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Hydrogen/deuterium exchange of electrosprayed ions in the atmospheric interface of a commercial triple–quadrupole mass spectrometer

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Abstract

A novel H/D exchange technique capable of the deuteration of electrosprayed ions has been developed. H/D exchange was carried out by introducing deuterating agent (e.g., d-MeOH) into the curtain gas flow of a commercial triple quadrupole mass spectrometer. In contrast to the widely used H/D exchange techniques, the ions are not trapped in this case. The main advantages of this technique are the ease of use and applicability on most commercial mass spectrometers, including quadrupole-type instruments.

Effect of various instrumental parameters was investigated in detail, including spray voltage, spray position, partial pressure of the deuterating agent in the curtain gas, gas flow rates, the orifice-to-skimmer potential and the source temperature. Among these only the partial pressure of the deuterating agent in curtain gas, orifice-to-skimmer potential and source temperature influenced the efficiency of H/D exchange. These suggest that the H/D exchange is likely to occur in the fore-vacuum region of the atmospheric interface. Analytical capabilities of the technique were demonstrated by differentiation of lysine and glutamine protonated molecular ions. Selective quantitation of lysine and glutamine mixtures was achieved, with a lower limit of detection of 1.5% for glutamine and 0.2% for lysine. H/D exchange of multiply charged macromolecular ions can also be carried out using this technique, which was demonstrated using cytochrome *c*.

Keywords: H/D exchange; Electrospray; Ion–molecule reactions

1. Introduction

H/D exchange between gaseous ions and suitable deuterating agents is a well-established method in mass spectrometry. It has been applied successfully to various types of molecules from singly charged small species to large, multiply charged protein ions to obtain structural information, in particular to distinguish between isomer ions and establish connection between the gas- and liquid-phase ion conformations [1–6].

In general, H/D exchange rate constants are influenced primarily by the type of the exchangeable proton (amine, amide, hydroxyl, carboxyl, guanidyl, etc.), the presence of intramolecular hydrogen bonds (or intermolecular hydrogen bonds in the case of molecular complexes), and various steric factors. The measured

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H/D exchange rates also depend on the type and pressure of the applied deuterating agent. These features provide insight into the role of various non-covalent interactions stabilizing the appropriate 3D structure of gaseous ions.

H/D exchange mechanism of amino acids, primary alkanols, oligopeptides and oligonucleotides has been studied in detail [7–13]. These investigations were focused on the structure and energetics of the deuterating agent–ion complex, including careful investigation of steric effects and thermodynamic parameters. Various mechanisms have been proposed for H/D exchange with ND_3, D_2O and DI. Theoretical expectations are, however, not always in agreement with experimental results [14–17].

The generally accepted mechanism [7,8] for H/D exchange between a protonated substrate and a deuterating agent in the gas phase consists of three steps (Fig. 1): formation of a loose, long-lived ion–molecule complex; exchange within the complex by reversible transfer of a proton between the two components; and dissociation of the complex to yield either the original or the exchanged species. Charge transfer is a possible side reaction, observed in the case of multiply charged ions. Another possible endproduct is the adduct of the sample ion and the deuterating agent.

The precise role of proton affinity difference ($\Delta PA = PA_{substrate} - PA_{reagent}$) between the reagent and the substrate in the exchange process is still unclear. Generally, the barrier to proton transfer within the complex is assumed to depend on the ΔPA value; the lower the ΔPA the higher the reaction rate. If ΔPA exceeds a limit of about $100\,kJ\,mol^{-1}$, no exchange is observed [4]. In most cases this assumption is valid, however, exchange rates of protonated amino acids with ND_3 and CH_3ND_2 showed a reversed behavior [15]. Several studies on this topic proposed that the most important factor on the exchange rate is the structure of the bimolecular complex, thus simple thermodynamic values of the reagent or the substrate cannot determine the activation energy of the process accurately [8].

The situation is analogous in multiply protonated species, but proton affinity differences have to be considered in the transition state (TS). Due to electrostatic repulsion between charges, proton affinity differences in the TS can differ significantly (in the order of 1–1.5 eV) from those determined at infinite distance [18]. This makes it more difficult to estimate the probability of H/D exchange for multiply than for singly protonated compounds.

Probably most H/D exchange reactions occur via a cyclic reaction complex stabilized by two hydrogen bonds, generally referred as the ‘relay mechanism’ [13]. This cyclic coordination could enhance the reactivity in three different ways: (I) the stabilization energy released by formation of hydrogen bonds is available for the system to overcome the barrier of

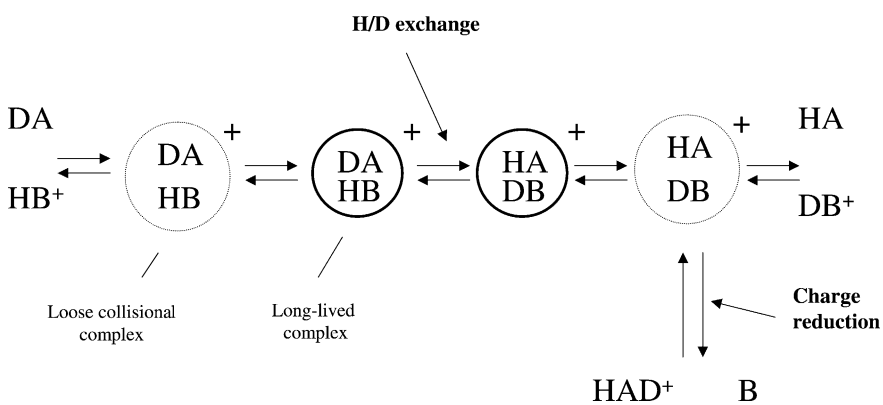

Fig. 1. Reaction scheme of gas-phase H/D exchange and related reactions.

proton transfer; (II) H-bridges can significantly lower the energy of the transition state; (III) the lifetime of the complex is increased.

Gas phase H/D exchange reactions are generally carried out on trapped ions [17–22]. Various types of trapping techniques require different reagent pressures and reaction times. Typical deuterating agent pressures are 1–5 $\times$ 10^{-7} mbar in FT-ICR mass spectrometers and 0.1–10 $\times$ 10^{-5} mbar in Paul traps or in linear octapole traps [10,17]. The timescale of experiments in FT-ICR (either in the ICR cell or in the octapole ion guide) is in the range of seconds to hours, in Paul traps it is in the range of 10 ms–10 s. A major disadvantage of long reaction times is that conformational changes of macromolecules may become significant, as demonstrated by ion-mobility studies [23,24].

In this paper, we demonstrate that high H/D exchange rates can be realized by performing deuteration in the atmospheric pressure interface of the mass spectrometer. Ions generated by atmospheric pressure ionization techniques (electrospray (ES) or APCI) can undergo subsequent H/D exchange immediately following gas-phase ion formation. The deuterating agent can be introduced into an unmodified ES or APCI source by evaporating small amounts of the appropriate reagent into the curtain (sheath) gas.

Table 1
Instrumental settings used for H/D exchange experiments

Instrumental parameter	Generally used	Optimized[a]
Spray voltage	4800 kV	4800 V
Nebulizer gas pressure	2 $\times$ 10^5 Pa	2 $\times$ 10^5 Pa
Auxiliary gas pressure	3 $\times$ 10^5 Pa	3 $\times$ 10^5 Pa
Curtain gas pressure	0.5–5 $\times$ 10^5 Pa	2 $\times$ 10^5 Pa
Source temperature	Ambient–300 °C	Ambient
Axial spray position	0–15 mm	0 mm
Orifice voltage	0–100 V	30 V

[a] For lysine and glutamine determination. Deuterating agent: 10 mL min^{-1} CD_3OD.

2. Experimental

An API 2000 triple–quadrupole mass spectrometer equipped with electrospray and APCI ion sources was used. Only a minor modification was made on the curtain-gas system to allow introduction of deuterating agents. These were introduced in the curtain gas flow just before the gas line was connected to the mass spectrometer (Fig. 2), using the built-in syringe pump on the instrument, a fused silica capillary (Supelco) and a stainless steel T element (Swagelok). Fittings and ferrules were purchased from Upchurch Scientific. Flow rate of deuterating reagents was varied in the range of 0.05–10 μL min^{-1}. Typical instrumental

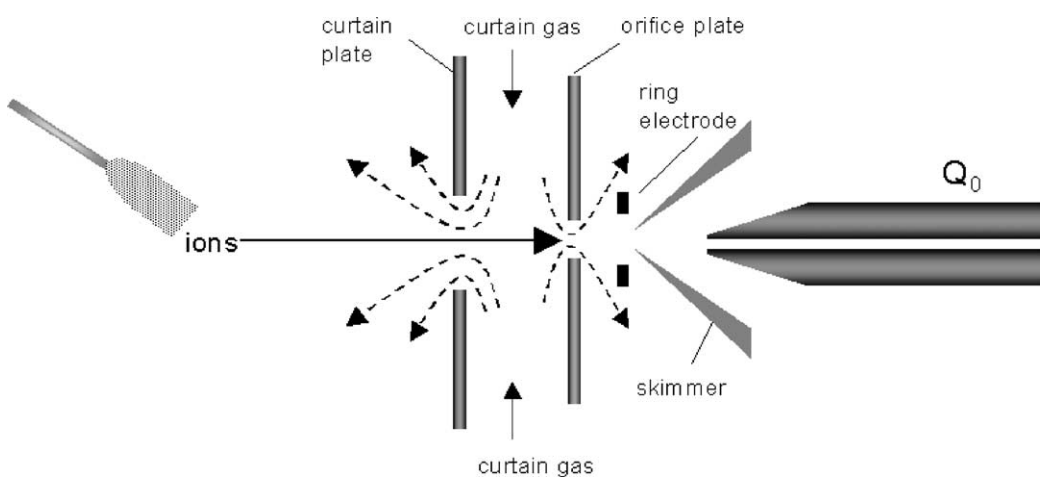

Fig. 2. Scheme of commercial SCIEX atmospheric interface. Deuterating agent (d-methanol) was evaporated into the curtain gas flow, as indicated by the dashed arrows.

settings are summarized in Table 1, it is stated in the text when other parameter values were used.

Amino acids were obtained from Sigma. CD_3OD and D_2O were purchased from Merck. Samples were introduced by flow injection at a flow rate of 20–100 μL min^{-1}. Amino acids and pyridine were dissolved in water or methanol/water 1:1. Cytochrome *c* was dissolved in water, containing acetic acid at a pH of 3.

3. Results and discussion

3.1. General features of the technique

Gas-phase H/D exchange of electrosprayed ions was carried out in the atmospheric interface of a commercial triple–quadrupole mass spectrometer. Dry nitrogen curtain gas is generally used in the atmospheric interface (schematics shown in Fig. 2) to prevent the formation of cluster ions. In its absence eluent/solvent vapors are present in this region, leading to the formation of cluster or adduct ions. If the curtain gas is not inert nitrogen, but some other compound, it may also play the role of a gas phase derivatizing agent. In such cases ion–molecule reactions can occur in the atmospheric interface. If vapors of a labile-deuterium containing compound are added to the curtain gas, various reactions may take place in the atmospheric interface region. These include H/D exchange, charge transfer (charge reduction) and adduct formation processes. The H/D exchange occurring in atmospheric interface is similar to other gas-phase H/D exchange processes described in the literature [7–17]. As examples, the spectra of glutamine (Fig. 3a) and lysine (Fig. 3b) and cytochrome *c* (Fig. 4) are shown using CD_3OD vapors were added to the nitrogen curtain gas.

Effect of various instrumental parameters on H/D exchange was tested, including partial pressure of deuterating agent, spray position, pressure of curtain gas, source temperature and orifice-to-skimmer potential (U_{or}). Among these the extent of deuteration is influenced by the partial pressure of the deuterating agent, the source temperature and the orifice-to-skimmer potential, other parameters have no effect. Fig. 5 shows the H/D exchange efficiency obtained for protonated pyridine as a function of d-methanol flow rate determining its partial pressure (Fig. 5a), source temperature (Fig. 5b) and orifice-to-skimmer potential (Fig. 5c).

The influence of U_{or} on H/D exchange efficiency and the lack of dependence on spray position strongly suggest that the process takes place in the curtain plate—Q_0 region of the mass spectrometer (Fig. 2). H/D exchange in the atmospheric region is unlikely, since the collision number in the atmospheric pressure regime of the mass spectrometer is directly related to the distance an ion has to drift from its place of formation in the atmospheric region, and this was not observed experimentally.

The extent of deuterium incorporation increases with increasing partial pressure of CD_3OD, since the collision number between ions and deuterating agent molecules is determined by the average pressure of the deuterating agent along the ion trajectory. Partial pressure of CD_3OD is determined by the infusion flow rate of CD_3OD into the curtain gas. The increase of H/D exchange with the flow rate is not linear, but approaches a limiting value. This is partly a characteristic of the H/D exchange process, and partly due to the fact that the partial pressure of CD_3OD cannot exceed the equilibrium vapor pressure.

When using low orifice voltage, H/D exchange efficiency undergoes a maximum with increasing source temperature (Fig. 5b). This indicates the effect of at least two factors depending on the temperature of the system. The general mechanism of H/D exchange (shown in Fig. 1) involves the sequential formation and decomposition of a tight, ‘long-lived’ complex, in which the exchange process takes place. At lower temperatures, the rate-determining step is likely to be the dissociation of this complex, which shows positive temperature dependence. At higher temperatures entropy effects become dominant. Formation of adducts is always unfavorable entropically, so at high temperatures formation of the ‘long-lived’ complex becomes the rate determining step, which reverts the sign of the temperature dependence.

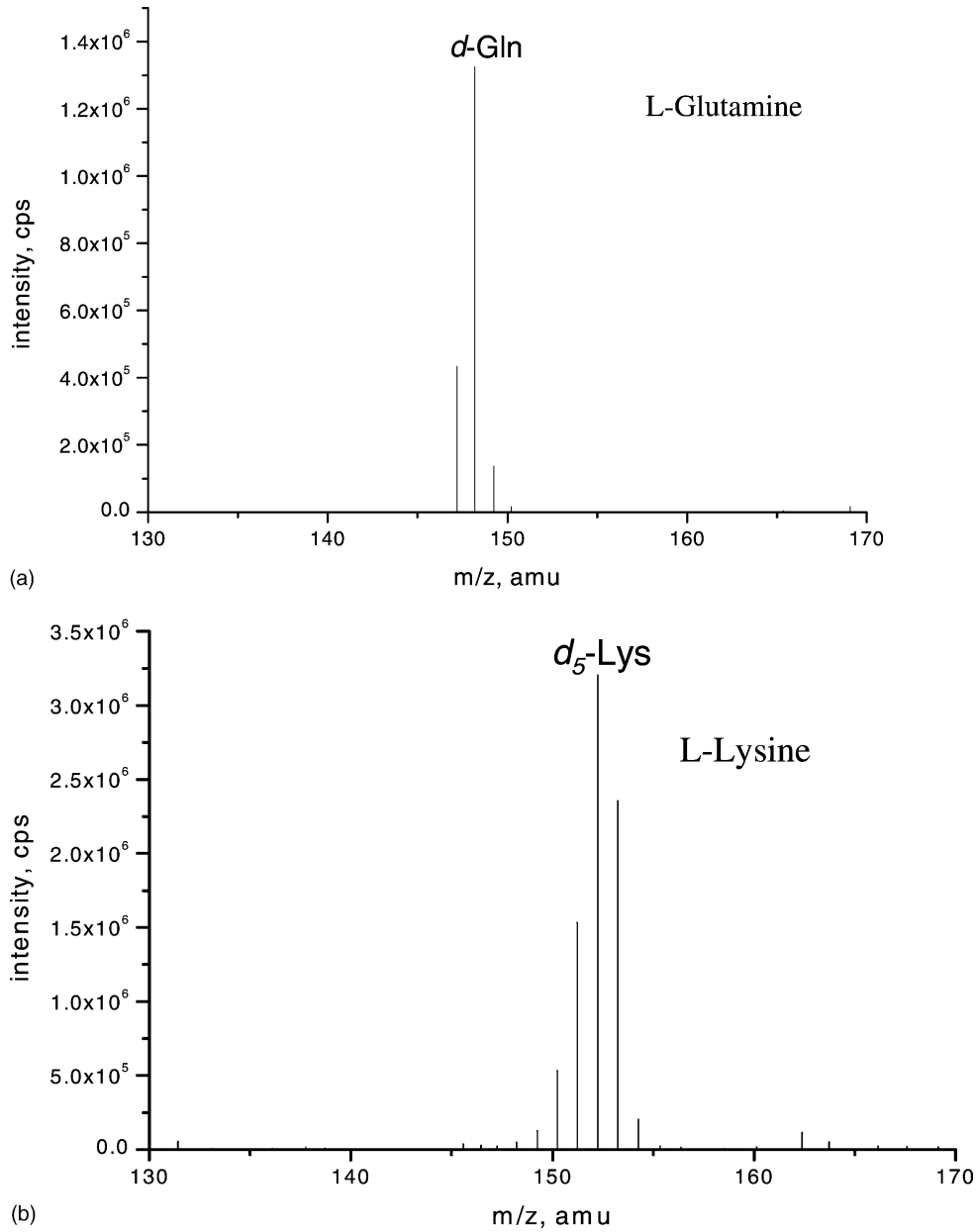

Fig. 3. Electrospray H/D exchange spectra of (a) L-glutamine, (b) L-lysine and (c) 1:1 mixture of L-glutamine and L-lysine. Deuterating agent is CD_3OD, experimental conditions are given in Table 1. Part (c) clearly indicates that the two isobars can easily be distinguished.

U_{or} has similar effect on the internal energy of the collision complex as the temperature, but changes the internal energy in a much wider range. H/D exchange, therefore, decreases with increasing U_{or}—like in the case of temperature, it is likely due to an entropy effect. Note that this observation is consistent with the work of Beauchamp, who observes slower exchange upon collisional heating [16]. U_{or} has also an effect

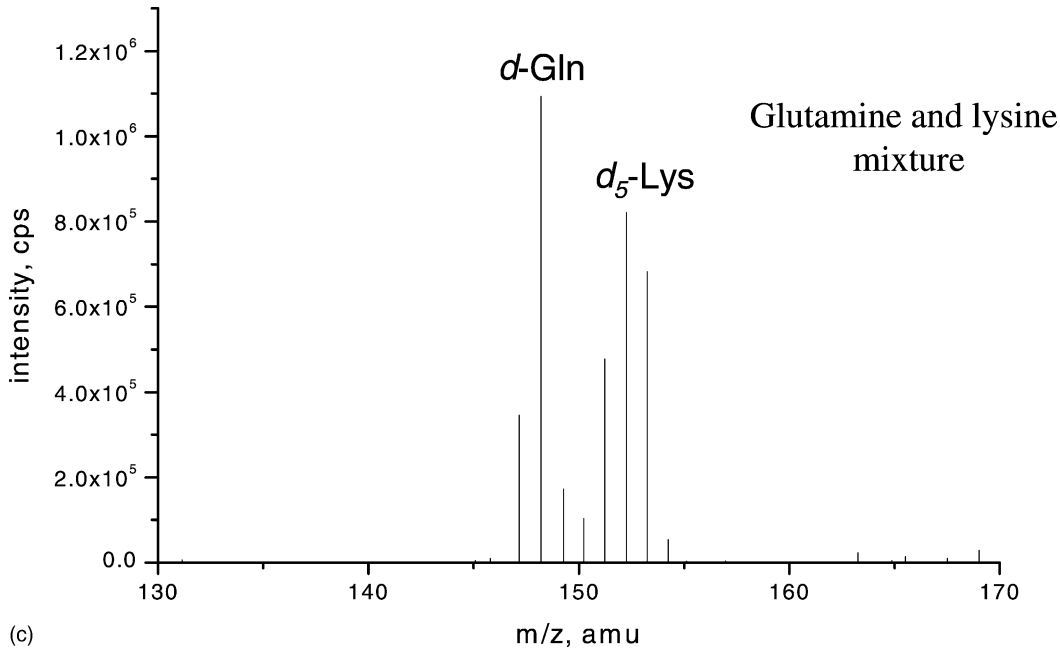

Fig. 3. (*Continued*).

on the timeframe on the H/D process, since at low U_{or} settings ions stay longer in the curtain plate—Q_0 region and may undergo more collisions yielding H/D exchange.

3.2. Applications to small molecules

H/D exchange in the atmospheric interface shows similar features to gas-phase H/D exchange performed in a quadrupole collision cell, both from the point of view of energetics and average collision numbers. To check the performance of our technique H/D exchange for various amino acids have been studied using CD_3OD deuterating agent. The results (shown in Table 2) are in good agreement with Dookeran and Harrison's data obtained in a quadrupole collision cell [15] though the latter were obtained using ND_3 as deuterating agent. According to Wyttenbach and Bowers' so called 'relay-mechanism' for the H/D exchange [13] (Fig. 6), the deuterating agent itself may play only a secondary role. The sole general difference between our and Harrison's data appears in the range of masses of exchanged ions. In our case, the half-width of the isotope distribution patterns is in the range of 1–2 Da, while Harrison's data shows 2–5 Da half-width values. This difference might be due to different ion internal energy distributions in the two cases, since the FAB ionization used by Harrison gives considerably wider internal energy distributions than ES. Alternatively, this could be due to the use of different reagent gases (ND_3 vs. MeOD), different number of collisions or different time-scale as well.

Another example of the analytical capabilities of the technique with regard to relatively small molecules (i.e., not macromolecules) is the distinction of amino acids lysine and glutamine. Distinction of glutamine and lysine is not straightforward using low-resolution mass spectrometry, not even in MS/MS mode, since the nominal mass of the two species is equal and the characteristic CID fragmentation process is, in both cases, the loss of ammonia. To distinguish these compounds, high-resolution mass spectrometry or multiple stage fragmentation (MS^3) is needed.

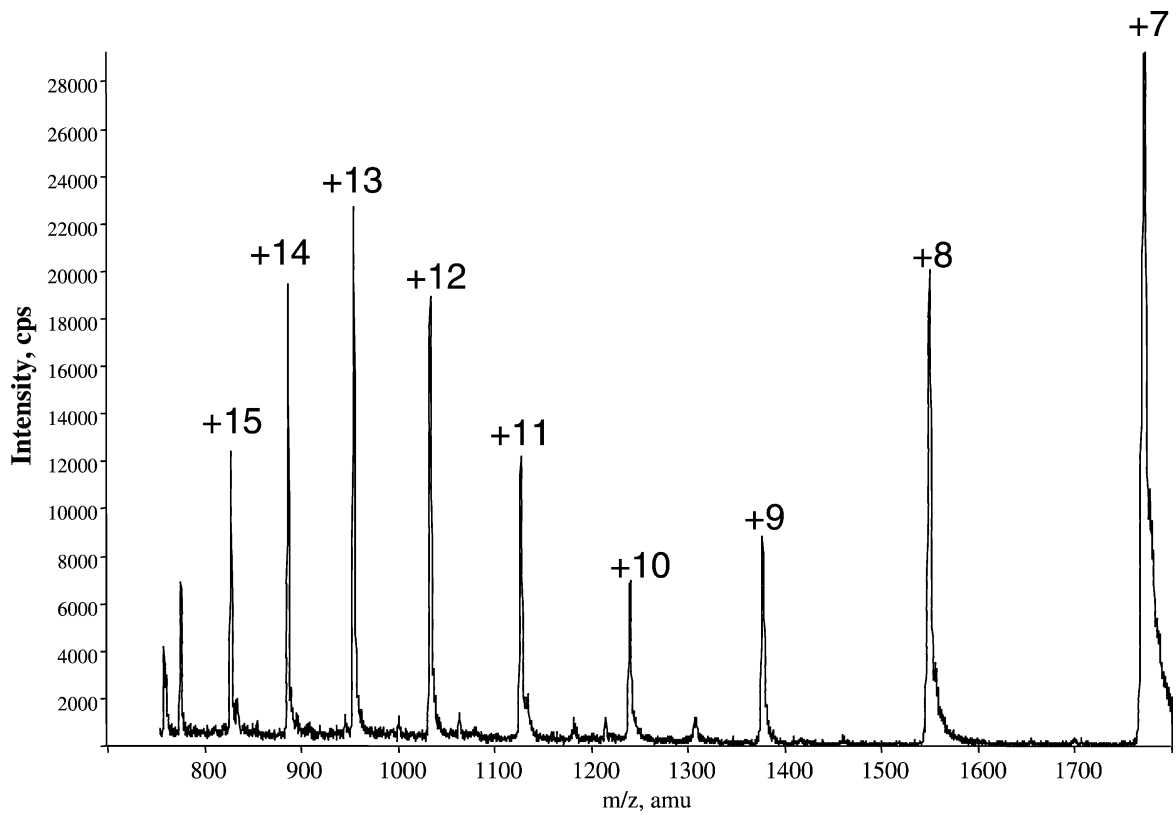

Fig. 4. Electrospray mass spectrum of cytochrome *c* after H/D exchange. Flow rate of CD_3OD was 10 mL min^{-1}.

On the other hand, there is a dramatic difference between the H/D exchange characteristics of the corresponding protonated molecular ions (Fig. 3). Molecules containing two or more heteroatomic groups generally undergo gas phase H/D exchange processes following the well-known 'relay mechanism' [7,13]. According to the mechanism, the deuterating agent forms a bridge between two functional groups of the molecule via two H-bonds and H/D exchange occurs by cyclic proton transfer reaction. Structure of the ion–deuterating agent complex is shown in Fig. 6. In this case the reaction enthalpy is determined by the proton affinity difference (ΔPA) between the two functional groups of the molecule, and the process involves an intramolecular charge transfer. α-Amino acids carrying heteroatomic functional groups on side chain can bind the deuterating molecule between the α-amino or carboxyl and the side chain group. The possible steps of H/D exchange process in the case of lysine and glutamine are shown in Fig. 7.

Since the ΔPA for the two amino groups in lysine is small, charge transfer and H/D exchange are facilitated on both groups. In the case of glutamine, the charge transfer is hindered by the higher ΔPA between the α-amino and the amide group. A further obstacle to H/D exchange in the case of glutamine is that the amide group is likely to be protonated on the oxygen atom, which excludes the amide-NH_2 group from the H/D exchange process. In accordance with characteristics of the 'relay mechanism', a large difference is observed between the H/D exchange kinetics of the two amino acids.

Using the parameters given in Table 1 and optimizing flow rate of CD_3OD (to 10 mL min^{-1}), spectra of

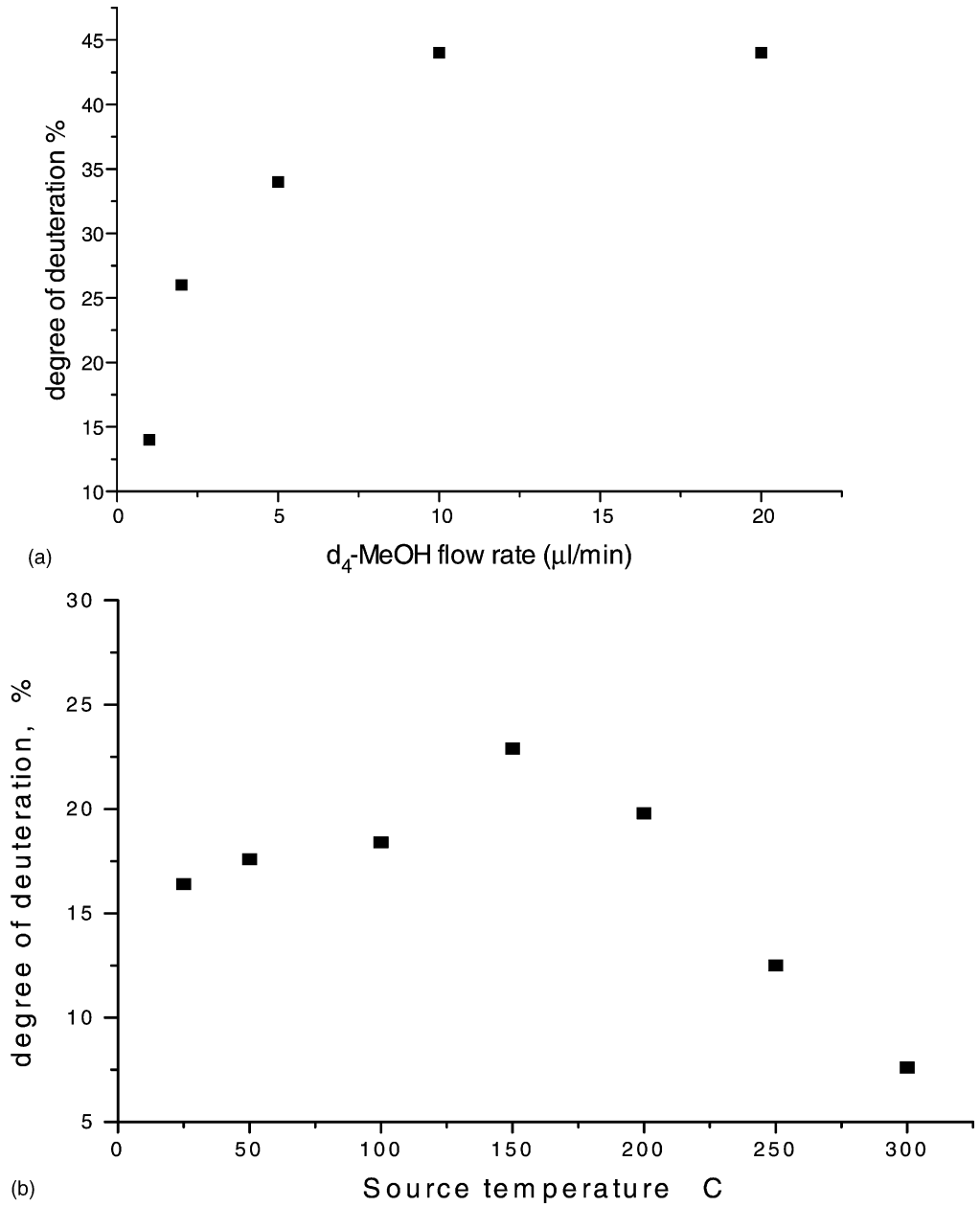

Fig. 5. H/D exchange efficiency as a function of (a) flow rate of CD_3OD, (b) source temperature and (c) orifice-to-skimmer potential.

glutamine and lysine exhibit quite different degree of deuteration, as shown in Fig. 3a and b. The most abundant peak for glutamine is 148 *m*/*z*, while for lysine it is 152 *m*/*z*. The spectrum of a 1:1 mixture of these amino acids (Fig. 3c) indicates very little overlap between the two ion populations. Mixing glutamine and lysine in various ratios the relative ion abundances of *m*/*z* 148 and 152 following deuteration were measured

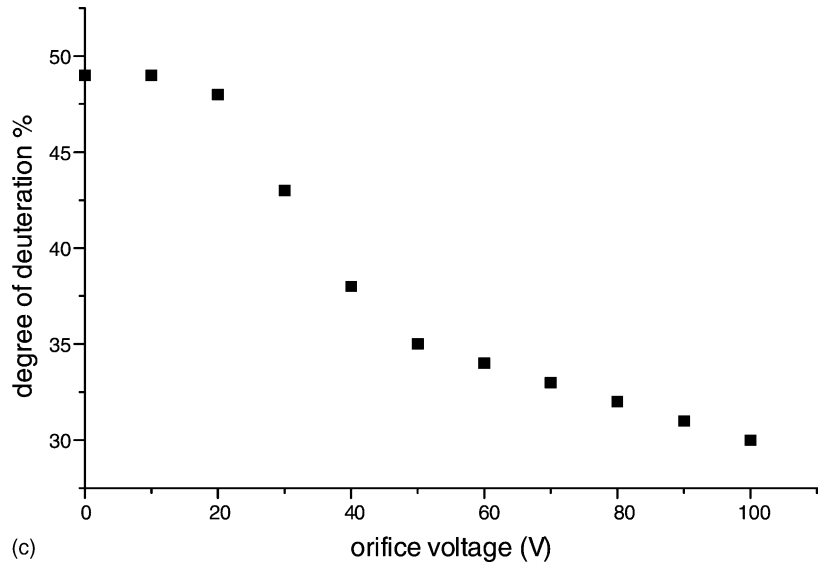

Fig. 5. (*Continued*).

(Fig. 8). These curves show excellent linearity from 0.2 to 100% for lysine and 1.5 to 100% for glutamine. This suggests that deuteration can be used to quantify these two isobars selectively.

3.3. Applications to macromolecules

Cytochrome *c* was chosen as a model compound to test the capabilities of H/D exchange in the atmospheric interface with regard to macromolecular systems. H/D exchange spectrum of cytochrome *c* is shown in Fig. 4, the number of H atoms exchanged to D is shown in Fig. 9 as a function of charge state. Instrumental parameters have similar effect in this case as it was described above, however the spray position also shows a slight influence on the efficiency of deuterium incorporation. This phenomenon indicates that ion formation and subsequent H/D exchange starts to occur in the atmospheric pressure region in the case of large molecules, which are likely formed following the charged residue model [25].

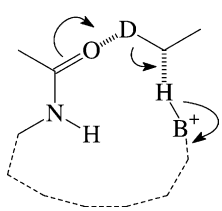

Fig. 6. Structure of the key intermediate in the relay mechanism of H/D exchange.

Electrospray H/D exchange spectra of cytochrome *c* show two multiply-charged mass envelopes, one with center of $[M + 13H]^{13+}$ and the other with center of approximately $[M + 6H]^{6+}$. The H/D exchange pattern of the individual charge states shows only one, relatively narrow, envelope. (Width of the isotope pattern is ca. 5–6 Da wider, than in the case of the undeuterated protein.) Conformers, showing widely different H/D exchange behavior (like that observed in FT-ICR [19,20] or in ion mobility instruments [23,24]), are not observed. H/D exchange in our experiments showed a saturation type curve with increasing amount of deuterating agent, like that shown in Fig. 5a. The degree of H/D exchange increased approximately linearly with the charge state of cytochrome *c*, as shown in Fig. 9. In the experimental setup discussed here the average number of exchanges was 55 for the 17+ ions, 48 for the 8+ ions. This shows that

Table 2
Deuterium incorporation in amino acid protonated molecular ions using 10 μL min^{-1} solvent flow added to the curtain gas

Amino acid	Deuterating agent	d0	d1	d2	d3	d4	d5	d6
Glycine	CH_3OD		6	48	100	92[a]		
	D_2O		1	33	100	85[a]		
Alanine	CH_3OD	4	17	64	100	78[a]		
	D_2O	7	21	58	100	86[a]		
Serine	CH_3OD	6	87	100	98	92	46[a]	
	D_2O	11	92	95	100	94	67[a]	
Asparagine	CH_3OD	26	100	35	8	2		[a]
	D_2O	32	100	26	2			[a]
Glutamine	CH_3OD	22	100	10	3			[a]
	D_2O	42	100	91	18	3		[a]
Phenylalanine	CH_3OD	2	18	42	100	57[a]		
	D_2O	14	64	100	98	67[a]		
Tyrosine	CH_3OD	6	25	66	94	100	19[a]	
	D_2O	15	33	71	100	97	11[a]	
Threonine	CH_3OD	14	68	92	100	96	45[a]	
	D_2O	8	42	76	95	100	61[a]	
Aspartic acid	CH_3OD		13	29	65	100	99[a]	
	D_2O		5	12	53	96	100[a]	
Glutamic acid	CH_3OD		10	18	27	55	100[a]	
	D_2O		2	14	24	34	100[a]	
Arginine	CH_3OD	84	100	12	2			
	D_2O	100	81	20	3			
Lysine	CH_3OD		1	5	13	54	98	100[a]
	D_2O			2	7	62	100	99[a]
Histidine	CH_3OD	64	100	21	14	5	[a]	
	D_2O	51	85	100	26	12	3[a]	
Cysteine	CH_3OD	4	19	74	100	88	61[a]	
	D_2O		13	28	67	100	75[a]	

Curtain gas pressure was set to 1.5×10^5 Pa.
[a] The maximum number of H/D exchange.

cytochrome *c* exchanges an average 38–40 hydrogen atoms plus all additional protons to deuterium in the H/D exchange in the curtain gas region.

This number can be tentatively associated with the 19 lysine residues present in the molecule. Since the molecule is charged most likely on lysine amino groups, the energetically desirable H/D exchange process may involve charge transfer between one charged and one uncharged lysine side chain, and the process follows the 'relay mechanism' [13]. According to solution phase NMR data [26], the lysine residues are readily available for H/D exchange, and each lysine amino group has at least one other in its neighborhood, which facilitates H/D exchange following the 'relay mechanism' mentioned above [13]. Note that these results are in good accord with those of Marshall's group, demonstrating that amine groups exchange more rapidly than carboxylic acid or amide hydrogens [10].

The two multiply-charged mass envelopes may indicate two conformers with very similar H/D exchange characteristics (and therefore likely of similar

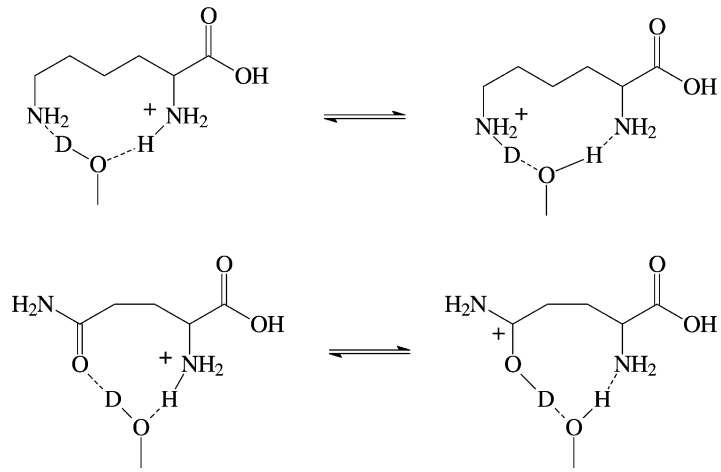

Fig. 7. Proposed H/D exchange mechanism of lysine and glutamine involving the side chain groups.

shape). The single H/D exchange envelope contrasts results obtained in FT-ICR and that in ion mobility [19,20,23,24]. For example, FT-ICR studies [19] indicate multiple ion populations centered at ca. 55, 70 and 110 exchanges. Ion mobility data [23,24] indicate that the evolution of many macromolecule conformations in the gas phase requires a time delay of ca. 10–1000 ms. On the other hand, some may be present in the solution or be formed in less than 1 ms. Previous ES studies indicated that exchange kinetics of the first approx. 100 protons is too fast to follow with ES-MS techniques [3].

A possible explanation of the observed behavior is that the time-frame of H/D exchange in the

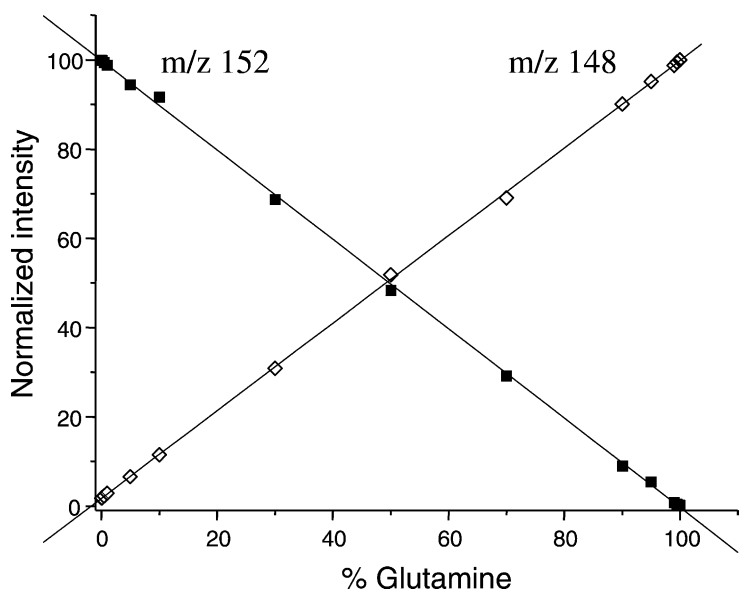

Fig. 8. Calibration curves for the selective determination of lysine and glutamine using intensity of ions at 148 *m*/*z* and 152 *m*/*z*, showing excellent linearity.

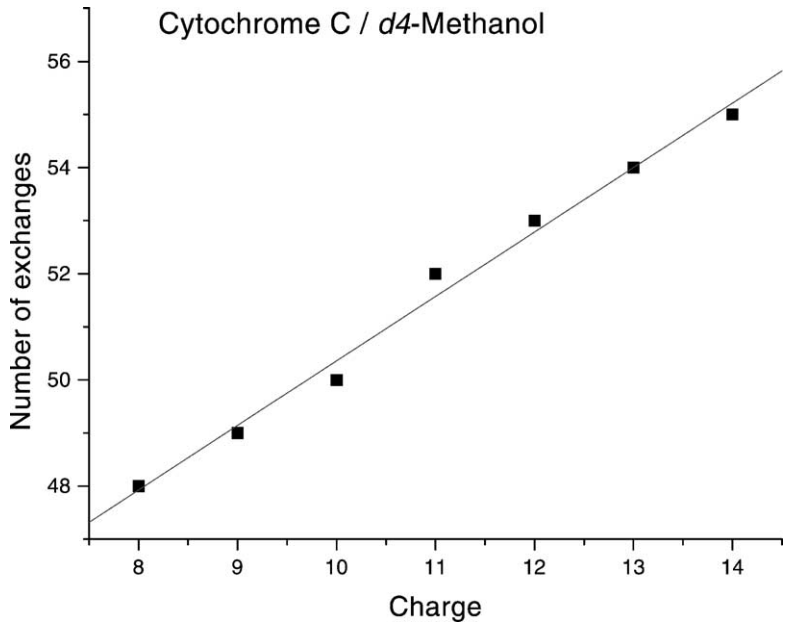

Fig. 9. Average number of H/D exchanges as a function of charge state in the case of cytochrome *c*.

curtain gas region (1 ms or less) is too short for isomerization to take place in the gas phase. In consequence, the H/D exchange is likely to relate to a conformation similar or identical to that in solution. An alternative explanation, suggested by one of the reviewers, is that isomer structures detected by FT-ICR may be present here as well, but the time-frame and pressure in the present instrumental setup is insufficient to lead to an extent of exchange necessary to observe them.

4. Conclusions

H/D exchange performed in the atmospheric interface of a triple quadrupole mass spectrometer does not provide an exhaustive deuteration of protonated molecules in all cases. The degree of deuteration depends strongly on the structure of the sample and on experimental conditions. Mechanistic considerations suggest that the low efficiency of H/D exchange reaction is mainly caused by the relatively high ion internal energy, and the low number of collisions. The latter, together with the fact that instrumental parameters influencing only the atmospheric region do not show any effect on the exchange process, imply that the reaction occurs in the fore-vacuum region of the mass spectrometer. This also implies that gas phase ion formation is also likely to take place in this region. While the method is not suitable to determine the number of mobile protons in a molecular ion, it is capable for distinction of species having similar molecular weight and different structure.

A further field of application of the method can be the structural characterization of multiply charged macromolecular ions, such as proteins, polysaccharides, etc. Conformational changes in these molecules are known to happen in the gas phase. In our technique, in contrast to most gas-phase H/D exchange requiring long trapping times, the timescale is very short, probably less than a millisecond. Based on results on cytochrome *c* we tentatively suggest that conformational changes may not occur in this period, so H/D exchange in the curtain gas region may provide information closer to the solution phase structure than other gas phase H/D exchange methods.

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