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Phosphate Stabilization of Intermolecular Interactions

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Abstract

Receptor heteromerization is an important phenomenon that results from the interaction of epitopes on two receptors. Previous studies have suggested the possibility of Dopamine D₂- NMDA receptors' interaction. We believe that the interaction is through an acidic epitope of the NMDA NR₁ subunit (KVNSEEEEEEDA) and a basic epitope of the D₂ third intracellular loop (VLRRRRRKRVN), which was shown to also interact with the Adenosine A_{2A} receptor. In previous work we highlighted the role of certain amino acid residues, mainly two or more adjacent arginine on one peptide and two or more adjacent glutamate, or aspartate, or a phosphorylated residue on the other in the formation of noncovalent complexes (NCX) between epitopes. In the present work, we use the phosphorylated (KVNSpEEEEEDA), non-phosphorylated (KVNSEEEEEEDA) and modified (KVNpSAAAAAAA) forms of the NMDA epitope that possibly interact with the D₂ epitope to investigate the gas-phase stability of the NCXs as a function of the nominal energy given to the NCX ion as it enters the collision cell. In addition to theoretical calculations, the experimental data was used to calculate the stability of each electrostatic complex versus that of the dimer of KVNSpEEEEEDA. Our results demonstrate the importance of the phosphate group in stabilizing molecular interactions and that appreciably higher collision energies are required to completely dissociate any of the three different NCX ions that are formed through electrostatic interaction in comparison to the energy required to dissociate the KVNpSEEEEEEDA dimer ion, which is mainly kept together by hydrogen bonding. This study emphasizes ionic bonds stability and their importance to protein structure as their potent electrostatic attractions can in the gas phase surpass the strength of covalent bonds.

Keywords

Phosphate groups; Guanidinium groups; Electrostatic interactions; Dimers; Binding energies; Heteromerization

Introduction

ATP the universal currency of energy is the source of phosphate for protein phosphorylation. Phosphorylation/dephosphorylation is a very effective means for switching target proteins on/off. Proteins that are phosphorylated/dephosphorylated are involved in regulating many of the more important aspects of a cell's life, thus explaining why abnormal phosphorylation often leads to disease¹, and highlighting the importance of the role of phosphate in chemical interactions.² Mass spectrometry has emerged as an important tool for the study of phosphorylated biomolecules³ as well as noncovalent complexes (NCX) that form between phosphorylated residues and the guanidinium group of arginine. In particular, electrospray

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ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) have been employed for the analysis of NCXs.^{4–7} One of the major motivations for these studies is the possibility that the structure, stability, and conformations of NCX gas-phase ions may yield data related to their formation in biological systems. NCXs created between the cationic guanidinium and the anionic carboxyl and phosphate groups has been addressed in a recent review.⁸ Furthermore, Coulombic interactions have been suggested to play an important and often controlling role in protein and peptides interactions.⁹

We have previously demonstrated using MALDI-MS, ESI-MS, and ion mobility (IM)-MALDI-MS that NCXs between peptides are formed through an electrostatic interaction between adjacent arginine (R) residues on one epitope and adjacent glutamate (E) or aspartate (D) or one phosphorylated residue on the other.^{6,10–14} The guanidinium group of Arg has a delocalized positive charge, which is distributed over the entire group, and a pKa of 12.5 and thus is ionized over the entire pH range in which proteins exist in nature. According to measurements made by Amekraz¹⁵, the basicity of Arg (GB = 237 kcal mol⁻¹) is even greater than that of guanidine (GB = 224.2 kcal mol⁻¹) probably due to stabilization by intramolecular hydrogen bonding.^{16,17} The carboxyl groups of aspartate and glutamate have resonance structures where one pair of electrons is delocalized over the π molecular orbital system, while the phosphate in phosphorylated serine $\delta\pi$ molecular orbital systems spreads its negative charge over two or more oxygens. Furthermore, we have demonstrated the occurrence of such interactions between the adenosine A₂A receptor and the dopamine D₂R in co-transfected cells using fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) techniques.¹⁸

In the current study, we observed that the molecular ions (M+3H)³⁺ of NCXs of a basic epitope **VLRRRRKRVN** from the Dopamine D₂ receptor and various forms of an acidic epitope from the NMDA receptor NR₁ subunit **KVNSEEEEEEDA** (non-phosphorylated), **KVNpSEEEEEEDA** (phosphorylated) and, **KVNpSAAAAAAA** (modified), when submitted to collision-induced dissociation, dissociate into the individual parent peptides rather than fragment. In addition the NCX intensities varied as a function of the nominal energy given to an NCX as it entered the collision cell. These results were used to compare the stability of the noncovalent interaction for **VLRRRRKRVN** with each acidic peptide. Further comparison between the NCXs and the dimer of **KVNpSEEEEEEDA** was conducted and it was observed that appreciably higher collision energies were needed to completely dissociate any of the three different NCX ions in comparison to the energy required to dissociate the (M+3H)³⁺ dimer of **KVNpSEEEEEEDA**.

Materials and Methods

Peptides

The basic peptide **VLRRRRKRVN** (MW = 1351.87 Da) and three acidic peptides: **KVNSEEEEEEDA** (MW = 1277.53 Da), **KVNpSEEEEEEDA** (MW = 1357.49 Da), **KVNpSAAAAAAA** (MW = 1023.48 Da), where pS is a phosphorylated serine, were synthesized at the John Hopkins School of Medicine Peptide Synthesis Core Facility. Stock solutions were prepared in water at a concentration of 1 nmol/ μ L. Sample mixtures, consisting of **VLRRRRKRVN** at 1 pmol/ μ L and one acidic peptide at 15 pmol/ μ L in water, were employed for mass analysis.

Instrument

A Q-TOF Global Ultima mass spectrometer (Waters, Milford, MA) was used for electrospray analysis. A flow rate of 5 μ L/min was used to introduce the sample into the mass spectrometer.

The mass spectrometer was operated in positive ion mode with a capillary voltage of 2.9 kV, a sampling cone voltage of 50 V, a source temperature of 100 °C, a desolvation temperature of 200 °C, a desolvation gas flow rate of 650 L/Hr, and a cone gas flow of 100 L/Hr. For MS/MS analysis, a selection mass window of 6 m/z with a collision gas (Argon) pressure of 7 psi was employed. Collision energies between 5–30 V were used in the collision cell for ion fragmentation. Table 1 contains the MS/MS experimental setup for each sample mixture analyzed in this work. Mass spectra presented are the sum of 50 consecutive 1-second scans. Average intensity values reported in this study are the result of three replicate sample runs.

Molecular Modeling

The non-covalent interactions between VLRRRRKRVN and KVNpSEEEEDA were modeled by Accelrys DS Modeling 1.1 Suite running CHARMMTM force field. The dielectric constant was set at 1 to neglect the solvent effect. An SGI Octane2 workstation was configured as the calculation server for the simulation. The two peptides were individually minimized. The minimized peptides were then arbitrarily arranged together to form a two-peptide system for the molecular dynamic simulation. The two-peptide system was first minimized by Steepest Descents (SD) method, followed by Adopted Basis-set Newton-Raphson (ABNR) method. The simulation temperature was increased from 50 °K to 300 °K, equilibrated for 5 nS, and the data collected for 6 nS at 300 °K. Wide ranges of duration for system equilibration and data collection were tested in the pilot studies. It was found that the durations used above were sufficient to allow the system to reach its minimum. The formation energy was estimated by subtracting the sum of the systemic energy of individual peptides from the systemic energy of the non-covalent complex system

Results

Initial studies were conducted to compare the stability of NCX complex ions with dimer complex ions. In particular, the NCX of VLRRRRKRVN + KVNpSEEEEDA was compared with the dimer of KVNpSEEEEDA. For both the NCX and dimer, the triply charged ion (mass difference less than 2 m/z) was dissociated in the collision cell with collision energies between 5–30 V. The resulting mass spectra are displayed in Figure 1. At a collision energy of 5 V, Figure 1a, both of the complex peaks are mostly intact and the major peaks in the mass spectrum are the triply charged NCX and dimer ions. Due to the instability of the dimer complex, small peaks associated with the singly and doubly charged ions of KVNpSEEEEDA were also observed. At a collision energy of 15 V, Figure 1b, the base peak in the mass spectrum is the triply charged NCX ion while the dimer complex has disappeared. The other major peaks in the mass spectrum are the singly and doubly charged ions of KVNpSEEEEDA, which are produced from the dissociation of the KVNpSEEEEDA dimer. The NCX complex does not dissociate until the collision energy is increased to 25 V and is still observed intact at a collision energy of 30 V (Figure 1c). The major peaks in this mass spectrum are the doubly charged ion of VLRRRRKRVN, resulting from the disruption of the NCX complex, and the singly charged KVNpSEEEEDA, resulting from the dissociation of the NCX and dimer complexes. Fragmentation peaks (b series ions) from the KVNpSEEEEDA are also observed. These results are in agreement with a previous MALDI mass spectrometry study of NCXs and dimers that demonstrated the stronger stability of NCXs through salt bridging as compared to the gas phase dimer ions resulting from hydrogen bonding.

Figure 2 illustrates a molecular model of the noncovalent complex formed between KVNpSEEEEDA (tubes) and VLRRRRKRVN (lines). The colors on the ribbons superimposed along the peptide backbones represent the secondary structure motifs of the peptide. Pink represents the coil motif, green the helix, and blue the turn. The dimension of

phosphate is slightly larger than that of the carboxyl groups in the glutamate and aspartate side chain. ChelpG atomic charge calculation by Gaussian 03W using DFT/B3LYP 6-31+ G(d,p) basis set on the HF 6-31+ G(d, p)-optimized model molecules $\text{CH}_3\text{CH}_2\text{COO}^-$ and $\text{CH}_3\text{CH}_2\text{OPO}_3^{-2}$ indicated that the APT atomic charges on the oxygen atoms of the PO_3^{-2} group range between -1.1254 and -1.1457 (with hydrogens summed into heavy atoms), while the same APT atomic charges on the oxygen atoms of the carboxyl group ranged between -0.998 and -1.042 . Geometrically speaking the phosphate group could protrude further away from the side chain; electrostatically speaking the oxygen atoms on the phosphate group carry more negative charge than those seen on the carboxyl group. Another factor is generated by the conformational change of the acidic epitope, caused by the negative charges on the phosphate group, which repulse the rest of the carboxyl groups further away from each other and “relax” the helical structure on this section of the peptide for interaction with the guanidinium groups on the basic epitope. Together, these factors synergistically strengthen the ionic bonds formed between the two epitopes, and account for most of the enhanced stability.

To better understand peptide-peptide interactions, the stability of NCXs made of different types of anionic groups (phosphate group, glutamate and aspartate residues) was examined. In these experiments, NCXs of **VLRRRRKR**VN, a basic epitope, with three acidic epitopes, **KVNSEEEEEEDA**, **KVNpSEEEEEEDA**, and **KVNpSAAAAAAA** and the dimer of **KVNpSEEEEEEDA** were analyzed by ESI-MS/MS. The specific selection mass used for each sample mixture along with the ions fragmented is listed in Table 1. Each of the triply charged NCX and dimer complex ions was fragmented using collision energies between 5 – 30 V. These observations, for each of the three acidic peptides as well as the dimer, are summarized in Figure 3. The relative ion intensities of each complex ion in Figure 3 are plotted as a function of collision energy, using the average of three replicate measurements of ion intensity of the triply charged ions. The stability of NCXs of **VLRRRRKR**VN with the acidic peptides from most stable to least stable was in the following order: **KVNpSEEEEEEDA** > **KVNSEEEEEEDA** > **KVNpSAAAAAAA**. The stability in terms of anionic groups was as follows: one phosphorylated residue + six acidic amino acid residues > six acidic amino acid residues > one phosphorylated residue. These results demonstrate the stability that the presence of a phosphate group imparts on NCXs. This is in agreement with a previous MALDI-MS study which demonstrated that a single phosphate group forms a more stable interaction with the arginine guanidinium group than two adjacent carboxyl groups from either aspartate or glutamate.¹¹ The similar stability between the NCXs of **VLRRRRKR**VN + **KVNSEEEEEEDA** and **VLRRRRKR**VN + **KVNpSAAAAAAA** was surprising suggesting that in this case one phosphate group contributes similar noncovalent attraction as six acidic residues, hence reinforcing the notion that biomolecular electrostatics is more than the sum of attractions and repulsions between fixed pairs of charges. Even more crucial are the molecular details of ion solvation and the factors that affect the strengths of the different types of intermolecular forces. In the case of phosphate its dimensions are larger than those of the carboxyl groups of glutamate and aspartate, leading to stronger attractive forces. In addition two pairs of delocalized electrons are found within the same molecule. Basically, bigger and closer charges equate to strength.

Discussion

In order to quantify the observed differences in mass spectral intensities of the NCXs of the **VLRRRRKR**VN epitope with each of the three acidic epitopes, the nominal energy given to an NCX as it entered the collision cell was varied and compared to the energy required to dissociate the dimer of **KVNpSEEEEEEDA**. Collision energies are taken in laboratory units. In order to estimate the binding energy of the NCX ions, we assume that the collision energy required to completely dissociate an ion complex is a measure of the enthalpy change of the reaction:

That is, we consider only the disappearance of the complex ion and do not follow the various reaction products. A similar approach has been used in previous studies of water cluster noncovalent complexes.^{20,21} In order to place these measurements on a rational energy scale, we used the dissociation curve of the KVNpSEEEEDA dimer as a standard for dissociation energy. We recognize that our approach is simplistic in that it neglects many issues of complex internal energy, temperature and center of mass coordinates, but at the same time it does employ an energy standard which is an internal one in the case of the VLRRRRKRVN + KVNpSEEEEDA complex and an external one in the case of the other two complexes.

Estimation of binding energies from the collision data was carried out in a manner analogous to a method used for the determination of ion appearance potentials, the critical slope method pioneered by Honig²² and described further by Field and Franklin.²³ In this approach, the rapidly changing portion of the dissociation curve is plotted as the logarithm of normalized ion intensity versus collision energy and fit with a linear regression. Figure 4 shows such a plot for the KVNpSEEEEDA dimer and the VLRRRRKRVN + KVNpSEEEEDA NCX. Energy values were assigned at the point on the abscissa crossed by the regression lines. The slopes of the NCX dissociation curves were consistent within 20% as seen from Table 2.

Table 2 summarizes the measurements of the differences in NCX dissociation curves relative to the KVNpSEEEEDA dimer ion. The most striking point about Table 2 is the energy required to disrupt the noncovalent complexes. For comparison, the carbon-carbon triple bond in acetylene is 234 kcal/mole and the ionization potential of Ar is 15.8 eV. Clearly the energies involved with the dissociation of these gas phase complexes are extraordinarily high. Since all of these energies are determined relative to a triply charged dimeric ion, the dissociation energies reported in Table 2 are not simply a consequence of dissociating a triply charged complex. The second striking aspect of the results in Table 2 is the relative strength of the phosphate interaction with the very basic VLRRRRKRVN peptide. While the highly acidic KVNSEEEEDA peptide yields an excess interaction energy of 246 kcal/mole, the addition of a phosphate group generated by the phosphorylation of the serine residue increases the strength by 60% and the presence of a p-Ser in a peptide otherwise containing only the neutral alanine residues shows an interaction almost as strong as the heavily glutamylated KVNSEEEEDA. Aside from the disruption of the three primary interactions shown in Figure 3, there appears to be a secondary disruption that occurs in the VLRRRRKRVN + KVNpSEEEEDA curve. That is, the dissociation of this particular complex appears to be a two-step process that dissociates completely at an energy of 396 kcal/mole greater than the KVNpSEEEEDA dimer. We carried out a critical slope analysis of what appears to be a lower energy dissociation for this complex using the points in the curve above the obvious plateau in the curve shown in Figure 3. Points for the regression line were obtained by subtracting the fractional ion signal at the plateau from the signal intensities measured at lower values of the dissociation energy. The regression analysis for this process is summarized on the fourth line of Table 2 again using the dissociation of the dimeric peptide ion as the energy standard. At present, we do not understand the origin of this lower energy dissociation, but simply report it. The molecular modeling calculations for the complex of KVNpSEEEEDA and VLRRRRKRVN gave the following value -1348.01 ± 11.17 kcal/mol. While the complex between KVNSEEEEDA and VLRRRRKRVN gave -1086.45 ± 4 kcal/mol. So the addition of a phosphate to the peptide resulted in a decrease of -261.56 ± 7.17 kcal/mol.

From previous work we know that a motif composed of two adjacent Arg "RR" or the "RKR" motif on one peptide and two adjacent Asp "DD", or Glu "EE" or one phosphate on the other peptide were sufficient to generate a stable NCX between two even larger peptides.^{7,11,12} In the present study we have four adjacent Arg followed by a Lys and an Arg on the

basic peptide and six adjacent acidic residue plus a phosphate or six adjacent acidic residue or just one phosphate on the acidic peptide. Hence the possibility, that at least some of the NCX formed by these two acidic peptides KVNpSEEEEEEDA and KVNSEEEEEEDA, could be involving not just two adjacent Arg (from the basic peptide), but multiple Arg interacting with not just two adjacent Glu or a phosphate, but multiple Glu and the phosphate when available. Thus we could be dealing with various populations of NCXs, where the energy of dissociation could possibly depend on the number of charges involved and the nature of the acidic charges involved. As the peptide containing just one phosphate shows a similar but slightly weaker pattern to the one containing five Glu, implying that the interaction involving just one phosphate is almost as stable as the one involving multiple Glu, and that disruption of interactions involving a phosphate proceeds in a biphasic fashion. In solution noncovalent complexes are in a flux of association-dissociation, the above data seems to imply that in the case of the availability of multiple charges the number of charges involved in the interaction as well as the orbital distribution of the charges might play a role in the kinetics of dissociation, mainly the larger the number of charges involved the slower the dissociation, however below a certain number the dissociation is faster, thus explaining the biphasic nature of our experimental results.

In this work, the gas phase stability of peptide-peptide NCXs was probed using ESI-MS/MS. These results demonstrated the significant increase in stability that the presence of a phosphorylated residue has on noncovalent interactions between epitopes, as phosphorylation is used by biological systems as an on/off switch, the stability of such interactions might play an important role in regulation of physiological and biochemical processes on a cellular level. Further probing of NCX using plasmon resonance and circular dichroism could also prove helpful.

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Figure 1.
ESI-MS/MS spectra of peptide mixture consisting of 1 pmol/uL of VLRRRRRKRVN and 15 pmol/uL of KVNpSEEEEEEDA at collision energies of (a) 5 V, (b) 15 V, and (c) 30V.

Figure 2.
Molecular model of the non-covalent complex between KVNpSEEEEDA (tubes) and VLRRRRKRNVN (line). The structural motifs of the peptides are color-coded on ribbons superimposed along their backbones: pink for coil, green for helix, and blue for turn.

Figure 3. Stability of NCXs of VLRRRRKRVN with KVNSEEEEEEDA, KVNpSEEEEEEDA, KVNpSAAAAA, and the dimer of KVNpSEEEEEEDA at varying collision energies.

Figure 4.
Plot of the log of normalized ion intensity for [Dimer of KVNpSE₅DA+3H]³⁺ and [VLR₄KRVN+KVNpSE₅DA+3H]³⁺ versus collision energy fit with linear regression.

Table 1**MS/MS Experimental Setup.**

Sample Mixtures	Selection Mass Window	Complexes Dissociated
VLRRRRKRVN+KVNpSEEEEEEDA	905 Da	$[\text{NCX}+3\text{H}]^{3+} = 904.13 \text{ Da}$ [Dimer of KVNpSEEEEEEDA+3H] ³⁺ = 906 Da
VLRRRRKRVN+KVNSEEEEEEDA	878 Da	$[\text{NCX}+3\text{H}]^{3+} = 877.47 \text{ Da}$
VLRRRRKRVN+KVNpSAAAAAAA	793 Da	$[\text{NCX}+3\text{H}]^{3+} = 792.79 \text{ Da}$

¹ Note: NCX = noncovalent complex formed between acidic and basic peptides.

² Masses are monoisotopic.

Table 2
Regression Parameters and Energy Differences for Dissociation Curves.

	a_0	a_1	x_0 (eV)	Δx_0 (eV)	Δx_0 (kcal/mol)
Dimer of KVNpSE ₅ DA	0.990	0.180	12.145		
VLR ₄ KRVN+KVNpSE ₅ DA	3.089	0.146	29.377	17.2	396.3
VLR ₄ KRVN+KVNSE ₄ DA	3.553	0.231	22.850	10.7	246.2
VLR ₄ KRVN+KVNpSA ₇	4.076	0.221	21.507	9.4	215.3
VLR ₄ KRVN+KVNpSE ₅ DA (adj)	0.122	0.095	11.321	-0.8	-18.9
Average a_1 *		-0.195			
Rel Std Dev		0.20			

* Excludes VLR₄KRVN+KVNpSE₅DA (adj)