The modest agreement between gas-phase and

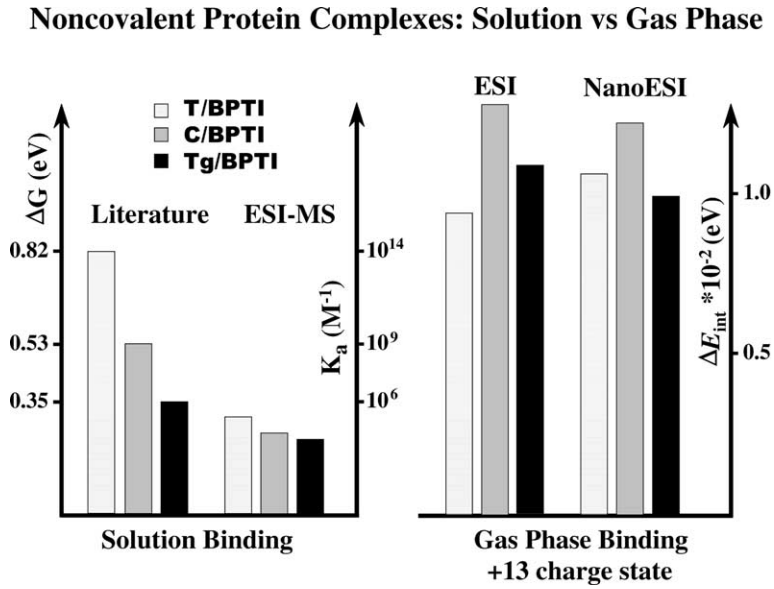


Fig. 6. Comparison of solution and gas-phase binding properties of noncovalent protein complexes evaluated by MS with known solution data. T: trypsin, C: chymotrypsin, Tg: trypsinogen.

solution data may be due to a number of experimental factors discussed in detail as follows.

*4.1.1. Effect of experimental parameters and assumptions made during analysis*

Differences in molecular weight and ionization efficiencies of individual proteins, changes in solution equilibrium occurring during the ESI process and partial dissociation of the complexes occurring in the interface region of the MS could govern the accuracy of the determination of solution binding values using ESI-MS.

Direct quantification of the solution affinities of the noncovalent complexes by ESI-MS was based on the assumption that the relative abundance of the gas-phase ions of free proteins and noncovalent protein complexes are reflective of the solution-phase equilibrium. Consistent with this assumption, and in accordance with solution equilibrium data, comparison of trypsin and trypsinogen signal intensities in the competitive binding experiment indicates on the abundance of free unbound trypsinogen. However, analysis of the mass spectra under noncompetitive conditions clearly indicates the presence of charge states corresponding to free unbound protein despite an excess of BPTI. A probable explanation is that the spraying process was not gentle enough to preserve solution equilibrium even in the presence of fivefold excess of inhibitor over protein. However, an even greater excess of inhibitor during the titration experiments shifted the equilibrium towards complexation, resulting in a situation more closely resembling solution behavior. This observation suggests that the relative abundance of the gas-phase ions of free proteins and noncovalent protein complexes are not reflective of solution-phase equilibria.

Moreover, deduction of accurate absolute binding constants from the relative abundance of gas-phase ions in the ESI-MS is not possible without additional assumption about the absence of the MS signal discrimination between respective charge states of free protein and its noncovalent complex. Thus, ionization factors should be taken into account during evaluation of the absolute solution binding affinities based on the MS measurements. A comparison of the respective charge states corresponding to free protein

and its noncovalent complex is often hampered by the low-signal intensity from either of them. Additional difficulties may appear from the inability to resolve ions of close *m*/*z* values. Much of the interpretation of the presented data depended on the extend of overlap existing between individual charge states corresponding to complexed and uncomplexed protein. As was stated above, the difference between +13 and +10 charge state signals corresponding to complexed and uncomplexed proteins was sufficient enough to overlook possible contribution of their signals toward each other during data analysis. At the same time inability to resolve +14 and +11 charge state signals corresponding to complexed and uncomplexed proteins may had an effect on the results of the analysis of titration experiments. A reduction in the accuracy of the integrated signal intensities, which should include as complete as possible charge state envelope, may also affect the evaluation of solution binding affinities.

#### *4.1.2. Effect of sample concentration and MS sensitivity*

Several recent reports described the possibility of directly evaluating the solution binding affinities of noncovalent complexes based on the results of titration and competitive binding experiments [9,10]. It is of note that in all these reports ESI-MS measurements of solution binding data were conducted on systems with micromolar binding affinities. It was already noted that accurate measurements of the solution binding constants should involve the use of ligand concentrations in the range below and above dissociation constants [11]. Thus, correlation between known solution affinities and those obtained by ESI-MS, observed for micromolar affinity systems, is not surprising. At the same time, the study of noncovalent protein complexes between BPTI and its target enzymes involves systems with dissociation constants ranging from micromolar to almost femtomolar concentrations. Therefore, it is possible that the minimal difference between calculated and known micromolar binding affinities, observed for trypsinogen-BPTI complex, is not accidental. It is known that measurements of the absolute binding constants of noncovalent systems with higher (submicromolar) affinities could be associated with limitations in instrumental sensitivity [7]. Thus, measurement of absolute affinity values much below micromolar levels is not likely to be quantitative with the triple quadrupole MS used in this work. However, application of experimental set-ups with greater sensitivity would address this requirement and allow evaluation of solution binding affinities of complexes presented in nano-, pico-, and femtomolar concentrations.

Nevertheless, despite these mentioned limitations and complexity of noncovalent protein interactions, the data presented here conclusively demonstrate the successful evaluation of the relative binding affinities in solution for noncovalent systems with submicromolar affinities using titration and competitive binding experiments.

### *4.2. Gas-phase binding properties evaluated by nano- and ESI-MS/MS*

Gas-phase ions of noncovalent complexes between BPTI and its target enzymes produced by either nano- or ESI sources showed significant differences in both absolute gas-phase binding values and relative order in comparison to their solution analogs (Fig. 6). Gas-phase ions of chymotrypsin-BPTI complex produced by both nano- and ESI source showed biggest binding energies followed by either trypsin- or trypsinogen-BPTI complexes depending on the ionization source (Fig. 6). Although a direct comparison of the characteristics of noncovalent protein complexes measured by nano- and ESI sources is beyond the scope of this manuscript, the available data allows to make a preliminary evaluation.

#### *4.2.1. Charge states and collision cross-sections*

Gas-phase ions of noncovalent protein complexes formed by nanoESI showed a shift of charge state distributions towards higher charge states in comparison to gas-phase ions of noncovalent complexes produced by the ESI source. This result may appear surprising, especially considering that the appearance of higher charge states quite often indicates unfolding

of a protein. However, according to model of Fenn, the specific surface-charge density of the droplet determining the charge state of the analyte ion is lower for a bigger droplet formed during ESI than for a smaller droplet formed during nanoESI [31]. In addition, operation of a conventional ESI source at a distance between the spraying needle and the sampling orifice greater than that of a nanoESI source may led to discrimination against higher charge states [32].

Comparisons of the collision cross-sections of the gas-phase ions of noncovalent protein complexes produced by nano- and ESI sources are shown in Table 1. Collision cross-sections of the gas-phase ions formed by ESI were similar. In contrast, collision cross-sections of the gas-phase ions produced by nanoESI showed greater variance and were generally smaller than those obtained using the ESI source. The observed difference in collision cross-sections of gas-phase ions formed by nano- and ESI sources may be attributed to the size of the initial droplets [31,33,34].

#### 4.2.2. Relative internal energies

In accordance with previous findings [16], noncovalent protein complexes with prevailing aromatic side chains in the interface region (chymotrypsin-BPTI) demonstrated a stronger gas-phase binding compared to complexes with an abundance of aliphatic side chains (trypsin-BPTI) [35,36].

Similarity of intermolecular interfaces between trypsin- and trypsinogen-BPTI complexes [37] lead to expect similarity in the gas-phase binding of these complexes. In fact, difference in relative internal energies observed for gas-phase ions of these complexes produced by the nanospray ionization source (7.5 eV) were smaller than that for gas-phase ions formed by the ESI source (14.7 eV). The observed difference in gas-phase binding for ions formed by nano- and ESI sources remained currently unexplained.

A comparison of relative internal energies required to dissociate the +13 charge state of the gas-phase ions of noncovalent complexes formed by nano- and ESI sources did not show drastic differences in absolute values (Table 1). It is not clear whether changes in the internal energy should be similar or different for the same complex. There are two major factors contributing to the value of the relative internal energy required to dissociate protein complexes: collision cross-sections and half-way dissociation voltages depending on pressure and nature of the collision gas. Variations in these parameters may cancel each other out or act cumulatively producing respectively similar (chymotrypsin-BPTI) or different (trypsinogen-BPTI) values of relative internal energies for gas-phase ions formed by either nano- or ESI sources (Table 1).

### 4.3. Evaluation of solution affinities based on the gas-phase measurements

#### 4.3.1. Effect of the evaluation method

MS evaluation of the solution and gas-phase binding properties for noncovalent BPTI-tryptic protein complexes yielded results significantly different from each other. Assessment of titration and competitive binding experiments by ESI-MS showed correlation with the *relative* binding order in solution. However, MS evaluation of the solution dissociation constants showed 2–8 orders of magnitude differences with absolute values, known from literature. At the same time, dissociation of the gas-phase complex ions by CAD-MS/MS resulted in relative gas-phase binding order different from that existed in solution, suggesting that the conformation of the binding pockets in these complexes were not preserved in the gas-phase. The results presented here are in accordance with previous observations made for drug-DNA complexes that show discrepancies between abundance of the gas-phase complex ion and the ease of their dissociation [38,39]. In contrast, such inconsistencies were not observed in two noncovalent protein systems studied by the combined approach used here [25,26].

A survey of a variety of noncovalent protein systems, studied to date, showed that the relative abundance of gas-phase ions always indicated a correlation with the known relative solution binding order (Table 2). However, correlation with absolute binding constants was observed in only 6 out of 17 reports. Moreover, three out of these six reports were

Table 2
Binding of noncovalent protein complexes evaluated by MS methods

| System | | Method | Correlation | Reference |
|---|---|---|---|---|
| Host | Ligand | | | |
| Trypsin | | Titration | Yes[a] | This work |
| Chymotrypsin | BPTI | Competitive binding | Yes[a] | This work |
| Trypsinogen | | CAD | No | This work |
| Protein | Drugs | Competitive binding | Yes | [25] |
| | | CAD-MS/MS | Yes[a] | |
| Peptides | Vancomycin | Negative mode: CAD | Yes[a] | [13] |
| | Antibiotics | Competitive binding | Yes[a] | |
| | | Positive mode: CAD | No | |
| Carbonic anhydrase | Sulfonamide drugs | Competitive binding | Yes[a] | [26] |
| | | SORI-CAD | Yes[a] | |
| Peptide | Antibiotics | Titration | Yes | [3] |
| Protein | DNA | Titration | Yes[a] | [2] |
| Protein | DNA | Titration | Yes | [4] |
| Protein | Carbohydrate | Titration | Yes | [18] |
| v-Src SH2 protein | Peptides | Titration | Yes[a] | [11] |
| | | Competitive binding | Yes[a] | |
| Protein | DNA | Competitive binding | Yes[a] | [20] |
| Protein | Peptides | Competitive binding | Yes[a] | [19] |
| Protein | DNA | Competitive binding | Yes[a] | [5] |
| Protein | DNA | Competitive binding | Yes[a] | [6] |
| Protein | Antibiotic | Competitive binding | Yes[a] | [7] |
| Peptides | Antibiotics | Competitive binding | Yes | [10] |
| Peptides | Antibiotics | Competitive binding | Yes | [9] |
| Myoglobin (Mb) | Heme | CAD-MS/MS, trapping | Yes[a] | [30] |
| Multimeric hemoglobins (Hb) | | CAD-MS/MS(Q) | No | [23] |
| Mb, Hb | Heme | CAD, front end | No | [15] |
| Peptides | Antibiotics | RF excitation | No | [17] |
| Mb, hemoglobin | Heme | BIRD | Yes[a] | [12] |
| Carbonic anhydrase | Sulfonamide drugs | SORI-CAD | No | [16] |
| Multimeric proteins (avidin, concanavalin A, Hb) | | Front-end dissociation | Yes[a] | [21] |
| | | Capillary heating | Yes[a] | |
| Cytochrome $b_5$, Mb | Heme | Front-end dissociation | Yes[a] | [41] |
| S92D/K45E Mb | Heme | Front-end dissociation | No | |
| Aldose reductase | Inhibitors | Front-end dissociation | No | [14] |

[a] Correlation was observed in relative rather than in absolute terms.

investigating binding of the same vancomycin and ristotetin antibiotics with peptides consisting of only two to three amino acid residues of a limited hydrophobicity (A, G, K) [3,9,10]. Thus, it is quite possible that the correlation described in these reports can be attributed to intrinsic properties of these particular systems that favor the observed phenomena. At the same time, dissociation of the noncovalent protein complexes in the gas-phase by a number of CAD methods did not always resulted in a correlation with known solution binding affinities [40]. Close inspection of Table 2 shows that out of eight studies using CAD in triple quadrupole instruments to investigate the correlation between solution and gas-phase binding properties, only three indicated any correlation between solution and gas-phase structures [13,25,30]. Investigations of solution properties of noncovalent complexes using a front-end dissociation approach yielded similar results. Analysis of four reports utilizing interface dissociation, suggested correlation between solution and gas-phase properties in only two cases [21,41]. Correlation between the solution and gas-phase binding

properties of noncovalent protein complexes were obtained in three out of five systems studied by SORI-CAD [26], blackbody infrared radiative dissociation (BIRD) [12], and resonant RF excitation in ion trap mass spectrometers [30]. Interestingly, in some cases application of different dissociation methods on the same system gave contradictory conclusions. Application of triple quadrupole MS/MS on the multimeric hemoglobin (Hb) molecule indicated differences with known solution behavior [23], while application of capillary heating and front-end dissociation suggested solution mirroring properties [21]. However, in latter case, this conclusion was made based on the comparison of the relative stability of tetrameric forms of a number of proteins. Application of the SORI-CAD method to the complexes formed between bovine carbonic anhydrase II and sulfonamide inhibitors resulted in mutually contradictory conclusions with regards to the similarity of solution and the gas-phase affinities [16,26]. Use of eight inhibitors with different affinities and different polar surface areas gave results inconsistent with known solution behavior [16], while use of two inhibitors with different affinities and minimal differences in electrostatic interactions indicated, that specific solution interactions were preserved in the gas-phase [26]. The reasons behind the differences between known solution affinities and those derived from the abundance of the gas-phase ions have been partially discussed earlier. In addition, the nature of noncovalent interactions could also contribute to the observed differences and explain the limited success of the CAD approach to probe solution affinities of the noncovalent complexes in the gas-phase.

#### *4.3.2. Role of hydrophobic and electrostatic forces to the gas-phase binding*

The observed differences in the relative order and absolute values of the solution and the gas-phase binding of the noncovalent protein complexes can be rationalized by taking into account thermodynamic components of the solution and the gas-phase binding. Initial estimation and computer simulations of the electrostatic and hydrophobic components to the free binding energy in solution for trypsin- and trypsinogen-BPTI complexes showed a major contribution from the hydrophobic component [27,42–44]. In view of this data, the relatively low value of gas-phase binding for the trypsin-BPTI complex, observed here, is not surprising. Analogous estimates for the chymotrypsin-BPTI complex are unavailable at this time. Use of the same computer algorithm to calculate hydrophobic and electrostatic components of the free binding energy between BPTI and its target enzymes may explain differences observed in solution and gas-phase binding. In addition, interactions strongly influenced by the presence of solvent, such as in trypsin-BPTI complexes [36], may have a different response to a change in solvation occurring during ESI process. Thus, it is quite possible that the observed difference between solution and gas-phase binding results from the change in the nature of interactions in the interface region occurring during the "jump" of the molecule from solution into the gas-phase environment. Therefore, predicting solution binding constants from the relative abundance of noncovalent protein complexes in the gas-phase may be difficult.

This study supports previous findings [7,11] that there is an emerging relationship between the abundance and ease of dissociation of the gas-phase ions of noncovalent protein complexes, and the type of interaction involved in their binding. A number of reports have established that the presence of an extensive hydrophobic component in the free solution binding energy, leads to the lack of correlation between known solution binding affinities and those derived from the abundance of gas-phase complex ions [14,16,40]. Thus, it is expected that the dissociation of gas-phase ions of noncovalent protein complexes stabilized by hydrophobic interactions might not closely reflect those found the solution [15,16,23]. On the other hand, it has been shown that the abundance of gas-phase ions and energetics of their binding closely reflect aqueous solution behavior, when electrostatic components play a major role in the formation of noncovalent complexes [4,26,41]. In addition, similar solution binding mechanisms may lead to similar binding in the gas-phase, which may have implications for the successful determination of absolute and relative solution

binding affinities by MS [9,10]. In view of this, close correlation of known solution affinities with those determined from the abundances of the gas-phase ions of noncovalent complexes between vancomycin group antibiotics and peptides is not surprising. However, puzzling differences in the gas-phase binding of negatively and positively charged vancomycin and dipeptide stereoisomeric complexes indicates that there are still questions remaining to be answered [13].

## 5. Conclusion

MS evaluation of the solution and gas-phase binding affinities of noncovalent protein complexes between BPTI and its target enzymes confirmed that correlation between solution and the gas-phase properties is not straightforward. Assessment of the solution affinities of these complexes from the abundance of the gas-phase complex ions showed correlation with relative binding order in solution, suggesting that, to some extent, MS measurements reflect solution equilibrium. This result indicates that ESI-MS titration and competitive binding experiments can be used to evaluate not only noncovalent systems with a micromolar affinities, but also complex protein–protein interactions with a wide spread in the affinity values. At the same time, correlation with known solution values was in relative, rather then absolute terms, indicating the limitations imposed by the destructive nature of the ESI process. Use of mass spectrometers with sensitivity limits in the range of affinities under evaluation and greater resolving power will provide further improvements in the quantitative evaluation of the binding constants. In contrast, gas-phase ions of these noncovalent protein complexes dissociated by CAD-MS/MS showed no correlation with relative binding order in solution. The observed lack of correlation suggests that solution interactions of these complexes were not preserved in the gas-phase, implying that solution structures may not be reflected by gas-phase ions. Reports presented so far clearly indicate the ability of MS to detect and characterize a wide variety of noncovalent protein complexes. However, the nature of noncovalent interactions can critically affect the extent to which the solution affinities of protein complexes can be evaluated by MS. Detailed calculations of the hydrophobic and electrostatic components of the free solution and the gas-phase binding energy may provide further understanding of limitations imposed by the nature of noncovalent interactions on the correlation between solution and gas-phase binding of noncovalent protein complexes.

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