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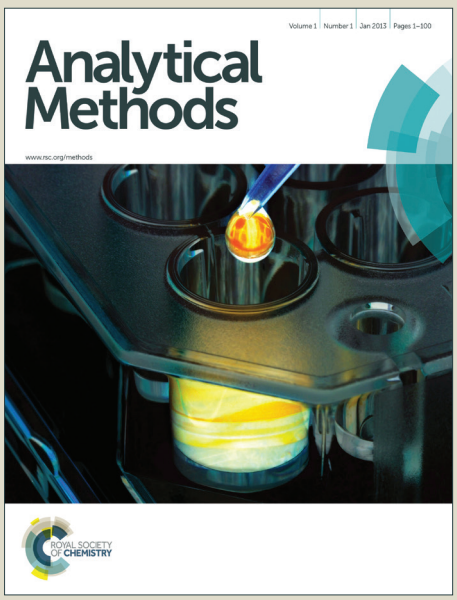

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Investigation of protonated and sodiated leucine-enkephalin by hydrogen/deuterium exchange and theoretical calculations

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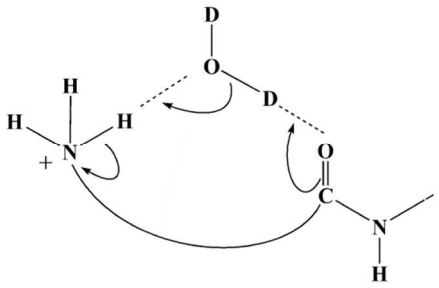
In this work, protonated and sodiated leucine-enkephalin (LE) were investigated by gas-phase hydrogen/deuterium exchange (HDX) performed on a linear ion trap time-of-flight mass spectrometer. It is found that more hydrogen atoms are exchanged in protonated LE than in sodiated LE, indicating the different conformations of the two peptide ions. To further clarify the experimental results, the conformations were calculated by using density functional theory that terminal amino group is the most thermodynamically stable protonation site, while the sodium ion coordinated four carbonyl oxygen atoms forms the most favourable sodium adduct. Limited HDX reactions of sodiated LE is explained by the rigid conformation and fewer exchangeable acidic hydrogen atoms from sodium coordination.

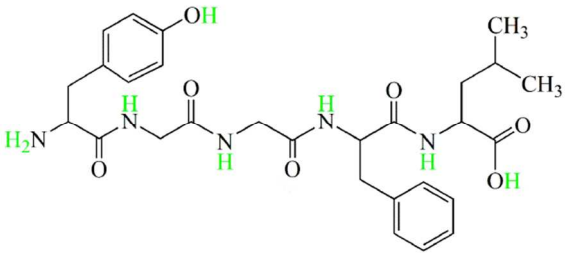
1. Introduction

Hydrogen/deuterium exchange (HDX) is an important tool for monitoring transitions and conformations of proteins by coupling with mass spectrometry (MS)[1-4] and ion mobility spectrometry techniques[5-7] with the introduction of electrospray ionization (ESI)[8]. Moreover, combined with theoretical calculations, it shows great advantages to investigate the intrinsic structure in gas-phase chemistry.[9-12] Previous researches indicate that various deuterating reagents exhibit distinct exchange efficiencies with their different proton affinities.[13,14] Beauchamp and co-workers investigated gas-phase HDX reactions of protonated glycine oligomers with various deuterating reagents and proposed several HDX mechanisms based on semi-empirical calculations.[15] Among those, D_2O favours the "relay" mechanism, in which D_2O coordinates between protonation site and a basic site via two hydrogen bonds (Scheme 1). HDX occurs by proton transfer from the N-terminus to the oxygen atom on D_2O coincident with a deuteron from D_2O to a less basic amide oxygen, and the process is chemically activated by hydrogen bonds. This mechanism is further supported by Bowers via ab. initio calculations and the surface accessibility of the charged sites and the basic sites and the associated distances between them are two important factors.[16]



Scheme 1 Relay mechanism of HDX reaction with D_2O.

Leucine-enkephalin (LE) is a pentapeptide composed of amino acids Try-Gly-Gly-Phe-Leu (=YGGFL) (Scheme 2). It is a standard compound to test the properties of new instruments and methodologies, and to tune instruments[17-20] with known fragmentation mechanisms and energetics.[21] What's more, the peptide can act as an agonist at opiate receptor sites in the brain.[22] Pert found that LE reveals a dual agonist-antagonist at opiate receptors with low "sodium response ratio"[23] and the effect may result from conformational change by sodium ion (Na^+) coordination like methionine-enkephalin.[24]



Scheme 2 Chemical structure of leucine-enkephalin.

Cationic ions of the protonated LE and sodiated LE have been wildly studied by MS. Different fragmentation processes of these two ions are revealed by tandem mass spectrometric analysis that dissociation of protonated LE produces the most fragments of $\boldsymbol{a_n}$ or $\boldsymbol{b_n}$ ion series and some ***y*** ions, while sodiated LE ion gives a sodiated *tetra-* or tripeptide via the loss of C-terminal amino acid residues[25-28] and more collision energy is required in the latter than the former.[20] Hydrogen/deuterium exchange mass spectrometry (HDXMS) experiments also have been performed to study the two adducts. Justice observed complete HDX reactions of protonated LE and sodiated LE by coupling HDXMS with capillary electrophoresis, but paid little attention to their reaction behaviours.[29] Williams found that sodium adduct exchanges slowly compared with the proton adduct, but did not attempt to explain the phenomenon.[30]

In this study, sodiated LE and protonated LE are investigated by HDXMS combined with theoretical calculations. Similar results are obtained that more hydrogen atoms are exchanged in protonated LE than the sodiated LE. The reactions are nearly quenched at 10 s in sodiated LE. It is supposed that the different exchange reactivities are caused by conformational difference of these adducts and the favourable conformations were calculated by using density functional theory (DFT) to further identify our proposal.

2. Experimental

2.1 Mass spectrometer

All experiments were performed on homemade linear ion trap time-of-flight (LIT-TOF) mass spectrometer, schematic diagram of which is shown in Fig. 1. The present resolution (FWHM) of TOF is about 2000 for the dissatisfactory machining accuracy. The resolution is required further optimization, but it is enough to perform HDX reactions of small molecules. Briefly, sample ions were produced from ESI (3500 V), passed through an aperture in curtain plate (950 V), suffered further desolvation in curtain gas (nitrogen, ⩾ 99.999% 0.5 L/min), then passed through orifice (200 V) and skimmer plates, and entered the first chamber with two quadrupole sets, Q0 ion guide and Q1, both operated in RF-only mode (frequency 768 kHz). Transmitted from the Q1 through a coupling lenses stack (L1-L4), ions were extracted and finally analysed by TOF mass spectrometer orthogonal to Q1. The ions can be trapped in Q1 for HDX reactions by applying timed DC stopping potentials to the entrance lens Q0/Q1 (4.0 mm aperture covered with a 90% transmitting 50 mesh grid) and exit lens L1 (0.7 mm aperture) axially and RF potentials simultaneously applied to Q1 radially. Usually the ion trap chamber was pumped by a turbomolecular pump to 3.3×10^{-3} Torr, measured with a precision capacitance manometer (MKS Instruments, Andover, MA model: 120AA) and in HDX experiments the pressure was about 8.3×10^{-3} Torr with 5×10^{-3} Torr gaseous D_2O unless other statements.

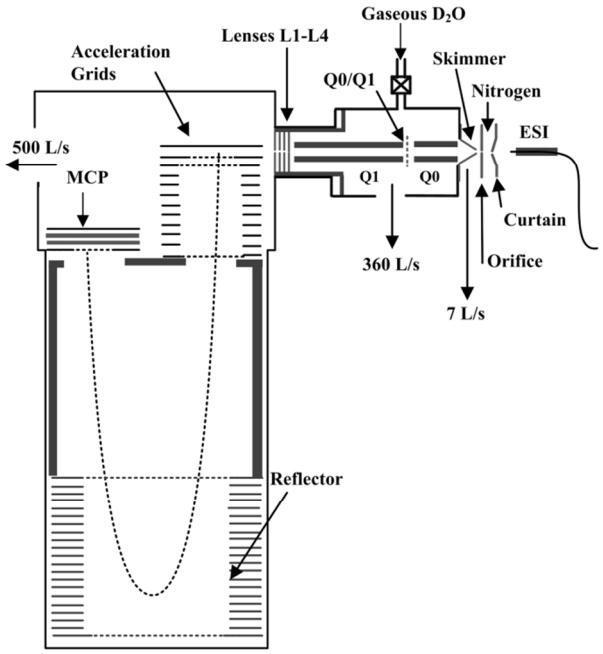


Fig. 1 Schematic diagram of LIT-TOF mass spectrometer.

The timing sequence and voltages applied to Q0/Q1 and L1 are illustrated in Fig. 2, controlled by an arbitrary waveform generator (AWG-312; PC Instruments Inc., Akron, OH, USA). High/low voltages on Q0/Q1 and L1 are +40 V/+11 V and +60 V/-15 V, respectively. Each test cycle includes four stages, ion draining (50 ms), injection (50 ms), trapping and detection (30 ms). Trapping time in the third stage, i.e. the HDX reaction time, is variable, ranging from 0 s to 20.0 s and so on. The product ions were finally introduced into TOF for detection.

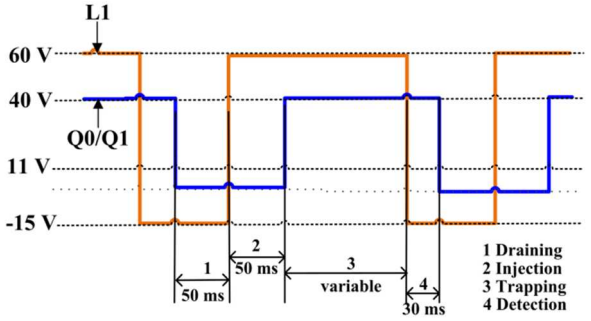


Fig. 2 Timing sequence and voltages on Q0/Q1 and L1 in HDX test.

2.2 Chemical reagents

Leucine-enkephalin (YGGFL, MW=555) was purchased from GL Biochem. Ltd. (Shanghai, China). Deuterium oxide (D, 99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol (CH_3OH, HPLC grade) was purchased from Merck (Darmstadt, Germany). Acetic acid ($C_2H_4O_2$, A.R. grade) and sodium chloride (NaCl, A.R. grade) were bought from Shanghai Chemical Reagent Co. All reagents were used without further purification. Deionized water was used in all experiments.

Leucine-enkephalin was dissolved in methanol/water (50:50 v/v, 0.1% $C_2H_4O_2$) to 20 μM with sodium chloride of 50 μM. The solution was pumped into a capillary (i.d. ~75 μM; Polymicro Technologies, L.L.C., USA) at a rate of 1 μL/min.

2.3 Computational details

Density functional theory (DFT) calculations were performed with the hybrid B3LYP functional[31-33] and Gaussian 09 program[34] to study the geometric and electronic structures of M, $[MH]^+$ and $[MNa]^+$ (M: neutral LE molecule). Conformational searching of this molecule or ions was performed using Open Babel[35] with a genetic algorithm. Additional initial structures of $[MH]^+$ and $[MNa]^+$ were obtained by putting H^+/Na^+ at various suitable sites in the low-lying structures of M. In DFT calculations, 6-31++G** basis sets were chosen for all atoms to include diffusion and polarization functions, which are essential to describe intramolecular hydrogen bonds well. Vibrational frequencies were calculated to verify that all stable structures had no imaginary frequency. Gas-phase acidities (ΔH_{acid}) were also calculated and the ΔH_{acid} are defined by the reaction MH = M^- + H^+. The calculated energies are reported after zero-point vibrational energy correction. Charge on each active hydrogen atom was calculated through natural population analysis (NPA) which is stable with respect to basis set changes.[36]

3. Results and discussion

3.1 HDX results

There are eight labile hydrogen atoms in the neutral LE molecule (hydrogen atoms in green shown in Scheme 2), so protonated LE and sodiated LE possess nine and eight exchangeable hydrogen atoms, respectively.[29] The HDX results of these two peptide ions are compared in Fig. 3. Obviously, the mass spectra extend to the high mass side with trapping time increased as the longer reaction time is favourable for the exchange reactions. Dramatically difference in mass spectral peak patterns occurs at 10 s. As the reaction time was increased to 20 s, eight and four hydrogen atoms were exchanged in protonated LE and sodiated LE, and relative intensity distributions in Fig. 3(l) are much similar to that in Fig. 3(g) without exchange, indicating the reactions of sodiated LE were nearly quenched after 10 s. Therefore, more hydrogen atoms are exchanged in protonated LE than the sodiated counterpart under identical experimental conditions and the two ions are conformationally different.

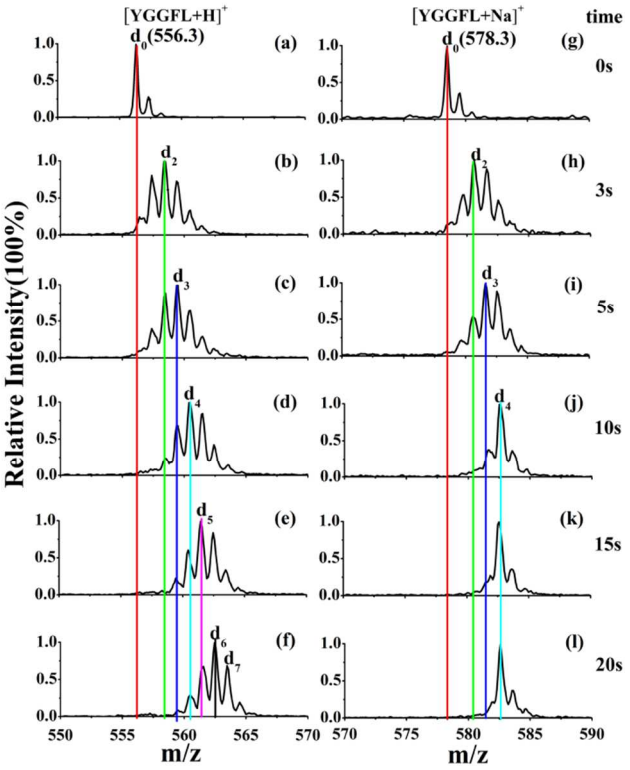


Fig. 3 HDX mass spectra of protonated LE ($[YGGFL+H]^+$) (left) and sodiated LE ($[YGGFL+Na]^+$) (right) at various reaction times with 5×10^{-3} Torr gaseous D_2O.

The time-intensity dependence behaviour of all ions in reactions is shown in Fig. 4. Relative intensity of d_0 decreases continuously and when it approaches zero, all initial precursor ions are exchanged. Compared with Fig. 4(a), d_0 decreases fast in Fig. 4(b) and maximum intensity of d_1 - d_3 occurs much early, suggesting faster exchange in sodiated LE than protonated LE in the primary reaction stage. In Fig. 4(a), eight labile hydrogen atoms are exchanged in protonated LE and product ions of d_1–d_5 display unimodal intensity distribution for predominant singly exchange with D_2O.[14,15] However, in Fig. 4(b) of sodiated LE, four hydrogen atoms are exchanged. Ions of d_1–d_3 exhibit unimodal distribution, while d_4 is in high intensity after 10 s coincident with very low intensity of d_5 and d_6, suggesting the reactions were nearly stopped after 10 s. As shown in Fig. 4, the two cationic ions show different HDX performances and the results are consistent with previous studies.[30]

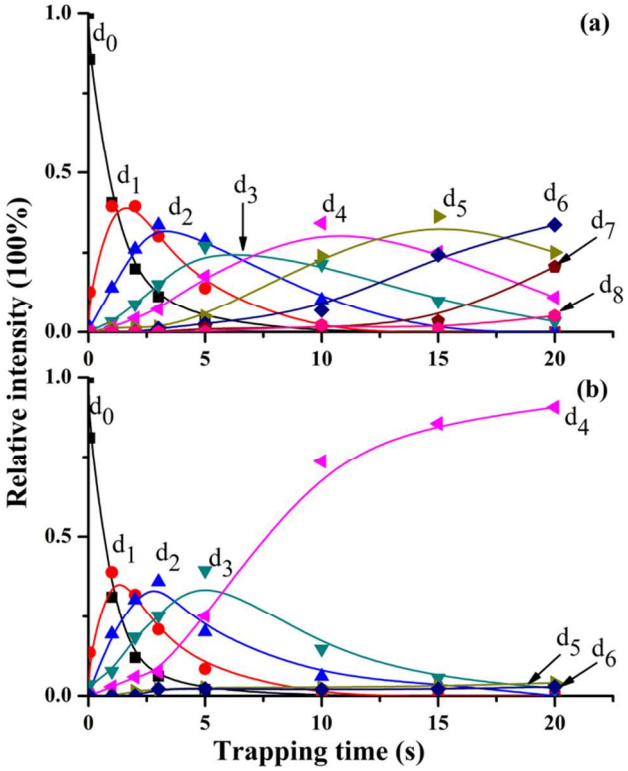


Fig. 4 The time dependence behaviour of ion species in HDX of $[YGGFL+H]^+$ (a) and $[YGGFL+Na]^+$ (b) with D_2O at 5×10^{-3} Torr. d_n refers to the number of exchanged hydrogens.

3.2 Conformations of protonated and sodiated leucine-enkephalin

The different HDX reaction behaviours reflect the conformational difference of these two ions, so their favourable conformations were calculated to further clarify the experimental results. All of the possible structures are optimized by high level DFT calculation at 6-31++G** basis sets to find the most stable conformations. As shown in Fig. 5, protonation site on the terminal amino group produces the most thermodynamically stable protonated LE (A), while protonation site on amide oxygen atom is less stable (B, 51 kJ/mol). For sodiated LE, the most favourable conformation is formed by Na^+ multi-coordinating to four carbonyl oxygen atoms (C), three from peptide bonds and one from a carboxyl moiety. When Na^+ is coordinated to one nitrogen atom and three oxygen atoms, the conformation (D) is 23 kJ/mol higher in energy than the lowest energy conformation (C). Based on the calculations, protonated LE and sodiated LE ions are formed by different binding types that a proton generally singly binds to the most basic site and sodium ion prefers multidentate coordination, so these peptide ions are of different conformations theoretically, which is in good agreement with their different performances in HDX reactions.

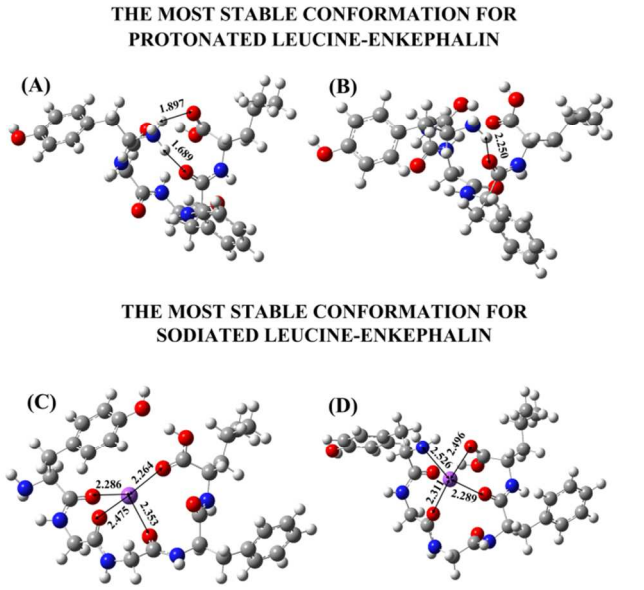


Fig. 5 Calculated conformations of $[YGGFL+H]^+$ and $[YGGFL+Na]^+$ using DFT (B3LYP/6-31++G**). (A) Protonation site is amino group on N-terminus (0 kJ/mol); (B) Protonation site is amide oxygen atom on backbone (+51 kJ/mol); (C) Sodium ion is coordinated to four amide oxygen atoms on backbone (0 kJ/mol); (D) Sodium ion is coordinated to three amide oxygen atoms and nitrogen on N-terminus (+23 kJ/mol). Bond length is in Å.

In electrospray mass spectrometry, protonation and sodiation are the two most common ways for generating cationic ions and the different binding types between these two ways usually induce different ion characters. Herein, it is confirmed that a proton generally singly binds to the most basic site in protonation and sodium ion prefers multidentate coordination in sodiation and these binding types are consistent with early studies.[37] Besides, the associated different characters in HDX reaction of cationic ions produced from protonation and sodiation are further displayed by comparing protonated LE and sodiated LE in our work.

3.3 Discussion

Discussion based on "relay" mechanism

HDX with D_2O is much more likely to participate in a "relay" mechanism presumably due to the low proton affinity.[15,37] Herein, the different HDX reactivities of sodiated LE and protonated LE are attempted to be explained by the "relay" mechanism, though other mechanisms including "salt-bridge" mechanism cannot be conclusively excluded.[30]

In the "relay" mechanism, a proton is shuttled from the protonation site to D_2O in coincidence with the transfer of a deuteron from D_2O to a distant, slightly less basic amide oxygen[15] (Scheme 1), and intramolecular deuterium-hydrogen rearrangement subsequently distributes deuterium to other sites of the molecule. It is believed that hydrogen atom is exchanged through hydrogen bonded intermediate and the proton transfer is viable within a molecule using the chemical activation provided by hydrogen bonds, so any factors hampering the formation of hydrogen bond and hydrogen bonded intermediate would block the exchange reactions. In sodiated LE, electron density on amide oxygen atoms is decreased from sodium ion coordination compared the protonated LE, so hydrogen bond between D_2O and oxygen atoms is weaker than that in proton adduct. In addition, hydrogen bond between labile hydrogen atom and D_2O is unfavourable (relative to the LE). Positive charge on each reactive hydrogen atom was calculated as Fig. 6, in which exchangeable hydrogen atoms are labelled in the most stable conformation of protonated LE (Fig. 5A) and hydrogen atoms on sodiated molecular ion are labelled in the same order (not shown). Obviously, atoms of 3-6 possess less positive charge in sodiated LE than in protonated LE, namely the active hydrogen atoms in sodium adduct are less acidic than those of analogous proton adduct, so compared with protonated LE hydrogen bond formed between D_2O and exchangeable hydrogen atom is weak in sodium adduct. Therefore, proton transfer is more difficult in sodiated LE for the weak hydrogen bonds and the associated lower HDX reactivity was performed.

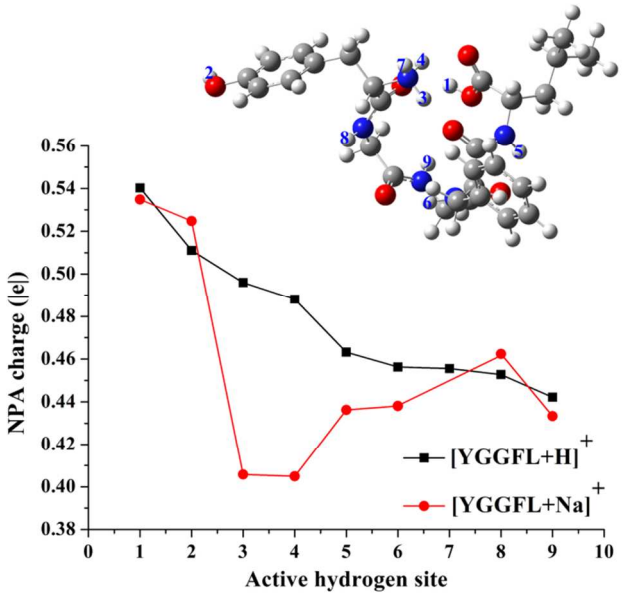


Fig. 6 Charge distribution on each exchangeable hydrogen atom in $[YGGFL+H]^+$ and $[YGGFL+Na]^+$.

The PKa of a molecular ion in the gas phase can be characterized by its proton affinity (PA), i.e. the energy needed to remove a proton from it. The higher the PA is (or less acidic), the more difficult to remove the proton, i.e. the more difficult for the exchange of the hydrogen. From Fig. 7, it can be seen that nearly 4 labile hydrogen atoms in $[YGGFL+Na]^+$ have higher PAs than those in $[YGGFL+H]^+$ and hydrogen atoms in sodiated LE are less acidic, consistent with Fig. 6. In agreement with experimental results, theoretical calculations predict that $[YGGFL+Na]^+$ has lower HDX reactivity than $[YGGFL+H]^+$.

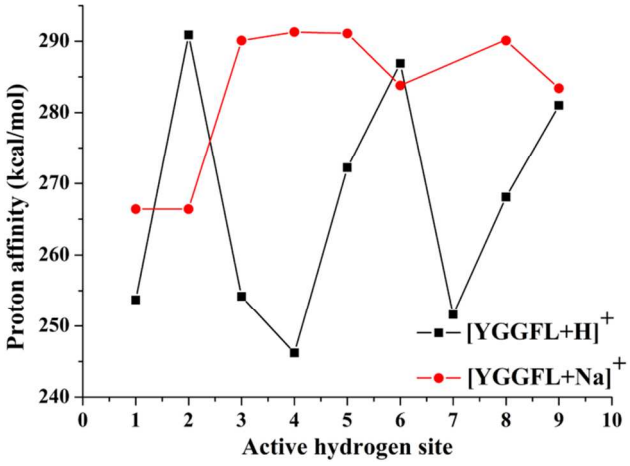


Fig. 7 Proton affinity distribution of the exchangeable hydrogen atoms in $[YGGFL+H]^+$ and $[YGGFL+Na]^+$.

Comparison with other researches

The HDX performance of LE is similar to polyamine[13] and peptides of gramicidin S[38] and Arg-Gly-Asp[39], but contrary to other peptides,[10,40,41] such as bradykinin, which is adducted a single metal ion can significantly increase the HDX reactivity. There are a few studies on HDX between sodiated peptides with D_2O.[9, 16, 30, 40-42], however, to the best of our knowledge, the HDX mechanism of these reactions is still unclear.

Williams et al. investigated the role of acidic residues and of sodium ion adduction on the gas-phase HDX of peptides with D_2O. In their studies of singly sodiated VEPIPY, there are five hydrogens exchanged, which could correspond to the two carboxyl hydrogens, two N-terminal hydrogens and one hydrogen atom from side chain of tyrosine, and the three amide hydrogens do not exchange. The similar results were obtained in single sodiated FLEEL with slow exchange of four amide hydrogen atoms on backbone and rapid exchange of three carboxylic hydrogens and two N-terminus hydrogens.[43] The results were attempted to be explained by the "salt-bridge" mechanism involving proton transfer from a carboxylic acid to D_2O and the stabilization of the resulting carboxylate anion by a nearby charge site.[15] For peptides adducted several metal ions, HDX reactions is decreased presumably due to no carboxylic acids available for proton transfer.[30]

Based on the finding of Williams[30] accounted with "salt bridge" mechanism, four hydrogen atoms exchanged in sodiated LE with D_2O probably correspond to one carboxyl hydrogen, two N-terminal hydrogens and one hydrogen atom from side chain of tyrosine with four backbone amide hydrogens exchange much slowly. However, as far as I known, the real exchange process between sodiated peptide and D_2O is still ambiguous and more work is required to explore the mechanism.

Besides, the above calculations demonstrated that sodium ion prefers multidentate coordination with LE to form rigid conformation compared with protonated LE.[6,38,39,44] Furthermore, conformational change can also affect exchange reactions.[2,13,38,39] Intramolecular rearrangement, the surface accessibility of the charged sites and the basic sites and distance between them might be influenced by the rigid conformation, lowing the associated reactivity.

Conclusions

Protonated LE has higher reactivity than the sodiated LE in the gas-phase HDX reaction and HDX reactions of sodiated LE might be limited by the conformational change and fewer acidic active hydrogen atoms from Na^+ coordination. Our investigation suggests protonated LE and sodiated LE are in different conformations and provides clues on dual agonist-antagonist at opiate receptors of LE in the presence of sodium salts. The work is helpful for better elucidation of HDX experiment in conformational study of peptides and the investigation on understanding of differences in protonation and sodiation in electrospray mass spectrometry. Additionally, HDXMS combined with theoretical calculations is proved to be an efficient method to study interaction between peptide and metal ions.

Acknowledgements

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Protonated and sodiated leucine-enkephalin compared by hydrogen/deuterium exchange mass spectrometry and theoretical calculations.