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High-energy collision-induced dissociation of histidine ions, $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$, and histidine dimer $[\text{His}_2+\text{H}]^+$

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Abstract**RATIONALE:**

Histidine (His) is an essential amino acid, which side group consists of aromatic imidazole moiety that can bind a proton or metal cation and act as a donor in intermolecular interactions in many biological processes. The discovery that deprotonation or protonation takes place at the His side group rather than the backbone has led to revisiting its unimolecular fragmentation reactions.

METHODS:

Using a new home build electrospray ionization (ESI) source adapted to a double focusing mass spectrometer of BxE geometry, we investigate fragmentation reactions of protonated and deprotonated His, $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$, and protonated His dimer $[\text{His}_2+\text{H}]^+$, accelerated to 6 keV in a high-energy collision with He gas. We have evaluated kinetic energy release (KER) for the observed dissociation channels.

RESULTS:

ESI of His solution in positive mode has led to the formation of His clusters $[\text{His}_n+\text{H}]^+$, $n = 1 - 6$, with notable enhanced stability of the His tetramer. $[\text{His}+\text{H}]^+$ dissociates predominantly by loss of $(\text{H}_2\text{O}+\text{CO})$ with KER of 278 meV, while the dominant dissociation channel of $[\text{His}-\text{H}]^-$ is due to the loss of NH_3 with high KER of 769 meV. Dissociation of $[\text{His}_2+\text{H}]^+$ is dominated by loss of monomer but smaller losses from the dimer are also observed.

CONCLUSIONS:

The KER for HCOOH loss from both, $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$, is similar of 278 and 249 meV, respectively, which suggest the collision-induced dissociation takes place via similar mechanism. The loss of COOH and $\text{C}_2\text{H}_5\text{NO}_2$ from the dimer suggests the dimer of His binds through a shared proton between the imidazole moieties.

Keywords: histidine; tandem mass spectrometry; collision-induced dissociation; electrospray ionization; amino acid clusters;

Running Title: Fragmentation of histidine and histidine dimer.

Introduction

Histidine (His, **1**, Scheme 1) is an essential aromatic amino acid, crucial for biological functioning of proteins and enzymes. His is a precursor of hormone histamine, an inflammatory agent in immune system response, and is a key residue in the active sites of number of enzymes.^[1,2] His also acts as a mediator for proton transfer in proteins.^[3] To understand its stability, structural preference, and dissociation reactions of His residue and its ionic variants is important in its own right.

The importance of the knowledge on the His amino acid can be seen by number of recent studies on the structure of neutral as well as charged His.^[4-6] Due to the presence of imidazole moiety in His, His can adopt two tautomeric conformations (Scheme 1) with H on either of the two N atoms in the imidazole moiety referred to as τ tautomer **1a** with H on N1 (tele), and π tautomer **1b**, where H is on N3 (pros). The structural preference of neutral His and its ionic forms $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$ has been investigated in the work of Huang *et al.*^[4] and Purushotham *et al.*^[5] and of His and $[\text{His}-\text{H}]^-$ by Stover *et al.*^[6] by means of quantum chemical calculations. They all found neutral His τ tautomer **1a** to be more stable than π tautomer in the gas phase^[4-6] and the two independent studies suggested similar population of $\sim 80\%$ of the τ tautomer and 20% of π tautomer at equilibrium in the gas phase at 298 K.^[4,6] The higher stability of the His τ tautomer **1a** is explained by the higher hydrogen bonding capabilities than that of the π tautomer.^[4,5] The deprotonation of His to form $[\text{His}-\text{H}]^-$ and protonation of His to form $[\text{His}+\text{H}]^+$ showed to take place predominantly at the imidazole ring,^[4,6] which results in identical ions and the notation of His τ or His π tautomer becomes irrelevant considering $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$ in the gas phase. It has been pointed out that imidazole ring is an excellent proton donor as well as a good proton acceptor, which makes His a unique amino acid due to the protonation and deprotonation taking place at the amino acid side group rather than the backbone,^[4] making imidazole in His free to bind a

proton or metal cation and act as a donor in intermolecular interactions and salt bridges,^[7] or mediate oxygen transporting function.^[8]

[Insert Scheme 1 here, please]

Fragmentation reactions of protonated amino acids including His has been investigated previously using variety of experimental methods.^[9-11] Metastable ion fragmentation reactions have been often complemented by high-energy collision-induced dissociation (CID) in a single collision,^[9] or by low-energy CID to obtain energy dependence of the fragmentation reactions.^[10] The most important fragmentations in the fast atom bombardment (FAB) mass spectra of protonated amino acids including His were reported to be loss of NH_3 , H_2O and $(\text{H}_2\text{O} + \text{CO})$,^[9] where the latter leads to the formation of iminium ions $[\text{H}_2\text{N}^+=\text{CHR}]$, where R is the amino acid residue. Iminium ions are fingerprints of amino acids in peptide sequencing. The loss of $(\text{H}_2\text{O} + \text{CO})$ has been reported to be associated with large kinetic energy release (KER), and in the case of metastable decay of $[\text{His}+\text{H}]^+$ it was measured to be 324 meV.^[9] This fragment showed to be dominant also in the case of low-energy CID,^[10] however, the loss of NH_3 from $[\text{His}+\text{H}]^+$ was not observed in the following studies.^[10,11]

Positive- and negative-ion fragmentation spectra may be very different and thus provide useful complementary structural information for peptides.^[12,13] Bowie pointed out in his review^[14] that major deprotonation of amino acids occurs at the carboxylate centre and most of the fragmentation reactions are preceded by specific proton transfers to the carboxylate centre. However, as already mentioned above the deprotonation of His takes place at the imidazole moiety.^[4,6] The FAB mass spectra of deprotonated His indicated major losses of NH_3 , H_2O , CO_2 , and $(\text{H}_2\text{O} + \text{CO})$.^[9,15] Interestingly, fragmentation of $[\text{His}-\text{H}]^-$

created in atmospheric pressure chemical ionization led to observation of a sole fragment due to the loss of NH_3 upon a low-energy CID in a quadrupole.^[16] Loss of NH_3 is characteristic of amino acids with long side group or a side amine (His) or phenyl group.

Non-covalently bound amino acid clusters have been subject of intense research over the past 20 years and covered diverse areas of interest, e.g., the use of fragmentation of proton-bound dimers to determine the gas-phase proton affinities^[17] or methylating abilities,^[18] peptide bond formation within amino acid clusters,^[19] formation and ionisation of neutral amino acid clusters in helium droplets,^[20] or formation and fragmentation reactions of multiply charged clusters using variety of activation methods.^[21-24] To the best of our knowledge there is no data on the fragmentation reactions of protonated His dimer $[\text{His}_2+\text{H}]^+$.

In the present work a new home build electrospray ionization (ESI) source was constructed and adapted to a double focusing mass spectrometer, where we investigate the fragmentation reactions of protonated and deprotonated His, $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$, and protonated His dimer $[\text{His}_2+\text{H}]^+$ after high-energy collisions with He atoms. We have also evaluated the KER for the most important dissociation channels.

Experimental

L-Histidine (purity $\geq 99\%$) was used as received from Sigma-Aldrich, Austria. All experiments were carried out using a 10 mM solution of His dissolved in methanol/water/acetic acid (50/50/1) on a double focusing mass spectrometer equipped with ESI source.

Since the ionisation process taking place in ESI was already explained in detail,^[25] only an overview about the novel experimental setup will be given. The ESI source shown in Figure 1 consists out of spray needle (A), an inlet capillary (B), an ion funnel (C), an octupole ion guide (D), and an einzel lens setup (E) assembled in a differential pumping system. The

spray solution was filled into a motor driven syringe which pumped sample solution between 300 nL up to 2800 nL per minute via a silica tube with an inner diameter of 150 μm towards the spray needle with an inner diameter of 120 μm . The spraying process was achieved by applying 4.00 kV to 4.88 kV for positive ion measurements and -3.8 kV to -4.5 kV in negative mode at the needle (A) relative to the inlet capillary (B) what causes the formation of an electric field between needle and the inlet capillary. The inlet capillary was a stainless steel needle with an inner diameter of 0.75 mm and length of 5 cm. Additionally the capillary was heated between 115 $^{\circ}\text{C}$ and 205 $^{\circ}\text{C}$ to prevent the capillary from blocking. Through the above mentioned inlet the ions emerge into the first differential pumping (1st) stage at a pressure of approximately 3.4 mbar accomplished by a rotary vane pump and arrived at the first electrostatic lens of the ion funnel system (C). The reduction of the ion kinetic energy and the focussing of the ion sample beam were achieved with a homemade ion funnel system designed after Julian *et al.*^[26] The ion funnel that reduces the kinetic energy of the ions by numerous collisions with ambient air consists of 36 electrostatic lenses with conical shaped inner diameter where a dc-voltage gradient and ac-voltage were applied to focus the beam and guide it towards the next differential pumping stage (2nd). The second pumping stage allows further reduction of the pressure by an order of magnitude which is realized with a rotary vane pump. To guide the sample ions from a low into a high vacuum regime a self-built octupole ion guide (D) was used in pumping stage number three (3rd). Therefore a turbomolecular pump was mounted above the octupole ion guide to reduce the pressure by three orders of magnitude and ensured a high vacuum at the einzel lens (E) and at the VG-ZAB double focussing mass spectrometer.

[Insert Figure 1 here, please]

Because the VG-ZAB 2double focussing mass spectrometer was described in detail before,^[27] just some brief overview about this mass spectrometer will be made. The mass spectrometer is built in reversed Nier-Johnson geometry, i.e. ions are analysed first by their momentum in a magnetic sector, then analysed regarding their energy in an electric sector and finally detected with a secondary electron multiplier. The acceleration voltage for the ions to be analysed in this instrument is 6 kV. Therefore, the complete ESI-source had to be isolated from the analyser to receive the required acceleration of the ion beam. A CF-160 ceramic flange was used at the end of the ESI-setup which provides the isolation and connects the ion source setup to the adjustable lens system of the analyser at the same time. As a result the required voltage approximately of 6kV for the ion acceleration was applied. For the investigation of CID a collision cell was mounted before the electric sector sector and was filled with helium as collision gas. The exact pressure in the cell is unknown. All pressure values mentioned in the Section Results and Discussion were determined with a pressure gauge mounted in the vacuum chamber of the collision cell. The stated values may be up to three orders of magnitudes lower than the real pressure in the cell.^[28]

The charged products formed in CID were probed using the mass analysed ion kinetic energy (MIKE) scan method. The ratio of the precursor ion mass m_p and the resulting fragment ion mass m_f are related to the ratio of the corresponding electric sector field voltages E_0 and E , where those ions are transmitted through the electric sector:

$$E/E_0 = m_f/m_p \quad (1)$$

For the most abundant product ions we calculated the KER of the reaction by the following formula:

$$KER = \frac{y^2 m_1^2 eV}{16 x m_2 m_3} \left(\frac{\Delta E}{E} \right)^2 \quad (2)$$

where m_1 denotes the mass of the parent and m_2 and m_3 respectively the masses of the fragments. The charge state of the parent ion and the fragment ions (in our case both +1) are denoted by x and y and V is the acceleration voltage (6 kV). E is the corresponding electric sector voltage and in the case of a Gaussian peak shape ΔE is the width of the peak in the MIKE scan (of which the width of the parent beam has to be subtracted). See reference [29] for further details.

Results and Discussion

ESI of a 10mM of His in MeOH/H₂O/AcAc (50/50/1) resulted in the observation of $[\text{His}+\text{H}]^+$ at m/z 156 as the most abundant ion in the spectrum shown in Figure 2. Figure 2a shows the lower m/z region depicting protonated His $[\text{His}+\text{H}]^+$, sodiated His $[\text{His}+\text{Na}]^+$, and protonated His dimer $[\text{His}_2+\text{H}]^+$. A closer inspection of the spectrum in Figure 2a reveals a considerable in-source fragmentation leading to the observation of fragment ions reported upon vibrational excitation of $[\text{His}+\text{H}]^+$,^[11] labelled in Figure 2a by ♣. Two ions labelled by * correspond to protonated methanol monomer and dimer, $[(\text{MeOH})+\text{H}]^+$ at m/z 32 and $[(\text{MeOH})_2+\text{H}]^+$ at m/z 65, respectively. Figure 2b shows ESI mass scan performed with focus on His clusters $[\text{His}_n+\text{H}]^+$, $n = 1 - 6$. It is noteworthy, the intensity of the His tetramer $[\text{His}_4+\text{H}]^+$ is obviously higher than preceding trimer $[\text{His}_3+\text{H}]^+$, which may indicate extra stabilisation forces in tetramer structure. However, this stability is not as pronounced as in the case of octamerization of serine.^[30,31]

[Insert Figure 2 here, please]

Fragmentation of [His+H]⁺

Figure 3 shows high-energy CID mass spectrum of [His+H]⁺ ions formed via ESI and accelerated to 6 keV and the collision cell filled with helium gas. Protonated His [His+H]⁺ (*m/z* 156) dissociates by the following pathways: loss of H₂O forming fragment ion at *m/z* 138 (eq. 3); loss of (H₂O + CO) forming iminium fragment ion at *m/z* 110 (eq. 4) and additional loss of NH=[•]CH radical resulting in fragment ion at *m/z* 82 (eq. 5); the loss of (H₂O + CO + NH₃) leading to the fragment ion at *m/z* 93 (eq. 6); loss of (NH₃ + CO₂) forms ion at *m/z* 95 (eq. 7); an additional loss of HCN associated with ring opening reaction was proposed^[11] to lead to the fragment ion at *m/z* 68 (eq. 8); and the cleavage of the C_α–C_β bond (see Scheme 1) leading to the fragment ion at *m/z* 74, where charge is retained by the backbone (eq. 9). The calculated KER for all the dissociation channels is summarised in Table 1.

[Insert Figure 3 here, please]

We observe similar losses as reported previously^[9-11] noting that the loss of single H₂O (eq. 3) was only observed in high-energy CID^[9] and metastable decay^[9,10] experiments

and not the low-energy CID. This decay channel in the present study has a low KER of 94 meV. The loss of (H₂O + CO) (eq. 4) has KER of 278 meV, which agrees well with the reported KER in a metastable loss of 324 meV.^[9] Even though there is still no evidence for the loss to be a single molecule HCOOH,^[11 and refs therein] it is now believed that the loss is concomitant loss of (H₂O + CO) (eq. 5).^[11] Nevertheless, it has been noted that CO₂H₂ loss can occur in one step, and also a two-step fragmentation mechanism.^[9] The formation of the ion at m/z 82 via eq. 5 has been calculated to have a KER of 226 meV. The following decay channels via eq. 6 and eq. 7 show to have KER of 262 meV and 246 meV, respectively. The proposed dissociation reaction involved with ring opening and release of HCN^[11] is here via eq. 8 observed with high KER of 507 meV. The loss of a side group through breakage of the C_α-C_β bond forming fragment ion at m/z 74 via eq. 9 has KER of 119 meV.

We do not observe the loss of NH₃ as reported in earlier high-energy CID and metastable scan study^[9] where the parent ions were formed by FAB ionization, however, NH₃ loss was neither observed in the low-energy CID of parent ions formed by chemical ionization,^[10] which suggests that the lowest conformer is formed here via ESI with protonation on the imidazole moiety of His.

Fragmentation of [His₂+H]⁺

Dissociation spectra for protonated His dimer [His₂+H]⁺ are shown in Figure 4. Figure 4a shows the main decay channel leading to [His+H]⁺ by loss of a His monomer with small KER of 48 meV (eq. 10), the formation of iminium ion at m/z 110 via eq. 11 with KER of 163 meV (lot smaller than in the CID of the protonated His discussed above), and the formation of fragment ion at m/z 83 via eq. 12 with KER of 172 meV. Figure 4b shows the CID scan with focus on the m/z region between protonated His monomer at m/z 156 and the

protonated dimer at m/z 311. Two lower abundant fragment ions are observed due to the loss of COOH group via eq. 13 forming ion at m/z 266 with KER of 205 meV, and the loss of the backbone H_2NCH_2COOH via eq. 14 leading to the fragment ion at m/z 236 with KER of 170 meV. These two fragment ions highlight the damage to one of the His, while the hydrogen bond between in the dimer is maintained. We note that such preference of *intramolecular* bond cleavage over *intermolecular* bond cleavage was recently observed in a variety of non-covalently bound clusters.^[32-34] The preference of His to be protonated at the imidazole ring,^[4,6] and the observation of both of these fragments may lead to the suggestion that the His dimer $[His_2+H]^+$ is bound through a shared proton between the imidazole moieties. However, further theoretical investigations would be desirable to prove our suggestion.

[Insert Figure 4 here, please]

Fragmentation of $[His-H]^-$

Figure 5 shows high-energy CID mass spectrum of $[His-H]^-$ ions accelerated to 6 keV and the collision cell filled with helium gas. The fragment ions observed are similar to those reported in FAB CID studies,^[9,15] however, no report on KER in the dissociation exists. The loss of NH_3 leading to the fragment ion at m/z 137 via eq. 14 has been evaluated to have

very high KER of 769 meV (see Figure 5, Table 1). Notably, this has been the only fragment observed upon a low-energy CID in a quadrupole, where precursor $[\text{His-H}]^-$ has been created in an atmospheric pressure chemical ionization source.^[16] The decarboxylation reaction (eq. 15) leads to fragment ion at m/z 110 with KER of 113 meV. The loss of HCOOH via eq. 16 leading to fragment ion at m/z 108 leads to a KER of 249 meV, similarly to the loss of ($\text{H}_2\text{O} + \text{CO}$) in the case of protonated His, which makes it intriguing to suspect that the elimination of HCOOH proceeds in a similar way for deprotonated and protonated His ions. The HCOOH loss is in line with the above mentioned probability of deprotonation of His at the imidazole moiety, however, HCOOH loss has been reported for other deprotonated amino acids as well.^[9] The loss of ($\text{CO}_2 + \text{NH}_3$) leading to fragment ion at m/z 93 via eq. 17 has KER of 168 meV. The loss of the backbone NH_2CHCOOH through $\text{C}_\alpha\text{-C}_\beta$ bond cleavage resulting in fragment ion at m/z 80 (eq. 18) and the charge retained by the side group has KER of 215 meV. On the other hand, when the charge is retained by the backbone, as in the case of formation of ion at m/z 72 (eq. 19), the KER is lot smaller of 64 meV.

[Insert Figure 5 here, please]

Conclusions

Gas-phase fragmentation of protonated and deprotonated His monomer, $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$, and protonated His dimer, $[\text{His}_2+\text{H}]^+$, have been investigated by high-energy CID in a double focusing mass spectrometer equipped with ESI source. The KER of the most abundant dissociation channels has been evaluated.

Observation of loss of H_2O from $[\text{His}+\text{H}]^+$ suggest that (HCOOH) loss proceeds in two steps, however, the similar KER values for the loss of HCOOH from $[\text{His}+\text{H}]^+$ and $[\text{His}-\text{H}]^-$ suggest the formation of the fragments via similar dissociation mechanism. The reaction involved with ring opening and release of HCN molecule from $[\text{His}+\text{H}]^+$ was measured to have high KER of 507 meV.

Deprotonated His $[\text{His}-\text{H}]^-$ dissociates dominantly through the loss of NH_3 that is associated with very high KER of 769 meV. The $\text{C}_\alpha-\text{C}_\beta$ bond cleavage resulting in fragment ion where the charge is retained by the side group has KER of 215 meV. Notably, the $\text{C}_\alpha-\text{C}_\beta$ bond cleavage in the case of positive ions $[\text{His}+\text{H}]^+$ leads to a fragment ion where the charge is retained by the backbone with slightly smaller KER of 119 meV.

Protonated His dimer $[\text{His}_2+\text{H}]^+$ dissociates mainly by the loss of a monomer, however, the loss of COOH and a loss of the backbone of His amino acid suggest that the His dimer interacts through hydrogen bond between the nitrogen atoms of the imidazole ring of the two His. These losses have KER of 205 meV and 170 meV, respectively. It is noteworthy, that electronic excitation of non-covalently bond molecular system can result in the breakage of a covalent bond while the hydrogen bond between the His monomers is still there.

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Table 1:

Summary of kinetic energy release for the observed dissociation channels of histidine and histidine dimer ions.

Parent	[Histidine + H] ⁺	[Histidine ₂ + H] ⁺	[Histidine - H] ⁻
Kinetic energy release [meV]			
Fragment <i>m/z</i>			
C ₁₁ H ₁₈ N ₆ O ₂ ⁺ 266	-	205	-
C ₁₀ H ₁₄ N ₅ O ₂ ⁺ 236	-	170	-
C ₆ H ₁₀ N ₃ O ₂ ⁺ 156	-	48	-
C ₆ H ₈ N ₃ O ⁺ 138	94	-	-
C ₆ H ₇ N ₃ O ⁻ 137	-	-	769
C ₅ H ₈ N ₃ ^{+/-} 110	278	163	113
C ₅ H ₆ N ₃ ⁻ 108	-	-	249
C ₅ H ₇ N ₂ ⁺ 95	246	-	-
C ₅ H ₅ N ₂ ⁻ 93	262	-	168
C ₄ H ₇ N ₂ ⁺ 83	-	172	-
C ₄ H ₆ N ₂ ⁺ 82	226	-	-
C ₄ H ₄ N ₂ ⁻ 80	-	-	215
C ₂ H ₄ NO ₂ ⁺ 74	119	-	-
C ₂ H ₂ NO ₂ ⁻ 72	-	-	64
C ₄ H ₆ N ⁺ 68	507	-	-

Scheme 1

Molecular structures of two tautomeric forms of L-Histidine.

L-Histidine 1

Figure captions

Figure 1: Schematic setup of the ESI-source with differential pumping stages. Spray needle (A), inlet capillary (B), ion funnel (C), octupole ion guide (D), and einzel lens (E) are shown.

Figure 2: Electrospray ionization mass spectrum of solution of 10mM of histidine in MeOH/H₂O/AcAc (50/50/1) sprayed a 4.88 kV: a) full mass scan up to the histidine dimer [His₂+H]⁺, ions labelled by ♣ correspond to fragments of [His+H]⁺ upon vibrational excitation, ions labelled by * correspond to [(MeOH)+H]⁺ at *m/z* 32 and [(MeOH)₂+H]⁺ at *m/z* 65; b) mass scan performed with focus on histidine clusters [His_n+H]⁺, *n* = 1 – 6.

Figure 3: High-energy collision-induced dissociation mass spectrum of [His+H]⁺ ions formed via electrospray ionization and accelerated to 6 keV. The collision cell was filled with helium gas.

Figure 4: High-energy collision-induced dissociation mass spectrum of [His₂+H]⁺ ions formed via electrospray ionization and accelerated to 6 keV (the collision cell was filled with helium gas): a) the most abundant fragments; b) focus on the lower abundant fragments in the *m/z* region between the monomer [His+H]⁺ and dimer [His₂+H]⁺.

Figure 5: High-energy collision-induced dissociation mass spectrum of [His–H][–] ions formed via electrospray ionization and accelerated to 6 keV. The collision cell was filled with helium gas.

Figure 1:

Figure 2:

Figure 3:

Figure 4:

Figure 5: