Gas-Phase Hydrogen/Deuterium Exchange as a Molecular Probe for the Interaction of Methanol and Protonated Peptides

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The gas-phase hydrogen/deuterium (H/D) exchange kinetics of several protonated amino acids and dipeptides under a background pressure of CH₃OD were determined in an external source Fourier transform mass spectrometer. H/D exchange reactions occur even when the gas-phase basicity of the compound is significantly larger (> 20 kcal/mol) than methanol. In addition, greater deuterium incorporation is observed for compounds that have multiple sites of similar basicities. A mechanism is proposed that involves a structurally specific intermediate with extensive interaction between the protonated compound and methanol. (*J Am Soc Mass Spectrom* 1994, 5, 623–631)

The production of ionic gas-phase macromolecules allows the possibility of studying these complex systems in the absence of solvent. Although the gas and solvated phases are intrinsically different, there is evidence to suggest that conformation may be retained by the molecule even in the gas phase [1–5]. Isolating the molecule makes it easier to investigate without the interference of solvent molecules and at significantly greater sensitivities. However, there is no effective probe for observing structures of complicated gas-phase biomolecules. Destructive probes such as collisionally activated dissociation (CAD) [6], which require vibrational excitation and subsequent fragmentation, have provided information on the structure of smaller ions. Unfortunately, not only is CAD ineffective with large macromolecules, it also provides essentially no information on the conformation of the molecules. In this regard, hydrogen/deuterium (H/D) exchange offers a unique opportunity because it is both a nondestructive and low energy molecular probe [7–17]. Indeed, several groups have shown that conformational effects may be observable by using gas-phase H/D exchange [3–5, 16].

Despite the utility of gas-phase H/D exchange and its potential as a tool for structural elucidation, its microscopic mechanism is not well understood. Elucidating the mechanism is, however, important if H/D exchange is to be a useful tool. In H/D exchange involving protonated species, the role of gas-phase basicity is apparent. H/D exchange has been shown to occur when the gas-phase basicities of the reagent and the conjugate base of the protonated species are similar

[10, 11, 18, 19]. Investigations by Ausloos and Lias [10] have shown that for protonated compounds H/D exchange reactions do not occur when the proton affinity of the neutral base is greater than the deuterated reagent by more than 20 kcal/mol. The correlation between basicity and reactivity has led many people to conclude that H/D exchange in protonated species proceeds by proton transfer reactions within the dimer complex as outlined in Scheme I [7]. The intermediates are commonly thought to reside in a multiple-well potential where proton transfer reactions occur [20]. For example, in the mechanism shown, the two species form an ion–dipole complex in which the proton migrates from the primary base site B to the deuterated reagent A. Reverse transfer of the deuterium produces the H/D exchange.

Recent exceptions to the 20-kcal/mol limit have been shown with peptides. H/D exchange reactions have been observed between protonated renin substrate and ND₃ despite differences in proton affinities of greater than 50 kcal/mol [16]. Similarly, the H/D exchange reactions of several protonated amino acids and CH₃OD occur despite basicity differences of between 30 and 40 kcal/mol [21]. We propose that the limit on the endothermicity of the reaction observed by Ausloos and Lias applies only to organic compounds that contain a single functional group (monofunctional compounds). Protonated species with multiple func-

Scheme I. The commonly proposed mechanism for H/D exchange involving proton transfer reactions between protonated species and deuterated reagents such as CH₃OD, D₂O, and ND₃. The mechanism involves the formation of a dimer (designated by dashed lines) and proton transfer within the complex.

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tional groups (multifunctional compounds) undergo H/D exchange with deuterated reagents even when differences in gas-phase basicities are significantly greater than 20 kcal/mol. We infer this to mean that dimeric interactions that involve monofunctional and multifunctional groups are different and that a unique interaction exists between the protonated multifunctional compounds and deuterated reagents that allows H/D exchange to occur even when the differences in basicity are large. Probing this interaction, particularly for compounds such as amino acids and peptides, is difficult but necessary for determining the mechanism of H/D exchange in complex gas-phase proteins.

In an earlier publication, we reported the H/D exchange behavior of amino acids containing alkyl side chains (**Structure 1**) [21]. We noted that although protonation occurs primarily on the terminal amine, the most rapid exchange occurs on the carboxylic acid. This observation was confirmed by comparing the rate constants of the amino acids and their methyl ester derivatives. Exchange on the amino group is slow for glycine methyl ester and nearly nonexistent (at least under the same reaction conditions) for the esters of amino acids with alkyl side chains such as alanine, valine, leucine, and proline. This situation already illustrates that the most favorable site of protonation does not necessarily become the sole or even primary site of exchange. We further observed that the rate of the first exchange does not vary considerably between the different amino acids in the group. The rate of the second exchange depends strongly on the basicity: increasing basicity results in decreasing exchange rates. Thus glycine, which is 3.2 kcal/mol less basic than alanine, has a rate constant for the second exchange that is three times greater.

In this article, we propose a mechanism for the H/D exchange reactions of CH_3OD and protonated amino acids and peptides that involves a structurally specific long-lived ion-molecule complex responsible for the H/D exchange both on the protonated basic sites and on less basic sites far removed.

Experimental

Experiments were performed in an external ion source Fourier transform mass spectrometer equipped with a quadrupole ion guide that uses commercially available compounds [22]. The experimental procedures used for H/D exchange have been described in an earlier publication [21]. Ions were produced via liquid secondary ion mass spectrometry (LSIMS), isolated via conventional ejection techniques, and allowed to react

with a background of CH_3OD (Aldrich Chemical Co., Milwaukee, WI; 99.5% isotopic purity) maintained by a leak valve between 1 and 5×10^{-7} torr. The actual abundance of CH_3OD was diminished by H/D exchange that occurred on the chamber wall. It would be ideal to "season" the chamber by allowing it to react with D_2O before the experiment and H_2O immediately after. This step would allow the subsequent use of the instrument for other experiments, for example, bracketing measurements, which require that the system be relatively deuterium-free. However, seasoning the instrument with D_2O followed by a bakeout requires a minimum of several hours to be effective and may still not provide totally hydrogen-free conditions, in which case it would still be necessary to determine the H/D ratio of the system. Furthermore, there is a desire to develop this technique for routine use without intensive preparation. Pressure calibration was performed by using parametrized ion gauge response factors [23]. All experiments were performed under ambient temperatures (300 K).

Analysis of the data and determination of the rate constants have also been described earlier [21]. Ion injection, detection, and data collection were carried out under the control of Omega (Ionspec, Irvine, CA) software. The data consist of relative intensities of the mass peaks for $\text{D}_0, \text{D}_1, \dots, \text{D}_n$, where D_n represents a protonated/deuteronated species with n deuterium atoms, measured at a number of time points (typically 10) that correspond to different reaction times evenly spaced over a preset interval. A program written in Mathematica (Wolfram Research, Champaign, IL) first normalizes the intensities for each time point and deletes mass peaks whose summed intensity falls below an arbitrary value (usually 0.03). A series of $n + 1$ coupled differential equations, of the form of eqs (1)–(5) [for a system with four exchangeable hydrogen atoms associated with the exchange reactions (6)–(9)], that incorporate initial estimates of the rate constants, is then solved numerically by using a built-in subroutine to yield $n + 1$ curves that describe the time behavior of the different deuterated species:

$$-\frac{d[\text{D}_0]}{dt} = k_1[\text{D}_0][\text{CH}_3\text{OD}] - k_{-1}[\text{D}_1][\text{CH}_3\text{OH}] \quad (1)$$

$$\begin{aligned} \frac{d[\text{D}_1]}{dt} = & k_1[\text{D}_0][\text{CH}_3\text{OD}] - k_{-1}[\text{D}_1][\text{CH}_3\text{OH}] \\ & - k_2[\text{D}_1][\text{CH}_3\text{OD}] + k_{-2}[\text{D}_2][\text{CH}_3\text{OH}] \end{aligned} \quad (2)$$

$$\begin{aligned} \frac{d[\text{D}_2]}{dt} = & k_2[\text{D}_1][\text{CH}_3\text{OD}] - k_{-2}[\text{D}_2][\text{CH}_3\text{OH}] \\ & - k_3[\text{D}_2][\text{CH}_3\text{OD}] + k_{-3}[\text{D}_3][\text{CH}_3\text{OH}] \end{aligned} \quad (3)$$

$$\begin{aligned} \frac{d[\text{D}_3]}{dt} = & k_3[\text{D}_2][\text{CH}_3\text{OD}] - k_{-3}[\text{D}_3][\text{CH}_3\text{OH}] \\ & - k_4[\text{D}_3][\text{CH}_3\text{OD}] + k_{-4}[\text{D}_4][\text{CH}_3\text{OH}] \end{aligned} \quad (4)$$

$$\frac{d[D_4]}{dt} = k_4[D_3][CH_3OD] - k_{-4}[D_4][CH_3OH] \quad (5)$$

Rate parameters are optimized by doing a least squares fit of the curves to the experimental points using the Marquardt [24] algorithm as described by Bevington and Robinson [25]. The differential equations are resolved at each iteration of the fitting procedure. Although reactions (6)–(9) are written as equilibrium reactions, in reality equilibrium is not obtained. Note that the reverse reaction accounts for the constant pressure of background CH_3OH during the experiment. The treatment, however, is strictly kinetic as would be observed clearly if the CH_3OH pressure in the instrument was zero.

A significant abundance of CH_3OH was present in the background gas. This impurity varied to as much as 30% of the total abundance and its contribution was treated as an additional unknown variable to be optimized during the curve fitting procedure. The d_0 -

methanol is believed to come from H/D exchange reactions that occur on the surface of the vacuum chamber. This hypothesis is confirmed by the decrease of the CH_3OH contribution in experiments performed later in the day. As further evidence, reference gases for bracketing measurements to obtain gas-phase basicity values subsequently introduced into the vacuum chamber also incorporated deuterium [26].

The deviation in the absolute values is approximately 40% due to uncertainties in the pressure determination. Deviations in the relative values, calculated from multiple determinations, were found to be significantly smaller and are listed in Table 1.

Isotope effects were assumed to be negligible in the calculation of the rate constants. This assumption is supported by earlier observations obtained in the reverse reaction that involves deuterated glycine and CH_3OH [21]. Rate constants of the reverse reaction did not vary greatly from the corresponding forward reactions. Similar conclusions concerning isotope effects have been reported for other gas-phase H/D exchange reactions [10, 12, 13].

Results

The time dependence behavior of the deuterated products is shown for the two protonated amino acids, serine and histidine, that react with a background pressure of CH_3OD (Figures 1 and 2, respectively). The two compounds illustrate the contrasting behavior

Table 1. Measured rates for the H/D exchange of selected protonated amino acids and peptides reacting with a background pressure of CH_3OD . For comparison, the ADO [28, 29] rate for glycine with CH_3OD is $1.59 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$. All rates are $k \times 10^{-11} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$. The letters "nrx" mean reactions not observed during reaction period

Compounds	Labile H ^a	k_1	k_2	k_3	k_4	k_5	k_6
Glycine ^b	4	10.6 ± 2.1	3.7 ± 0.7	2.6 ± 0.4	1.2 ± 0.1	—	—
Glycine ester ^b	3	1.9	1.2	0.7	—	—	—
Alanine ^b	4	8.4 ± 1.5	0.8 ± 0.3	nrx	nrx	—	—
Alanine ester ^b	3	1.3	nrx	nrx	—	—	—
Threonine	5	12.7 ± 2.5	0.4 ± 0.2	nrx	nrx	nrx	—
Serine	5	11.7 ± 5.2	0.7 ± 0.2	nrx	nrx	nrx	—
Cysteine	5	18.7 ± 5.6	0.5 ± 0.1	nrx	nrx	nrx	—
Lysine	6	9.5 ± 1.8	8.1 ± 1.8	4.7 ± 1.5	4.7 ± 1.9	2.1 ± 0.5	1.5 ± 0.8
Histidine	5	14.2 ± 3.2	9.2 ± 0.4	3.6 ± 0.1	2.0 ± 1.0	1.3 ± 0.8	—
Histidine ester	4	15.3 ± 5.4	13.0 ± 1.7	3.2 ± 1.0	1.9 ± 1.0	—	—
Histamine	4	9.8 ± 1.9	6.2 ± 0.5	2.3 ± 0.4	1.0 ± 0.6	—	—
Diglycine	5	20.8 ± 1.2	20.4 ± 2.9	10.2 ± 3.5	4.8 ± 3.0	1.5 ± 0.4	—
Dialanine	5	15.4 ± 4.9	13.7 ± 3.0	5.4 ± 1.3	4.1 ± 1.3	0.4 ± 0.2	—
Gly-Ala	5	33.3 ± 5.0	45.8 ± 4.6	13.3 ± 1.6	6.3 ± 0.4	4.2 ± 3.0	—
Ala-Gly	5	31.2 ± 3.2	36.6 ± 5.0	7.5 ± 1.0	4.4 ± 1.0	1.8 ± 0.8	—
Arginine	8	nrx	nrx	nrx	nrx	nrx	nrx
4-Amino benzoic acid	4	nrx	nrx	nrx	nrx	—	—
N-N-Dimethyl-4-aminobenzoic acid	2	nrx	nrx	—	—	—	—

^a Number of hydrogen atoms bound to hetero atoms including the proton.

^b Values obtained from ref. 21.

Figure 1. The time dependence behavior of the relative intensities of ions in the molecular ion region of protonated serine reacting with a background pressure of CH_3OD ($\sim 3 \times 10^{-7}$ torr) up to a reaction time of 11 s. During this time period only the singly and the doubly deuterated products are observed. The curves represent the fitting functions used to determine the values of k .

Figure 2. The time dependence behavior of the relative intensities of ions in the molecular ion region of protonated histidine reacting with a background pressure of CH_3OD ($\sim 3 \times 10^{-7}$ torr) up to a reaction time of 10 s. Deuterated products corresponding to exchange of all labile hydrogen atoms are observed. The curves represent the fitting functions used to determine the values of k .

within the group of amino acids and peptides investigated for this report. Both contain the same number (five including the proton) of exchangeable hydrogen atoms, that is, hydrogen atoms bound to hetero atoms. Protonated serine undergoes rapid exchange of a single hydrogen atom followed by a slow exchange of a second hydrogen atom with no further exchange observed during the 10-s reaction time. By comparison, protonated histidine rapidly exchanges a single hydrogen followed by similarly fast second, third, and fourth exchanges. Exchange of the fifth hydrogen occurs but is not readily observed during this reaction period.

A series of partial mass spectra of the two compounds around the molecular ion region is shown after various reaction times under pressures of about $3 \times$

Figure 3. Partial mass spectra of serine in the molecular ion region under a pressure of 2.0×10^{-7} torr CH_3OD after a reaction time of (a) 0.20 s, (b) 1.29 s, (c) 2.38 s, (d) 3.47 s, and (e) 4.55 s. Ion abundances are scaled relatively.

10^{-7} torr CH_3OD (Figures 3 and 4). Other ions produced during LSIMS ionization were typically ejected to prevent interference. Amino acids with polar weakly basic side chains including serine [$\text{R} = \text{CH}_2\text{OH}$], cysteine [$\text{R} = \text{CH}_2\text{SH}$], and threonine [$\text{R} = \text{CH}(\text{CH}_3)\text{OH}$] have H/D exchange behavior similar to amino acids with alkyl side chains such as alanine, valine, leucine, etc., despite the presence of an additional exchangeable hydrogen. We have shown previously that protonated amino acids with alkyl side chains have the fastest exchange on the carboxylic acid, although protonation occurs primarily on the amine. The behavior of the rate constants for serine, cysteine, and threonine is similar to glycine, alanine, and other amine acids with alkyl side chains. Although no direct evidence has been found yet, we feel that the similarities in kinetics are indications of similar mechanistic behavior, that is, H/D exchange also occurs initially on the carboxylic acid.

Amino acids that contain strongly basic side chains, such as lysine and histidine, constitute a separate group. Protonated lysine exchanges all six labile hydrogen atoms whereas histidine (as already shown) rapidly exchanges four out of five labile hydrogens. These compounds do not necessarily exchange fastest on the carboxylic acid positions as shown by histidine

Figure 4. Partial mass spectra of histidine in the molecular ion region under a pressure of 3.8×10^{-7} torr CH_3OD after a reaction time of (a) 0.30 s, (b) 1.34 s, (c) 2.39 s, (d) 3.96 s, and (e) 5.00 s. Ion intensities are scaled relatively.

and its derivatives, histidine ester and histamine. All three have comparable rates for the first exchange despite the absence of carboxylic acid hydrogens in the latter two compounds.

Rate constants (k_n) associated with the incorporation of the n th deuterium for selected amino acids and dipeptides are tabulated (Table 1). Statistical factors were not included because the contributions of exchange on specific sites to the overall rates are not known. The deviations in the reported values correspond to 1 standard deviation from a set of multiple determinations. For compounds that undergo rapid multiple exchanges, rates of fourth or fifth deuterium incorporation were often slow and could not be calculated with high precision because of the diminishing intensity of the signal with time. Significantly higher pressures, which would allow slow reactions to be better represented, could not be used because they tended to degrade the quality of the spectra. Alternatively, increasing the trapping times at lower pressure did not allow the reactions to be better represented because ion loss also eventually degraded the spectra.

The time plots of serine and histidine (Figure 1 and 2) show the fitting curves from the calculated values of k 's. The fits, though satisfactory, are not exact and appear to vary depending on the degree of deuteria-

tion, the number of deuterated products, and the individual deuterated products. For example, a better fit is obtained with serine, which has only three different mass species, than histidine, which has six. It should be noted that the fit for histidine is possibly the worst, for reasons yet unknown, and this compound was selected only to illustrate the contrasting behavior. The values of k reported for all compounds are averaged from both short and long reaction times. The deviation between the fit and the experimental points may originate from several factors including the relaxation of ions in the cell during the reaction and the correlation of ion motion, particularly between ions with similar masses and frequencies.

The pressure dependence behavior of H/D exchange reactions was determined for protonated glycine between CH_3OD pressures of 1 and 5×10^{-7} torr. This pressure range represents the practical experimental range with this instrument. At lower pressures, the reactions proceeded too slowly to be observed and at higher pressures, collisions in the cell increase the magnetron radius of the ion cloud, which eventually produces ion loss [27]. Within this pressure range, the k values varied randomly by less than 20%, indicating the independence of the exchange to the pressure of CH_3OD .

Within the group of the amino acids, H/D exchange reactions of the first hydrogen (k_1) occur with rate constants between 10 and $20 \times 10^{-11} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$. When the values are compared to the theoretical ADO (average dipole orientation) [28, 29] rate of $1.6 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$, they correspond to reaction efficiencies of 10–20%. The dipeptides have consistently higher k_1 values compared to the amino acids, a likely consequence of higher collision rates and collision cross sections due to the larger size of the ion. These factors are not considered in the calculation of ADO rates.

The major differences in H/D exchange behavior between the amino acids and dipeptides are observed in the higher order exchanges, that is, k_n where $n > 1$. For compounds observed to undergo rapid multiple H/D exchange, the values of k_2 , for example, are equivalent to k_1 . With protonated compounds that are not observed to undergo rapid H/D exchange, k_2 values are small and typically less than $0.05k_1$. In this regard, k_2/k_1 is a general and simple measure of multiple H/D exchange. For example, the k_2/k_1 ratio for histidine is approximately 0.6, whereas for serine the corresponding value is 0.04.

The reactions of glycine and alanine methyl esters have been presented earlier and are included for direct comparisons [21]. The similarities of the k_2 values of the acids to the k_1 of the esters and the statistical nature of k_2 , k_3 , and k_4 of glycine provide strong evidence that the first exchange in these amino acids occurs with the carboxylic acid hydrogen. The results also indicate that exchange on basic and acidic positions proceeds independently.

The reaction behavior of the dipeptides, lysine, and histidine, which all undergo facile multiple exchange, suggests similarities in the interactions of these compounds with the methanol reagent. The dipeptides glycylalanine and alanylglycine have k_2 values greater than k_1 . The significance of this is not known, but the results are consistent with rapid multiple deuterium exchange. The contrast between alanine and the alanine-containing peptides is unexpected but provides insight. Protonated alanine incorporates, at most, two deuterium atoms, whereas protonated dialanine incorporates five deuterium atoms—the total number of labile hydrogen atoms—during the same reaction period. This trend is readily evident in the tabulated rate constants. It further appears that the position of the alanine in the dipeptide is unimportant. Both alanylglycine and glycylalanine compounds undergo rapid multiple exchange.

The lack of correlation between the extent of H/D exchange and molecular gas-phase basicity is in apparent contradiction to the results of Ausloos and Lias. For example, histidine and lysine are more basic than methanol by 50 and 48 kcal/mol [18], respectively, but they also exchange a greater fraction of their labile hydrogens compared to serine (34.9 kcal/mol). Similarly, alanine, which is 32.5 kcal/mol [24, 25] more basic than methanol, exchanges a smaller fraction of its labile hydrogen compared to dialanine, which is 38.2 kcal/mol more basic than methanol [26, 30]. Gas-phase basicity again does not appropriately explain the trends observed with multifunctional compounds. The exception is perhaps arginine, estimated to be over 60 kcal/mol more basic than methanol [29], which does not undergo significant H/D exchange [31]. The structural similarities between arginine and lysine suggest that structural factors may not be the primary reason for the lack of reactivity, but may instead represent an ultimate limit for the differences in gas-phase basicity between the parent compound and the deuterated reagent for H/D exchange to occur.

To obtain further information on the importance of individual functional groups in H/D exchange reactions, several model compounds were investigated. From the failure of 4-aminobenzoic acid and its dimethyl derivative *N,N*-dimethyl-4-aminobenzoic acid to undergo H/D exchange [Reaction (10)], it was concluded that neither an isolated protonated amine nor an isolated carboxylic acid group readily undergoes H/D exchange:

These results also preclude a simple four-center mechanism for the exchange. Close contact between the amine and the carboxylic acid is apparently necessary for H/D exchange to occur. Ranasinghe [23] have shown that protonated 4-aminobenzoic acid formed under chemical ionization conditions undergoes sig-

nificant H/D exchange. However, reaction conditions in the triple quadrupole instrument used by Ranasinghe et al. differ from those in Fourier transform mass spectrometry (FTMS). The ions in that study were produced by both electron impact and chemical ionization methods, which are believed to produce ions more energetically excited than fast-atom bombardment produced ions. In addition, the pressures of CH_3OD used during the reaction (3 mtorr) are nearly 4 orders of magnitude larger than in this investigation, producing a significantly greater number of two-body collisions and even allowing three-body collisions. Mixed protonated dimers and in some cases trimers are observed in the spectra. In contrast, dimers are not observed in the FTMS spectra. This means that conditions in the FTMS favored interactions of the protonated species with a single methanol molecule, whereas simultaneous interactions with several methanol molecules were possible in the high pressures used in the triple quadrupole instrument.

Other model compounds with features similar to amino acids were investigated to determine the importance of the amine-carboxylic acid combination. The compound 2-piperidinemethanol is analogous to proline [Reaction (11)] without the carboxylic acid:

This compound [Reaction (12)] does not undergo exchange suggesting the importance of the amine-carboxylic acid combination:

These results are further supported by the H/D exchange behavior of serine (and to some extent cysteine and threonine), which has similar 1-amino, 2-hydroxyl functionalities and does not appear to undergo H/D exchange on the hydroxyl side chain.

Discussion

The rapid exchange of the carboxylic acid hydrogen suggests a single reaction site within the protonated molecule. Initial exchange of the carboxylic acid hydrogen followed by rapid intramolecular H/D exchange between the amine and carboxylic acid sites is a possible mechanism. This mechanism, however, requires the carboxylic acid always to be present for the reaction to occur, inconsistent with the reactivity of the ester analogs.

Alternatively, proton migration may occur from the N-terminus to the carboxylic acid where it undergoes

exchange. Exchange rates, in this situation, would depend on the proton migration aptitude and, in turn, on the gas-phase basicity difference between the amine and carboxylic acid group. For glycine, theory predicts a reaction that is about 12 kcal/mol endothermic [26]. Amino acids that contain alkyl side chains are expected to increase this difference because the amine groups are more susceptible to the inductive effects of the alkyl groups than the acid [18]. Consequently, basicity should play a stronger role by allowing less basic compounds to be more reactive than more basic compounds—a trend not supported by experimental results. Proton migration could be extremely rapid, particularly with vibrationally hot ions, and significantly faster than the collision rate. This scenario could be consistent with the observed independence of the rates on pressure over the narrow range investigated, which would mean, however, that all labile hydrogens are equivalent, making the values of the rate constants statistical.

Although exchange can occur away from the site of protonation, results still point to the importance of the relative proximity of the functional groups. An isolated carboxylic acid and a protonated amine do not undergo H/D exchange with CH_3OD as illustrated by 4-aminobenzoic acid, its dimethyl derivative, and 2-piperidenemethanol. The least basic of these compounds (4-aminobenzoic acid) is estimated to be 26 kcal/mol more basic than methanol and would not be expected to react according to the results of Lias and Ausloos. Many of the weakly basic amino acids fall in the same basicity range as the benzoic acids but are significantly more reactive. Similarly, there appears to be no H/D exchange reactivity for an unprotonated carboxylic acid group under these reaction conditions, even when it is nearly 10 kcal/mol more acidic than glycine, for example, 4-aminobenzoic acid. Steric interference also does not explain the low reactivity of compounds with alkyl side chains such as alanine, valine, isoleucine, etc. because the dipeptides alanyl-alanine, alanylglycine, and glycylalanine all undergo extensive H/D exchange in their protonated form.

The importance of the spatial relationship between the carboxylic acid and amine functional groups has already been shown by Ranasinghe et al. [32] in the reaction of polyfunctional compounds with CH_3OD and ND_3 . The authors observe greater multiple deuteration when the amine and carboxylic acid groups are ortho rather than para. When the two groups are isolated, they are subject to the same constraints found in the H/D exchange of compounds that contain a single functional group. This suggests that H/D exchange in compounds such as amino acids and peptides must involve multiple interactions between the deuterated reagent and the protonated peptide.

An intermediate involving extensive hydrogen bonding and dipolar interactions between the protonated amino acid and CH_3OD is proposed for glycine (Intermediate I). This intermediate takes advantage of the true amphoteric nature of methanol, that is, acting

both as a proton acceptor and a proton donor. A similar bicoordinated intermediate is predicted by ab initio calculations to occur in complexes of NH_4^+ and 1,2-diols [33]. With the amino acids, protonation occurs primarily on the terminal amine, which is predicted to be 12 kcal/mol more basic than the carbonyl position for glycine [25]. Hydrogen bonding interactions can occur between the protonated amine and the oxygen atom of the methanol ($\text{N}-\text{H}\cdots\text{O}$) and between the deuterium and the carbonyl group of the carboxylic acid ($\text{C}=\text{O}\cdots\text{D}$). From Intermediate I, H/D exchange can occur on the carboxylic acid and the terminal amine with independent rates. Replacing the carboxylic acid with a methyl ester does not significantly affect the rates of exchange on the amine. This intermediate further allows exchange to occur on the carboxylic acid even though the site of protonation is the terminal amine. A reasonable mechanism for exchange on the carboxylic acid involves breakage of the $\text{CH}_3\text{O}-\text{D}$ bond in Intermediate I to produce the ring open Intermediate II (Scheme II). In this configuration, the CH_3O group can rock back and forth while attached to the protonated amine. Rotation along the bond α to the carbonyl allows exchange of the carboxylic acid hydrogen to occur (Scheme II, pathway a). This mechanism explains the similarity in k_1 for all the amino acids, particularly those containing a single dominant base site.

Multiple H/D exchanges on the terminal amine produce multiply deuterated products. We have already shown for amino acids with $\text{R} = \text{H}$ and alkyl that the rate of H/D exchange on the terminal amine decreases with increasing amino acid basicity [21]. For example, the k_2 value of protonated glycine is nearly five times greater than that of protonated alanine. This behavior is possible given Intermediate I and the relative strength of the $\text{N}-\text{H}$ bonds and $\text{C}=\text{O}\cdots\text{D}$

Scheme II. The proposed mechanism for the H/D exchange reaction of CH_3OD and protonated glycine.

interactions. The weaker N—H bond of a less basic compound allows movement of the proton from the amine to the methanol. The methanol then loses the deuterium which result in exchange on the terminal amine.

A strong C=O...D interaction can similarly weaken the N—H interaction through charge induction, facilitating the breakage of the CH₃O—D bond. Loss of CH₃OH and migration of the deuterium on the carbonyl group to the more basic amine completes the process (pathway b). Indeed, this is the likely scenario present in the H/D exchange of dipeptides and explains the difference in exchange behavior between alanine and the dipeptides dialanine and alanyl-glycine. The carboxylic group is converted to a more basic amide, thereby increasing the CO...D interaction. Semiempirical calculations predict that the basicity of the carbonyl amide is only about 4 kcal/mol less than that of the terminal amine [26].

Similar situations arise when at least two equally strong base sites are present on the molecule, producing a cyclic intermediate as shown with histidine (Intermediate IV). The strong N...D interaction weakens the CH₃O—D bond, allowing the CH₃O—H bond to form. This situation likely exists in the H/D exchange reactions of protonated lysine, histidine, and histidine derivatives and accounts for the reactivity of the histidine derivatives lacking a carboxylic acid group. Additionally, protons can migrate between base sites in these compounds, producing, for example, Intermediate V, which can also undergo exchange.

The relationship between multiple H/D exchange and the presence of base sites with similar proton

Table 2. Differences in proton affinities between the two most basic sites estimated by the use of model compounds with known values. Values for proton affinities are obtained from ref. 18

Compound <i>Proton affinities of model compounds</i>	Differences in proton affinity between the two most basic sites in the molecule
Serine <i>Serine 216.8</i> <i>CH₃CH₂CO₂H 191.8</i>	25.0 kcal/mol
Cysteine <i>Cysteine 214.3</i> <i>CH₃CH₂CO₂H 191.8</i>	22.5 kcal/mol
Alanine <i>Alanine 214.8</i> <i>CH₃CH₂CO₂H 191.8</i>	23.0 kcal/mol
Glycine <i>Glycine 211.6</i> <i>CH₃CH₂CO₂H 191.8</i>	19.8 kcal/mol
Histidine <i>Alanine 214.8</i> <i>n-Imidazole 219.8</i>	5.0 kcal/mol
Dialanine <i>Alanine 214.8</i> <i>n-(CH₃)₂NCHO 211.4</i>	3.4 kcal/mol
Lysine <i>Valine 217.0</i> <i>n-Propylamine 217.9</i>	0.9 kcal/mol

affinities can be illustrated when the proton affinities of the actual sites are known. This is possible only by performing time-consuming and expensive high level molecular-orbital calculations. Alternatively, proton affinities of various base sites can be estimated by using model compounds with known proton affinities and having functional groups similar to those found on the amino acid or peptide. These values are listed in Table 2 along with the model compounds (denoted by italic type) used to represent the peptides. For example, serine has a known proton affinity associated with protonation on the amine. The two base sites are the amine and the carbonyl group of the acid. The two model compounds selected were serine and ethanoic acid, which correspond to a proton affinity difference of 25.0 kcal/mol. Lysine is composed of two amine base sites, one of which is similar to an amino acid containing a large alkyl side chain (valine) and the other is similar to an alkyl amine (*n*-propylamine), giving a difference of only 0.9 kcal/mol. This treatment cannot accommodate intramolecular interactions that increase the basicity of individual sites. Intramolecular interaction would decrease the differences in basicity, because weakly basic sites become significantly more basic through interaction with strongly basic sites.

A plot of the ratio k_2/k_1 , a measure of multiple exchange, versus proton affinity difference in the model

Figure 5. Plot of k_2/k_1 versus the differences in proton affinities (estimated by using model compounds) of the two most basic sites in the molecules.

compounds is shown in Figure 5. It is readily apparent from Figure 5 that the smaller the basicity difference, the greater the degree of multiple exchange. Lysine, diglycine, and dialanine, which have two base sites with nearly equal basicities, have significantly higher k_2/k_1 ratios than serine, cysteine, and alanine, which all have a single dominant base site. Glycine and histidine provide intermediate points in which one base site is slightly more basic than the other. The H/D exchange behavior of the two compounds dialanine and lysine shows a large variation in through-bond distances over which one base site can facilitate H/D exchange on another base site. In dialanine, the two base sites are separated by two carbon atoms, whereas in lysine they are separated by five carbon atoms. Histidine is an intermediate case with three carbon atoms between the two base sites.

Strong intramolecular interactions such as those found in protonated lysine and histidine translate to strong intermolecular interactions in the methanol-protonated peptide complexes. Presumably, the reverse is also true so that strong intermolecular interactions in the methanol-protonated peptide dimer imply the capability for strong intramolecular interactions. In this regard, H/D exchange can be a useful probe for strong intramolecular interactions because sites that undergo rapid H/D exchange are also sites capable of forming strong intramolecular interactions. This situation provides a unique method for determining which base sites within the peptide are able to "communicate" via strong intramolecular interactions.

Finally, the results presented provide evidence for unique and highly defined complexes in the interaction of methanol and protonated amino acids and peptides. Complexes such as these could be extremely important in other gas-phase ion-molecule reactions, and H/D exchange provides a viable probe of their structures. The general presence of these complexes in similar reactions will be the subject of future investigations.

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