ION-MOLECULE REACTIONS AS PROBES OF GAS-PHASE STRUCTURES OF PEPTIDES AND PROTEINS*

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A review with over 100 references describes the recent applications of ion-molecule reactions to the study of gas-phase protonated peptides and proteins. The topic is focused specifically on the proton transfer and hydrogen-deuterium exchange reactions of amino acids, peptides, and proteins. A brief background is given of the various methods used for assigning proton affinities and gas-phase basicities. The methods used for measuring the kinetics of deuterium incorporation of charged ion in the presence of a background pressure of deuterating reagents are also described. Ion-molecule reactions are used to determine, among other things, the gas-phase basicities and proton affinities of amino acids, peptides, and proteins, the sites of protonation, intra- and intermolecular interactions, and conformational differences and changes in gas-phase ionic species. Singly charged and multiply charged ions are both covered. © 1997 John Wiley & Sons, Inc., Mass Spectrom Rev 16(2), 53–71, 1997

I. INTRODUCTION

Ion-molecule reactions have long been applied to organic compounds to obtain detailed information on ionic structures. Unlike collision-induced dissociation or collisionally activated dissociation, which probe the primary structure during collisional activation, ion-molecule reactions are much gentler probes that provide information on the ions' secondary structure, often under the conditions of thermal equilibrium. Furthermore, ion-molecule reactions can provide key information to help identify the intrinsic factors that determine gas-phase ion structures.

Gas-phase ion structure as a subject covers a broad area, and the subject has been of considerable interest for several decades. A recent perspective on ion-molecule equilibria has been published by Kebarle (Kebarle, 1992). The study of gas-phase biomolecular polymers is more recent, but its roots extend almost 20 years to the first gas-phase studies of amino acids by the groups of McIver (Bartmess et al., 1979; Locke et al., 1979, 1983) and Moet–Ner (Meot–Ner et al., 1979). The earliest applications of ion-molecule reactions with amino acids showed that neutral amino acids in the gas-phase do not adopt the zwitterionic structure often found in solution (Locke et

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al., 1979; Locke et al., 1983). At the time, it was not possible to produce large thermally labile species; instead, small organic compounds were used to model peptides and proteins [Meot–Ner (Mautner), 1988; Meot–Ner (Mautner), 1988]. The recent studies involving proteins represent a major step, made possible by recent innovations in ionization and mass analysis. The development of ionization sources such as fast atom bombardment (FAB) and the similar technique liquid secondary ion mass spectrometry (LSIMS) (Barber et al., 1981; Barber et al., 1982), electrospray ionization (ESI) (Yamashita et al., 1984), and matrix-assisted laser desorption-ionization (MALDI) (Karas et al., 1988) have made the study of relatively large macromolecules now common. Of these ionization methods, LSIMS and ESI have been more widely used, whereas MALDI and laser desorption (LD) have been used in a relatively small number of studies. The development of the external source in Fourier transform mass spectrometry (FTMS) and the quadrupole ion trap have similarly contributed to the study of large macromolecules. Both methods are capable of trapping ions over a long period of time. This capability has always been an important feature that allows the study of ion-molecule reactions with slow kinetics.

In this review, we discuss the use of ion-molecule reactions, specifically proton transfer reactions and hydrogen/deuterium exchange, in the study of gas-phase ion structures of peptides and proteins. By focusing its scope, we unfortunately leave out several on-going studies of ion structures such as alkali metal attachment to peptides and proteins and proton transfer reactions involving other biopolymers such as nucleotides and oligosaccharides. However, the broadness and richness of the field makes it difficult for a single review to adequately discuss all the important contributions. Even in this focused area, we still may have left out some important contributions.

II. METHODS

A. Proton Transfer Reactions

The most important thermodynamic parameters of a gas-phase proton transfer reaction are the free energy and the enthalpy of the reaction:

$$B + H^+ \rightarrow BH^+. \quad (1)$$

By convention, the negative of the free energy ($-\Delta G$) of the reaction is called the gas-phase basicity (GB), and the negative of the enthalpy ($-\Delta H$) is the proton affinity (PA). Because the reaction is always exergonic and exothermic, GB and PA are both always positive numbers. For small organic bases, it has been customary to provide the PA, because this parameter is less dependent on temperature. For large biomolecules, the choice of PA vs. GB is a difficult one. For compounds containing a large number of base sites and extensive intramolecular interactions, obtaining the PA often requires large approximations regarding intramolecular interactions. In this situation, it is advisable to provide GB rather than PA values. The most direct method for obtaining GB and PA is to perform the proton transfer experiment between the protonated unknown base B and a base R of known GB. By using several reference bases, the GB of the compound can be determined.

$$BH^+ + R \rightleftharpoons RH^+ + B. \quad (2)$$

This ''equilibrium'' method was extensively used to assign GB and PA values for numerous small organic compounds (Aue et al., 1979; Bartmess et al., 1979). The reaction is performed in the presence of gaseous R and B. The equilibrium constant is determined from the expression

$$K = \frac{[RH^+][B]}{[BH^+][R]},$$

where $[RH^+]$ and $[BH^+]$ are the relative intensities of the protonated reference and base, respectively, and [R] and [B] are the respective partial pressures. The GB is determined from the expression

$$\Delta G^\circ = -RT \ln K.$$

The problem with the equilibrium method for peptides and proteins is that no appreciable pressure of the neutral compound can be obtained in the mass spectrometer. To deal with this problem, two approaches are used. A simple approach is to produce the protonated species of unknown basicity and to react it with a background pressure of a reference base. This method is commonly called *bracketing:*

$$BH^+ + R \rightarrow RH^+ + B. \quad (3)$$

Simply, the bracketing method involves monitoring the disappearance of BH^+ as a function of the appearance of RH^+. If the intensity of RH^+ grows to an appreciable extent during the reaction time, then GB(R) $>$ GB(B). Measuring the kinetics and comparing efficiencies provides a more accurate method for determining where endergonic and exergonic transitions occur in a series of reference bases (Bohme et al., 1980; Büker et al., 1991). Problems arise when the basic site in either R or B is hindered. A hindered base can lower the efficiency of the reaction and cause a higher GB assignment (Meot–Ner (Mautner)

et al., 1991; Sunner et al., 1989; Wu et al., 1995). A variation of this approach is performed by Amster, where the compound of unknown basicity is formed as neutrals by laser desorption and reacted with the protonated reference base (Bliznyuk et al., 1993; Gorman et al., 1993).

As a side note, the analytical application of proton transfer reactions has been illustrated by Fenselau and coworkers in a process named neutralization-chemical reionization mass spectrometry. In this method, a basic gas such as ammonia is introduced into a collision cell of a tandem instrument to be used as both neutralization and reionization media. The proton transfer from the substrate to the gas is an endothermic reaction that is driven by the kinetic energy of the more basic ion. Fragmentation resulting from the subsequent reverse proton transfer reaction provides structural information on the initially formed ion (Orlando et al., 1991; Orlando et al., 1990a,b).

An alternative method was introduced by Cooks and Kruger and relies on the formation of the mixed dimer ($B\cdots H\cdots R^+$) (Cooks et al., 1977). The reaction monitored is the disproportionation reaction shown. The method is commonly referred to as the ''kinetic'' method:

$$BHR^+ \begin{array}{l} \xrightarrow{k_1} RH^+ + B \\ \xrightarrow{k_2} R + BH^+. \end{array} \qquad (4)$$

The kinetic method employs the following equation to obtain proton affinities:

$$\ln \frac{k_1}{k_2} = \ln \frac{Q_1^*}{Q_2^*} + \frac{\Delta PA}{RT},$$

where k_1 and k_2 are rate constants for the competing reactions whose ratio is obtained from the relative ion abundance. Q_1^* and Q_2^* are partition functions for the activated complexes. The method works best if the reference (R) and the base (B) are both chemically and structurally similar (Suzuki et al., 1996) and, with the assumption that reverse barriers are close to zero so that

$$\ln \frac{Q_1^*}{Q_2^*} \cong 0,$$

the above equation becomes

$$\ln \frac{k_1}{k_2} \cong \frac{\Delta PA}{RT}.$$

Thus, from the relative intensity, the relative proton affinity is obtained. In this method, the equation is applied primarily to compounds with a single basic site and very weak or absent intramolecular interactions. In the event that the proton transfer reaction is accompanied by a zero entropy or zero free-energy barrier, the term $R \ln \frac{Q_1^*}{Q_2^*}$ is equal to ΔS and $\Delta S^{\#}$:

$$\Delta S = \Delta S^{\#} = R \ln \frac{Q_1^*}{Q_2^*},$$

where ΔS is the difference between the entropy of protonation of the reference base and the unknown base, and $\Delta S^{\#}$ is the entropy difference between activated complexes (Majumdar et al., 1992; Wu et al., 1994). Thus, if intramolecular interactions occur, then ΔS is nonzero and the ratio of the rates is related instead to the difference in GB as shown by the equation:

$$\ln \frac{k_1}{k_2} \cong \frac{GB(1) - GB(2)}{RT}.$$

Discussions on the restrictions and the limitations of the kinetic method are also given in a recent article by Bliznyuk et al. (Bliznyuk et al., 1993). A very recent application was to determine alkali metal binding to DNA and RNA nucleobases (Cerda et al., 1996). Further discussions on bracketing and kinetic methods, as they apply to specific experiments, are given in greater detail in the following sections.

B. Gas-Phase Basicity Scale

The gas-phase basicity scale is currently in a state of revision. The most comprehensive compilation is by Lias, Liebman, and Levin (LLL) (Lias et al., 1984). Recently, two other scales have been suggested that reassign the more basic part of the scale that contains the amines. A scale proposed by Moet–Ner and Sieck (Meot–Ner et al., 1991) increases the GB value assignments for many organic amines. Szulejko and McMahon (SM) (Szulejko et al., 1993) also re-evaluated the high end, with many of the values supported by high-level *ab initio* calculations of Smith and Radom (Smith et al., 1993), and produced values closer to the LLL scale. In addition, a recent report by Hillebrand et al., using high-level *ab initio* calculations to model proton affinities of several amines, supports many of the values tabulated by LLL (Hillebrand et al., 1996). The use of both scales in assigning PA values of selected amino acids is illustrated by Li and Harrison (Li et al., 1993). The SM scale, though more recent, is not as extensive as the LLL scale, and direct comparison of the values between the SM and the LLL scales indicates that differences are typically less than 1 kcal/mol. Therefore, for consistency and convenience, we suggest using the LLL scale until all the values are revised.

C. Hydrogen/Deuterium Exchange

The rates of gas phase H/D exchange reactions are most conveniently measured in an ICR cell in which the ions can be trapped for extended periods of time in the presence of a background pressure (10^{-8}–10^{-6} Torr) of the exchange reagent. Generally, multiple exchanges are possible, and one observes the decay of the parent peak and the growth of peaks at $M + n$ corresponding to the products containing n deuteriums. One approach (Campbell et al., 1994; Gard et al., 1994) to analyzing the kinetics is to treat the system as one of successive exchanges with apparent rate constants k_n for each exchange, as illustrated with the deuterated reagent CH_3OD:

$$\text{(Reaction 1)} \quad D_0 + CH_3OD \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D_1 + CH_3OH$$

$$\text{(Reaction 2)} \quad D_1 + CH_3OD \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D_2 + CH_3OH$$

$$\text{(Reaction 3)} \quad D_2 + CH_3OD \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} D_3 + CH_3OH$$

$$\text{(Reaction 4)} \quad D_3 + CH_3OD \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} D_4 + CH_3OH$$

$$\vdots$$

Where D_n represents the ion containing n deuteriums. The k_{-n}'s are included to account for any protonated impurity in the deuterating reagent, but are assumed to be equal to the corresponding k_n's. The rate expressions are a series of coupled differential equations:

$$-\frac{d[D_0]}{dt} = k_1[D_0][CH_3OD] - k_{-1}[D_1][CH_3OH] \quad (1)$$

$$\frac{d[D_1]}{dt} = k_1[D_0][CH_3OD] - k_{-1}[D_1][CH_3OH] - k_2[D_1][CH_3OD] + k_{-2}[D_2][CH_3OH] \quad (2)$$

$$\frac{d[D_2]}{dt} = k_2[D_1][CH_3OD] - k_{-2}[D_2][CH_3OH] - k_3[D_2][CH_3OD] + k_{-3}[D_3][CH_3OH] \quad (3)$$

$$\frac{d[D_3]}{dt} = k_3[D_2][CH_3OD] - k_{-3}[D_3][CH_3OH] - k_4[D_3][CH_3OD] + k_{-4}[D_4][CH_3OH] \quad (4)$$

$$\vdots$$

The series of equations can be solved numerically and the rate constants extracted from the experimental data in an iterative fashion.

Alternatively, the system may be treated as one of x independent sites (Green et al., 1995), each following a simple first order rate law:

$$S_j(H) + CH_3OD \underset{k_{-j}}{\overset{k_j}{\rightleftharpoons}} S_j(D) + CH_3OH,$$

where S_j corresponds to site j. In this case, the observed populations of the D_n species are related to the populations of the individual sites. For example, the D_2 species observed in a system of three independent sites, a, b, and c, is actually the sum of three species, $d_{a,b}$, $d_{a,c}$, and $d_{b,c}$. Again, the rate constants may be determined iteratively, but, in this case, they represent the rates of exchange at individual sites.

A comparison of the results given by the two treatments on a data set for the reaction of glycineH^+ with CH_3OD is given in Fig. 1. Although the apparent rate constant treatment indicates that there is one exchange that is much faster than the other three, with k_1, k_2, k_3, and $k_4 = 10 \times 10^{-11}$, 3.1×10^{-11}, 1.9×10^{-11}, and 0.7×10^{-11} $cm^3 \cdot molec^{-1}sec^{-1}$, respectively, the independent site treatment indicates one fast-reacting site, $k_a = 6.6 \times 10^{-11}$ $cm^3 \cdot molec^{-1}sec^{-1}$, and three equivalent slow sites, $k_b = 0.8 \times 10^{-11}$ $cm^3 \cdot molec^{-1}$-

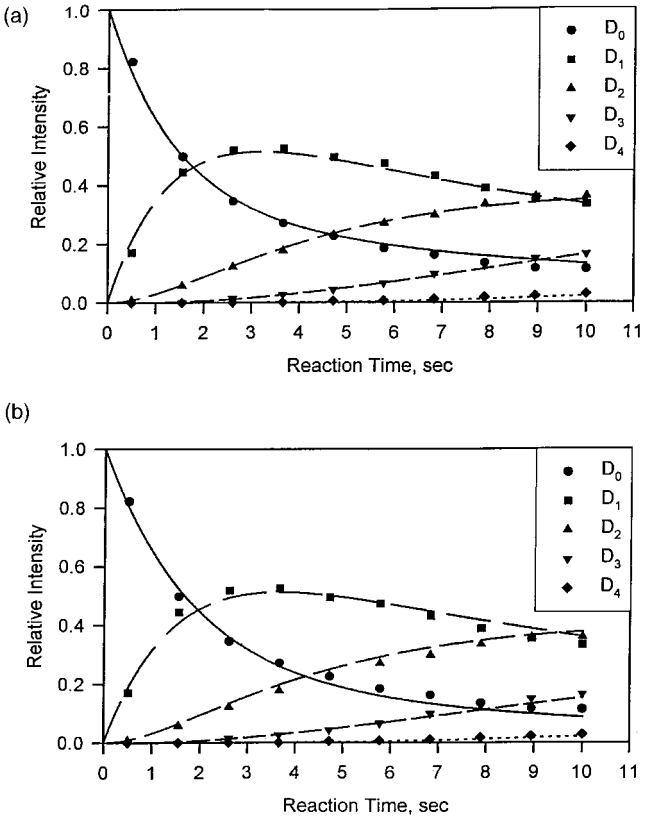


FIGURE 1. H/D exchange of GlycineH^+ reacting with 3.1×10^{-7} Torr CH_3OD. (a) Fit obtained with the apparent rate constant model. (b) Fit obtained with independent site model. For both fits, the ratio CH_3OD/CH_3OH was fixed at 3:1.

sec^{-1}. The latter result provides strong supporting evidence for a structure of glycineH^+ in which the proton is localized on the amino group, resulting in three equivalent amine hydrogens and one carbonyl hydrogen.

One difficulty with both of these treatments is that they are computationally intensive to the point where they become impractical for more than about 15 exchanges. An approach that is perhaps less precise but certainly more simple to carry out is to assume a number of exchangeable hydrogens in the ion (the number of protons + the number of hydrogens attached to heteroatoms) and to plot the logarithm of the number remaining vs. time. If the resulting plot shows one or more straight sections, then each rate constant and the number of sites associated with it may be obtained from the slope and the intercept, respectively (Wagner et al., 1994). The number of hydrogens remaining may be calculated from the individual peaks observed, or, in the event that isotopic resolution is not available, from the shift of the centroid of the unresolved peak. This approach, of course, may be applied to any number of exchanges. However, a relatively large number of time points are required, particularly if there are several distinct rate constants.

Recently, Zhang et al. (Zhang et al., 1996) reported extracting rate constant distributions from plots of deuterium incorporation vs. reaction time using a maximum entropy method. Although this method seems promising, data on the reliability and computational requirements of this treatment have yet to be published.

III. GAS-PHASE PROTON TRANSFER REACTIONS

A. Gas-Phase Basicities of Singly Protonated Amino Acids and Peptides

Proton affinities and gas-phase basicities of the most thermally stable α-amino acids were first determined by Moet–Ner and co-workers (Meot–Ner et al., 1979) and by McIver and co-workers (Lias et al., 1984; Locke et al., 1983), using the equilibrium method. Subsequently, values for nearly all amino acids were determined by McIver using the equilibrium method, and the results are tabulated in the LLL compilation (Lias et al., 1984). By comparing the intrinsic GB and gas-phase acidity to carboxylic acids and amines, it was determined that the site of protonation was the more basic amino group rather than the carboxyl group (Aue et al., 1979). The intrinsic basicities of amino acids were revisited recently by Bojesen (Bojesen, 1987; Bojesen, 1986), Isa et al. (Isa et al., 1990), Wu et al. (Wu et al., 1992), and Li et al. (Li et al., 1993) using the kinetic method with ions produced by FAB. Competition experiments were performed between various amino acids to initially obtain an ordering of the amino acids and later GB and PA values. Li and Harrison later used amine bases as references (Li et al., 1993). GB and PA values for the 20 amino acids, with the exception of Arg, were assigned by Amster and co-workers using the bracketing method in deprotonation reactions of protonated reference bases by neutral amino acids produced by laser desorption (Gorman et al., 1992). The PA of Arg was later assigned by Fenselau and co-workers using the kinetic method. The kinetic method as applied by Bojesen, Isa et al., and Wu et al. produced consistent values that corresponded well with those obtained by McIver using the equilibrium method (Table 1) (Lias et al., 1984). The bracketing method as applied by Amster and the kinetic method generally produced similar ordering with several exceptions, the most notable being His. Some disagreement is expected given that the PAs of most amino acids fall in a range of less than 20 kcal/mol, with the exception of Gly, the least basic, and Arg, the most basic. A majority of the amino acids have GB and PA values that differ by less than 1 kcal/mol from the next most basic amino acid.

The relative position of lysine and histidine on the basicity scale has been a source of some controversy. In protonated lysine and histidine, cyclic proton-bridged species are believed to form due to the interaction of the two amino groups. The behavior of lysine and histidine is expected to be very similar to other diamines, where strong intramolecular interactions between the two amines significantly increase the GB relative to monoamines (Aue et al., 1973; Aue et al., 1972; Bowers et al., 1971; Gorman et al., 1993; Meot–Ner et al., 1991). The GB of both amino acids has been obtained by using equilibrium, kinetic, and bracketing methods. The results of experiments utilizing the kinetic method are in agreement and suggest that histidine and lysine have similar ($<$1 kcal/mol difference) GB and PA. The equilibrium method applied by McIver and co-worker finds histidine to be 1.6 kcal/mol more basic (GB) (Lias et al., 1984), whereas Amster and co-workers find that lysine is more basic by 5.6 kcal/mol (Bliznyuk et al., 1993). The most recent report by Carr and Cassady, employing bracketing and kinetic methods, gives the same GB values for histidine and lysine (Carr et al., 1996).

Proton affinities of peptides of glycine polymers were first reported by Fenselau and co-workers (Wu et al., 1992) using the kinetic method. Subsequently, Lebrilla and co-workers used the bracketing method to determine proton affinities and gas-phase basicities (Wu et al., 1993), whereas Cassady and co-workers used a combination of both methods to also determine proton affinities and gas-phase basicities (Zhang et al., 1993). Proton affinities of polyglycines determined by Fenselau and co-workers increased continuously from glycine to diglycine, the largest polyglycine in the study. The largest increase is observed between glycine and diglycine (7.5 kcal/mol), with each of the larger homologs adding an additional 4.0 kcal/mol

TABLE 1. All values adjusted to the LLL scale. LLL values are a compilation of several values from several research groups. See references therein. This table is adapted from one published in [Wu et al., 1992]. LLL = Lias, Liebman, and Levin [Lias et al., 1984]; Bojesen [Bojesen, 1987; Bojesen, 1986; Bojesen et al., 1994]; Isa [Isa et al., 1990], GSTA = Gorman, Speir, Turner, and Amster [Gorman et al., 1992]; LH = Li and Harrison [Li et al., 1993]; and WF = Wu and Fenselau [Wu et al., 1992; Wu et al., 1993]. Multiple values are provided for some of the amino acids by LH (see original reference). These values were averaged in the table.

LLL	PA[a]	Bojesen	PA	Isa	GSTA	PA	LH	PA	WF	PA
Gly	211.6	Gly	—	Gly	Gly	206.5–207.6	Gly	211.0	Gly	—
Ala	214.8	Ala	—	Ala	Cys	209.1	Ala	213.6	Ala	—
Cys	—	Cys	—	Cys	Ser	214.2	Cys	213.8	Cys	—
Phe	216.5	Ser	217.2	Ser	Asp	214.2	Ser	215.0	Ser	—
Glu	216.5	Val	218.1	Asp	Ala	215.6	Val	215.7	Val	—
Asp	216.7	Asp	218.1	Val	Val	215.6	Leu	216.1	Asp	—
Ser	216.8	Leu	218.7	Leu	Leu	217.8	Phe	216.6	Leu	—
Val	217.0	Thr	219.2	Thr	Ile	217.8	Thr	216.8	Thr	—
Leu	218.1	Ile	219.2	Ile	Phe	219.7	Ile	217.4	Ile	—
Gln	218.4	Phe	229.9	Phe	Tyr	219.7	Met	218.2	Phe	—
Thr	218.6	Tyr	220.7	Met	Asn	219.7	Tyr	217.8	Tyr	—
Ile	218.9	Met	221.0	Tyr	Thr	220.8	Asn	218.7	Met	—
Asn	219.8	Asn	222.1	Glu	Met	220.8	Pro	219.5	Asn	—
Pro	220.2	Glu	222.3	Asn	Gln	220.8	Trp	220.8	Glu	—
Met	221.4	Pro	222.4	Trp	His	220.8	Gln	222.1	Pro	—
Tyr	222.3	Trp	223.5	Pro	Pro	223.0	Lys	222.9	Trp	—
Trp	225.4	Gln	226.9	Gln	Trp	223.0			Gln	—
Lys	230.3	Lys	228.7	Lys	Glu	225.5			Lys	—
His	231.9	His	230.5	His	Lys	226.7			His	—
Arg	—	Arg	>242.8	Arg	Arg	>234.8			Arg	245.2

[a] PA = proton affinity (kcal/mol).

per additional residue up to pentaglycine, and 2.5 kcal/mol per residue for larger homologs up to octaglycine. Lebrilla and co-workers used the bracketing method and FTMS to determine GB values that increased 5.4 kcal/mol between glycine and diglycine. Between diglycine and triglycine, the increase was only 1.1 kcal/mol and no increase was observed between tri- and tetraglycine. Between tetra- and pentaglycine, an increase of 1.9 kcal/mol was observed. In this study, molecular orbital calculations using AM1 (Dewar et al., 1985), a semi-empirical method, were used to predict that protonation on the N-terminus of diglycine yielded the most stable tautomer, with protonation on the amide oxygen unfavorable by only 2–3 kcal/mol. Cassady and co-workers obtained a similarly large increase between glycine and diglycine of 6.6 kcal/mol and further increases of 4.0 kcal/mol for triglycine, 5.3 for tetraglycine, and 0.1 for pentaglycine also using FTMS (McKiernan et al., 1994; Zhang et al., 1993). Rigorous *ab initio* calculations performed on glycine, diglycine, and triglycine (Zhang et al., 1994) supported the earlier conclusion that the most basic site in diglycine is still the terminal amine. The high-level calculation, however, predicted a difference in GB of nearly 9 kcal/mol between glycine and diglycine; that difference is consistent with their experimental results. Cassady in the same study used the kinetic and bracketing method to obtain similar GB values for glycine and polyglycines up to tetraglycine. For the larger penta- and hexaglycine, the kinetic method yielded larger values. The GB assigned for pentaglycine was 2.0 kcal/mol less with the bracketing method than the kinetic method, and for hexaglycine the difference increased to 3.4 kcal/mol.

The variations in GB values for the glycine homologs are due to several factors. In addition to the differences between the kinetic and bracketing methods in assigning GB values, there are variations due to differences in internal energy due to the combination of FAB ionization and various modes of cooling, including radiative. Ngoka et al. have shown that FAB-produced ions in FTMS are vibrationally excited and that a large fraction has sufficient internal energy to undergo slow metastable dissociation (Ngoka et al., 1993).

Peptides composed of amino acids without a basic side chain exhibit behavior similar to the polyglycines. Polyalanines and polyvalines also show increases in basicity as a function of chain length, with all the basicities shifted from the glycine analogs by an amount equal to the difference between the GB of glycine and the respec-

tive amino acid (Wu et al., 1995; Wu et al., 1993). For the alanine and valine homologs and triglycine, semi-empirical studies again predict that the terminal amine is the most basic site. With protonated alanine and valine, calculations predict a compact structure in which the alkyl side chains are positioned radially. This conformation may hinder access to the protonation site and decrease the rate of proton transfer reactions. In the same study, we further show the difficulty in assigning GB for large peptides using the bracketing method (Wu et al., 1995). The rates of proton transfer are severely attenuated in polyvalines, making assignment of GB difficult.

Peptides of mixed amino acids have also been studied. For example, McKiernan et al. have reported that the addition of more basic residues such as serine (McKiernan et al., 1994) to glycine has a stronger affect on basicity when serine is the N-terminus. Elsewhere in the chain, the more basic residue produces only slight effects, a 1–2 kcal/mol increase, over a similar compound containing only glycine. A combination of high-level *ab initio* calculations and experimental results were reported for Ala, Gly, GlyGly, AlaGly, GlyAla, and AlaAla (Cassady et al., 1995). Carr and Cassady also find that the placement of lysine and histidine in peptides, where the remainder of the residues are glycines, also vary the basicity of the peptide. For example, the GB of GlyLysGly is 5.4 kcal/mol less basic than either of the isomeric peptides GlyGlyLys or LysGlyGly (Carr et al., 1996). By contrast, Gorman and Amster found that, with residues containing basic side chains, the position of residue in the peptide is less important. The peptide takes on the basicity of the most basic residue, indicating that protonation occurs at the most basic site and not necessarily the N-terminus (Gorman et al., 1993). They also suggested an interesting interaction involving the protonated N-terminus and the aromatic side chain to explain the increase of 4.5 kcal/mol between Phe and Val-Phe and a similar increase between Tyr and Val-Tyr.

Kaltashov and Fenselau showed that N-alkylation of the N-terminus of the peptide Phe-Gly-Gly-Phe-Leu increases the basicity, depending on the size of the alkyl group. These results are consistent with the terminal amine as the site of protonation and as likely the most basic site of peptides not containing strongly basic side chains (Kaltashov et al., 1995).

The relationship between gas-phase and solvated peptides has also recently been studied. Klassen et al. have investigated the thermodynamics of solvation of protonated peptides (Klassen et al., 1995).

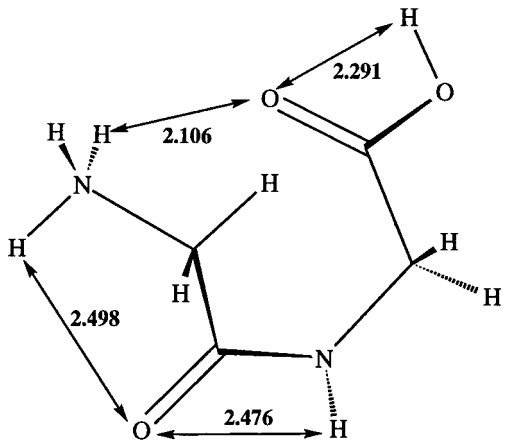
B. Sites of Protonation in Singly Protonated Amino Acids and Peptides

With singly charged species, the site of protonation plays the major role in the structure of the ion. In this regard, proton transfer reactions can provide strong indications of where the proton resides in the molecule. Molecular orbital calculations, employing either semi-empirical (Wu et al., 1993; Wu et al., 1995) or *ab initio* (Zhang et al., 1994; Zhang et al., 1993) models and, in some cases, molecular mechanics (Gorman et al., 1993; Kaltashov et al., 1995) have similarly been very useful in determining the possible sites of protonation and the relative gas-phase basicities of individual basic sites (Bliznyuk et al., 1993).

The most basic site of all but a handful of the 20 commonly occurring L-α-amino acids is the N-terminus. The exceptions are lysine, histidine, and arginine. For glycine, experimental and theoretical evidence show unambiguously that the amine is the most basic site of protonation (Bouchonnet et al., 1992; Jensen, 1992; Locke et al., 1983; Somogyi et al., 1994; Zhang et al., 1993). Using neutralization reionization mass spectrometry (NRMS), Wesdemiotis and co-workers have found that protonation on the amine and carboxylic acid occurs during FAB ionization, producing the corresponding tautomeric species (Beranová et al., 1995). *Ab initio* calculations predict O-protonation on the carbonyl oxygen to be 13.8 kcal/mol less favored than N-protonation (Zhang et al., 1993). This energy difference is readily accessible under FAB ionization conditions. However, evaluation of the kinetics for protonated amino acids in this laboratory and in others shows simple exponential behavior, consistent with a single protonated species with protonation on the amine. In FTMS, the longer time scale, compared to that of sector instruments where NRMS is performed, may allow conversion of the higher energy tautomer to the lower energy one, resulting in the behavior observed in our laboratory and by others using FTMS for proton transfer reactions.

The relatively higher basicity of lysine and histidine as well as the large ΔS value (10.1 and 4.2 cal/K-mol for lysine and histidine, respectively) (Wu et al., 1994) for protonation are the result of the formation of a cyclic structure bridged by the proton and involving the two amines. The protonated species of these amino acids behave like those of other diamines that have been studied in detail. The formation of a cyclic intermediate is supported by rigorous *ab initio* calculations (Bliznyuk et al., 1993). In contrast, arginine is clearly protonated on the guanido group, which is much more basic than the terminal amine. When one base site is significantly more basic than another, the formation of hydrogen bridging is significantly attenuated (Meot–Ner, 1983; Meot–Ner (Mautner), 1984).

Intramolecular interactions play a strong role in peptides, even when residues containing basic groups are absent. In glycine, it has been shown that interaction between the protonated amine and the carboxylic acid group, the second most basic site on neutral glycine, is essentially nonexistent (Cassady et al., 1995; Wu et al., 1993; Zhang



STRUCTURE 1

et al., 1993). Molecular orbital (MO) calculations (Wu et al., 1993; Zhang et al., 1993) and gas-phase basicities of polyglycines (Wu et al., 1993; Wu et al., 1995; Wu et al., 1992; Wu et al., 1993; Zhang et al., 1993) indicate that the interaction between the protonated terminal amine and the neighboring carbonyl amide is strong and the major reason for the large increase in GB between glycine and diglycine. This interaction is structurally limited in diglycine, and replacing the N-terminus glycine with a β-amino acid, as in β-Ala-Gly, allows a much stronger interaction between the protonated amine and the adjacent carbonyl amide (Wu et al., 1995). Experimental and theoretical evidence suggest that the most basic site in larger peptides like polyglycines is the N-terminus (Wu et al., 1995; Zhang et al., 1994). Shown above is the lowest energy structure of triglycine found with the semi-empirical method AM1. The selected distances shown between hydrogen and oxygen illustrate the high degree of intramolecular interactions (Structure 1).

Polyalanines and polyvalines follow similar GB trends as polyglycine. In these peptides, the most basic site remains the terminal amine. Semi-empirical calculations predict that intramolecular interactions in trialanine and trivaline are similar to those found in triglycine, with the large alkyl groups in alanine and valine arranged radially to minimize steric interactions (Wu et al., 1995).

C. Multiply Protonated Peptides and Proteins

Multiply protonated peptides and proteins are produced primarily with ESI. An excellent recent review by Williams on proton transfer reactions of large multiply charged ions covers much of the recent work dealing with proteins (Williams, 1996). Proton transfer reactions are complicated by a reverse activation barrier that results from the electrostatic repulsion of protons. Deprotonation reactions involving multiply charged cytochrome c were first performed by McLuckey et al. on a quadrupole ion trap (McLuckey et al., 1991; McLuckey et al., 1990). The 8+ to 15+ charge states of cytochrome c were reacted with dimethyl amine, and rate constants were determined to decrease with decreasing charge state. The largest rate constant, corresponding to the reaction of the 15+ charge state, was two orders of magnitude smaller than the collision rate constant. Cassady et al. performed deprotonation reactions of multiply protonated ubiquitin ions. With four amines ranging in GB from 210.1 kcal/mol to 232.6 kcal/mol, the deprotonation rate constants showed high efficiencies ($k_{experiment}/k_{theory}$ = 0.2−1.4) for the reactions involving the 13+ charge state (Cassady et al., 1994). Interestingly, with highly basic amines, the reactivity of all charge states varied only slightly. N,N,N′,N′-Tetramethyl-1,4-diaminobutane, which has a GB similar to lysine and histidine—the expected sites of protonation reacted with similar efficiencies for charge states 13−6, suggesting similar sites of protonation in each charge state. Furthermore, the lower charge states were found to react with the weaker bases under nonlinear pseudo-first-order kinetics, suggesting more than one reacting ionic species.

The bracketing method was applied by Gross and Williams to gramicidin S to determine the GB for the doubly and singly charged species (Gross et al., 1995). That article represents the first treatment of the role of coulombic energy in proton transfer reactions. Unlike singly charged species, multiply protonated peptides have a reverse activation barrier due to repulsion of the like charges. Coulombic energy in the doubly charged ion was determined to be >27.9 kcal/mol. Furthermore, based on a distance of 9.5 Å obtained from molecular modeling, an intrinsic dielectric polarizability of less than 1.2 was obtained. In this article and in others (Gross et al., 1995; Gross et al., 1996; Kaltashov et al., 1995; Zhang et al., 1991), the utility of molecular modeling with simple force fields for large peptides and even proteins has been clearly demonstrated, just as *ab initio* and semi-empirical calculations have been useful for amino acids and small peptides. A study by Gross et al. on diprotonated diaminoalkanes illustrated the pronounced effect of electrostatic interactions on the chemistry of multiply protonated ions (Gross et al., 1995). A further study on doubly charged gramicidin S ions showed that protonated gramicidin S has a slightly lower GB (by 4.2 kcal/mol) when the proton is replaced by an alkali metal (Gross et al., 1996). This difference was attributed to the greater charge separation between the proton and an alkali metal ion than between two protons. The alkali metal is believed to reside on the exterior surface of the peptide.

Kaltashov and Fenselau modified the kinetic method to account for the reverse activation barriers in proton

transfer reactions involving multiply charged ions (Kaltashov et al., 1995; Kaltashov et al., 1996; Kaltashov et al., 1995). An effective temperature (T_{eff}) of the mixed dimer was proposed and obtained from kinetic energy release measurements. The T_{eff} was input into the modified equation of the kinetic method

$$\ln \frac{k_1}{k_2} = \frac{GB_{app}(MH^+) - GB(B)}{RT_{eff}}$$

to obtain the difference in GB between the protonated peptide and the reference base. This calculation was first applied to des-Arg^9-bradykinin. They concluded that, although the first protonation site is the guanido group of arginine, the second protonation site may be the C-terminus, presumably the carbonyl group, to minimize coulombic interactions between the two charges.

The study of proteins in proton transfer reactions has yielded important clues regarding their gas-phase structure. Ion-molecule reactions have been used to predict the maximum charge state of the ion observed in ESI. The reaction between the multiply charged species and methanol, the commonly used solvent for ESI, was postulated by Williams to determine the highest observable charge state (Schnier et al., 1995; Schnier et al., 1995). Furthermore, the results from ion-molecule reactions were used to determine a dielectric polarizability for a fully denatured conformation of the ion corresponding to $\varepsilon_r = 2.0 \pm 0.2$. This value is nearly double that obtained by the authors for peptides.

Because of intensive intramolecular interactions, the gas-phase basicity of an amino acid residue in a protein is significantly larger than that of the isolated amino acid (Schnier et al., 1995) but slightly more that of the amino acid residue in a small peptide. The greater basicity is even more notable given that a significant Coulombic repulsion exists in multiply charged species. Coulombic repulsion decreases the observed GB of the molecule. Thus, the GB of proteins decrease with increasing charge state (Williams, 1996).

The detection of conformational differences is of special interest in the study of proteins formed by electrospray. Further discussions of conformations are presented in the next section, when hydrogen/deuterium exchange as probes of ion structure is discussed. Proton transfer reactions have been used to probe conformational differences in gas-phase proteins. Ions of several proteins, including cytochrome c generated from different solution conformations and under various ionization conditions (including several desolvating temperatures), did not exhibit significantly differing deprotonation rates with bases such as ammonia, triethylamine, and hexanediamine (Ogorzalek–Loo et al., 1994; Ogorzalek–Loo et al., 1994). However, when accurate rate measurements were performed on multiply protonated ubiquitin, more than one rate constant was necessary to fit the data; the multiple rate constants suggest multiple reacting species for the charge state (Cassady et al., 1994). Similar observations were reported for the reaction of intact and denatured hen egg-white lysozyme (HEWL) ions (Gross et al., 1996). Chemical degradation, as in reduction of disulfide bonds in proteins, is the most direct method for changing the protein's secondary structure. Ogorzalek–Loo et al. observed different rates of proton transfer for the same charge states of disulfide-reduced and native HEWL (Ogorzalek–Loo et al., 1994; Ogorzalek–Loo et al., 1994). Similarly, HEWL has different apparent basicities for same charge species, depending on whether the disulfide bonds are reduced or kept intact (Gross et al., 1996).

More recently, the question of chirality in sites of protonation has been addressed. There have been several notable examples of chiral ion-molecule reactions. Chu et al. have observed chiral selectivity in the complexation of a host molecule containing two stereocenters with a chiral guest (Chu et al., 1993). Nikolaev et al. have shown chiral effects in the unimolecular dissociation and ligand exchange of proton-bound dimers of dimethyl tartrates (Honovich et al., 1992; Nikolaev et al., 1995). The first reported chiral reaction involving proton transfer was performed in this laboratory using (2R)- and (2S)-2-butylamine and various charge states of cytochrome c (Camara et al., 1996). The R-isomer is found to be more reactive towards proton transfer than the S-isomer for the 9+, 8+, and 7+ charge states of cytochrome c (Table 2) by nearly an order of magnitude. Furthermore, in some cases such as the reaction of 8+ and 7+ states, the kinetics indicate the existence of more than one reacting species; these results are consistent with a similar behavior observed in H-D exchange (Suckau et al., 1993; Wood et al., 1995) and ion-mobility (Clemmer et al., 1995) experiments.

IV. HYDROGEN/DEUTERIUM EXCHANGE

A. Amino Acids and Peptides

H/D exchange is an essentially energy-neutral reaction, if isotope effects are ignored. Thus, with appropriate control of experimental conditions, these reactions can probe the reaction surface and barrier without the energy differences between reactants and products complicating the picture. One advantage of H/D exchange over proton transfer is that, whereas proton transfer reaction generally only involves a single site, H/D reaction can potentially probe several sites in a molecule, if the multiple exchanges observed can be correlated to the different available reactive

TABLE 2. Rate constant of deprotonation reactions involving the respective amines and the 9+, 8+, and 7+ charge states of cytochrome *c* produced by electrospray ionization. Units for rate constants are $cm^3 \cdot molecule^{-1}\ second^{-1}$. Rate constants involving the two isomers of 2-butylamine were determined from three separate experiments performed on different days.

Charge state	R-2-ButylNH_2 GB[c] = 211.7	%[d]	S-2-ButylNH_2 GB[c] = 211.7	%[d]	n-PrNH_2 GB[c] = 210.1	t-ButylNH_2 GB[c] = 213.0
9^+-8^+ [a]	1.5×10^{-11} ($\pm$0.3)		2.5×10^{-12} ($\pm$0.2)		2.2×10^{-12}	6.1×10^{-13}
8^+-7^+ [b]	2.3×10^{-12} ($\pm$0.5)		4.6×10^{-13} ($\pm$1.1)		2.9×10^{-13}	3.8×10^{-14}
8^+-7^+ [a]-FAST	1.0×10^{-11} ($\pm$0.3)	45	1.9×10^{-12} ($\pm$0.4)	46	3.1×10^{-13}	3.7×10^{-13}
8^+-7^+ [a]-SLOW	1.4×10^{-12} ($\pm$0.1)	55	3.7×10^{-13} ($\pm$1.0)	54		
7^+-6^+ [b]	2.3×10^{-13} ($\pm$0.1)		8.4×10^{-14} ($\pm$3.6)		7.2×10^{-14}	
7^+-6^+ [a]-FAST	1.1×10^{-11} ($\pm$0.1)	21	1.4×10^{-12} ($\pm$0.3)	30	1.4×10^{-13}	5.1×10^{-14}
7^+-6^+ [a]-SLOW	1.3×10^{-13} ($\pm$1.1)	79	1.4×10^{-13} ($\pm$1.9)	70		

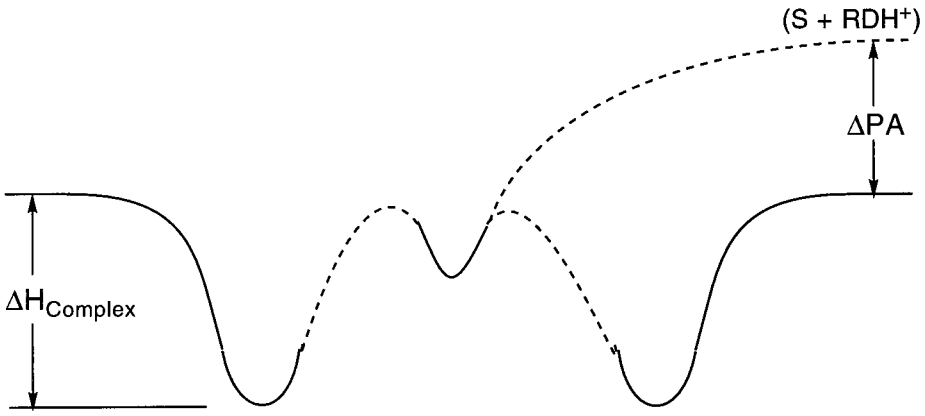
[a] Higher charge rate is isolated directly from ESI source with reaction proceeding towards the low charge state.
[b] Further reaction of the product ion in (a) to a lower charge state.
[c] Values obtained from Reference 44.
[d] Contribution of each rate constant to overall rate constants when two or more are observed.

sites. One drawback is that exchange reactions give no direct information on GB or PA.

The generally accepted simplified mechanism (Brauman, 1979; Lias, 1984) for H/D exchange between a protonated substrate (S) and a deuterating reagent in the gas phase consists of three steps: the initial formation of a loose hydrogen-bonded complex, complete or partial transfer of the proton to the reagent, resulting in isotope scrambling, and finally, dissociation of the complex to yield either the original or the exchanged substrate species (Scheme 1).

For exchange to be observed, the energy made available by complex formation must be sufficient to overcome the barrier to internal proton transfer. This barrier will depend mainly on the proton affinity difference ($\Delta PA = PA_{substrate} - PA_{reagent}$) between the two unprotonated species. This mechanism is supported by the observation that, for a large range of substrates and deuterating reagents, there is an approximately inverse correlation between ΔPA and the observed rate of exchange (Ausloos et al., 1981). Furthermore, when ΔPA exceeds a limit of ca. 20 kcal/mol, no exchange is observed, presumably because the energy made available by the exothermicity of complex formation is insufficient to overcome the barrier to endothermic proton transfer within the complex. It should be noted that this mechanism assumes thermodynamic, rather than kinetic control; that is, it is necessary to have a complex lifetime that is long compared to the time scale for proton transfer. This assumption is probably a good one for complexes with hydrogen bonding: Adams and co-workers (Adams et al., 1982; Henchman et al., 1991) have estimated that, for the proton-bound dimers of H_2O

$$SH^+ + RD \rightleftharpoons SH^+\cdots RD \rightleftharpoons S\cdots RDH^+ \rightleftharpoons SD^+\cdots RH \rightleftharpoons SD^+ + RH$$



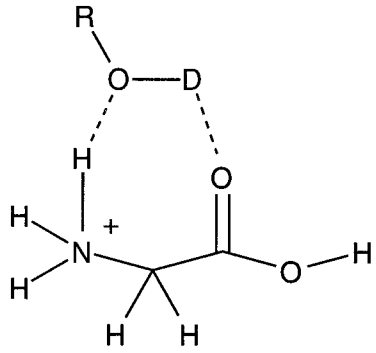
SCHEME 1

and NH_3, complex lifetimes at room temperature are 1–2 ns, as compared to a ''proton jump'' time of $<$100 ps. In contrast, the proton-bound dimer of CH_4 has a complex lifetime of about 8 ps, much less than the time for intramolecular proton transfer (30 ps) (Henchman et al., 1991).

Whereas the proton affinity difference between the substrate and reagent is clearly of paramount importance for simple monofunctional ions, structural effects can also play an important role even in relatively small ions. This notion was first demonstrated in a study by Freiser et al. (Freiser et al., 1975), who saw large differences in exchange reactivity with D_2O between protonated xylene isomers, and later by Ranasinghe et al. (Ranasinghe et al., 1992), who examined the reactions of a selection of deuterating agents with a series of aromatic compounds. In the latter study, it was observed that in many cases, exchange patterns varied with different isomers, and that smaller proton affinity differences between reagent and substrate resulted in greater reaction specificity. Some of the differences in exchange patterns could be readily explained in terms of the relative positions of the functional groups. For example, *meta*-hydroxybenzoic acid exchanges three protons in reaction with CH_3OD, whereas the other two isomers exchange only two, presumably because the two functional groups are more remote from each other. However, some of the other patterns observed had no ready explanation.

More recently, a number of studies have shown that amino acids and peptides are much more reactive than would be suggested by the trends observed in the earlier studies; significant exchange was observed even with PAs much greater than 30 kcal/mol (Campbell et al., 1994; Cheng et al., 1992; Gard et al., 1993; Hemling et al., 1994; Winger et al., 1992). Cheng and Fenselau (Cheng et al., 1992) examined H/D exchange of ND_3 and several protonated peptides in the collision cell of a sector instrument. Exchange was observed in all cases, although estimated ΔPAs ranged from 30–50 kcal/mol. In fact, ΔPAs were probably somewhat greater than this range, because the estimated PA in each case was simply the gas-phase PA of the free amino acid corresponding to the most basic residue in the peptide. Pressure and collision energy dependencies supported the importance of complex formation.

Hemling et al. (Hemling et al., 1994) examined the H/D exchange of a selection of peptides by using ND_3 as a nebulizer or a curtain gas in electrospray, with the aim of developing a facile method for counting the number of exchangeable H's in small molecules. They examined a variety of compounds, and achieved high levels of exchange in all cases. For ions with less than 25 active H's, all possible exchanges were observed, and in the worst case, oxidized insulin β chain, 45 out 51 possible exchanges were observed. In this last case, the maximum



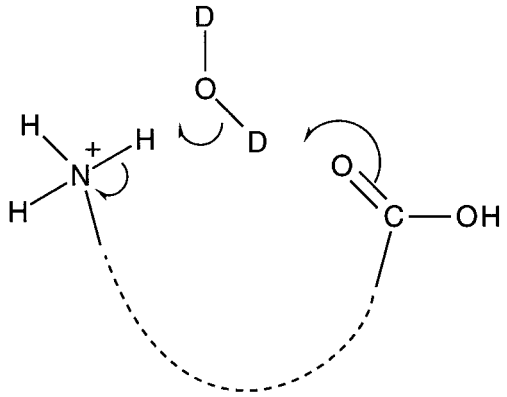
SCHEME 2

number of exchanges was probably somewhat higher, because in the absence of isotopic resolution, the authors were forced to report an average number of exchanges rather than the maximum. The authors speculated that incomplete exchange could be due to the gas-phase conformation, which would limit access of the reagent to some of the exchangeable hydrogens. Although there is the possibility that some of the exchange observed may have taken place in the liquid rather than the gas phase (because the reagent was added at a point where the electrosprayed droplets had not completely evaporated), these results suggest that, in the gas phase, even very basic sites on a peptide can be induced to undergo exchange, provided that the reagent concentration is high.

The occurrence of an exchange reaction between species whose neutrals differ greatly in PA can be attributed to a lowering of the barrier to proton transfer within the complex due to the formation of multiple hydrogen bonds (Campbell et al., 1994; Gard et al., 1993; Green et al., 1995; Gur et al., 1995). For the reactions of CH_3OD with simple amino acids, Gard et al. (Gard et al., 1993) proposed a complex in which the alcohol forms a bridge between the protonated amino group and the carboxyl carbonyl (Scheme 2).

Campbell et al. (Campbell et al., 1995) examined the reactions of glycine oligomers with D_2O, CD_3OD, CD_3COOD, and ND_3. Based on their experimental results and on extensive semi-empirical modeling, they proposed several mechanisms for H/D exchange that depend on the basicity of the reagent and on the nature of the hydrogen involved. Thus, in reaction with the N-terminus, more basic reagents such as ND_3 were postulated to gain a proton and be solvated by the peptide (the 'onium' mechanism), whereas less basic reagents such as D_2O, which PM3 calculations showed to be incapable of deprotonating the peptide ion, were postulated to exchange via a ''relay mechanism,'' in which the reagent simultaneously gains a D while losing an H (Scheme 3).

Nibbering and co-workers (Gur et al., 1995) examined



SCHEME 3

the reactions of D_2O, CD_3OD, and ND_3 with a series of dipeptides. Again, they rationalized their results in terms of exchange within a multiply hydrogen-bonded complex. Exchanges of hydrogens at different sites were attributed to isomerizations of the complex to different hydrogen-bonded forms. These authors quoted *ab initio* calculations by others (Hillenbrand et al., 1986; Scheiner et al., 1992) on related species to support their assertion that, even in the case of D_2O, the proton can be transferred to the reagent within the complex.

Dookeran and Harrison (Dookeran et al., 1995) examined H/D exchange reactions of ND_3 with the mass-selected ions of a series of amino acids and peptides in the collision cell of a sector/quadrupole instrument with the objective of counting the number of exchangeable hydrogens. They concluded that, under their conditions, gas-phase exchange was not suitable for counting labile hydrogens, because some types of hydrogen exchanged only very slowly. These types included side-chain amide and arginine hydrogens, phenolic H in tyrosine, and N-bonded H on the histidine imidazole ring. They proposed that exchange of the amine hydrogens proceeded via proton transfer to the reagent, and suggested that exchange at other positions was due to migration of the protonated reagent from the amine group to other sites on the peptide ion. Thus, lack of reactivity of some hydrogens was ascribed to a combination of high proton affinity at those sites and an inability of the protonated reagent to migrate there, due either to remoteness or steric factors.

A number of kinetic studies have been done on amino acids and simple peptides (Campbell et al., 1995; Gard et al., 1993; Green et al., 1995; Gur et al., 1995). Because all three groups have examined the diglycine + CH_3OD (CD_3OD in the case of Campbell et al.) reaction, it is interesting to compare their results for this case. In Table 3 are shown the apparent rate constants obtained by the Lebrilla and Beauchamp groups, as well as the specific site constants obtained by our group (*vide supra*), and the first apparent rate constant reported by Nibbering's group (the latter group reported a value only for the first exchange). Although the trends shown by the data of the first two groups are in agreement, with three fast exchanges and two much slower ones, there is considerable variation in the magnitude of the rate constants for the first exchange reported by the three groups. Some of this inconsistency can be ascribed to difficulties in calibrating the ion gauge used to measure the reagent pressure. Another factor is the effective ion temperature. The ions in all three studies were produced by FAB, and likely possessed excess internal energy when they entered the analyzer cell. Excess energy is expected to shorten the lifetime of the reagent-substrate complex, reducing the possibility of exchange, and slowing the apparent rate constant. Lebrilla and Beauchamp both noted some curvature of their kinetic plots at early time as a result. Nibbering cooled the ions with a pulse of argon before reaction. Eliminating this source of error would result in higher measured values for the rate constants.

Although the above results all point to a strong dependence of exchange reactivity on ion structure, care is needed in interpretation. Thus, in protonated glycine, the amine H's exchange very slowly relative to the carboxylic hydrogen (Gard et al., 1993); however, in diglycine, the amine H's exchange much more quickly than the carboxylic hydrogen. The differences in kinetic behavior are further borne out by the trends observed in exchange rate as a function of proton affinity difference between reagent and substrate for a series of alcohols in reaction with protonated amino acids and dipeptides (Figure 2). The presence of a third, highly basic, group in histidine, lysine, and the dipeptides results in dramatically increased reactivity relative to that observed for the simple alkyl amino acids.

TABLE 3. Apparent and site-specific reaction efficiencies, k/k_{ADO}, for H/D exchange of deuterated methanol and diglycineH^+

Apparent rate constants	Exchange of 1D	2D	3D	4D	5D
Nibbering [Gur et al., 1995]	0.87	n.d.[a]	n.d.	n.d	n.d.
Beauchamp [Campbell et al., 1995]	0.35	0.61	0.39	0.12	0.05
Lebrilla [Green et al., 1995]	0.14	0.14	0.07	0.03	0.01
Site-specific rate constants	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**
Lebrilla [Green et al., 1995]	0.042	0.042	0.042	0.017	0.005

[a] n.d. = not determined.

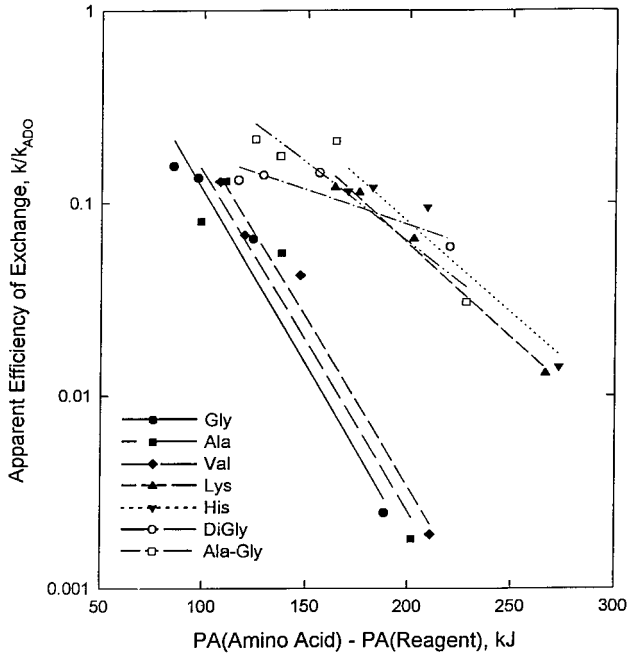


FIGURE 2. Correlation of exchange efficiency with proton affinity difference for a series of amino acids and dipeptides. The presence of a second highly basic group shifts the correlation to higher ΔPA. Adapted from reference (Green et al., 1995).

Williams and Gross (Gross et al., 1995) examined the H/D exchange reaction between D_2O and singly and doubly protonated gramicidin S. Their results (the rate constants for the doubly charged species were 3–4 times faster than for the singly charged species, but significantly slower than those reported for protonated amino acids) were used to support a model for gas-phase gramicidin S ions in which the protonation sites are highly solvated and are located on the two ornithine residues on opposite sides of the ion.

B. H-D Exchange of Proteins

A number of groups have examined gas-phase H/D exchange of proteins (Cassady et al., 1996; Green et al., 1995; Wood et al., 1995; Zhang et al., 1996). There is a large body of work documenting the use of exchange in the liquid phase to elucidate structural and conformational details of proteins (Bai et al., 1995; Katta et al., 1993; Wagner et al., 1994). Mass spectrometry has found particular application in monitoring the dynamics of folding and unfolding. Compact, folded forms tend to exchange more slowly, because labile hydrogens are tied up by hydrogen bonding and also, to some degree, are less accessible to solvent. A comparison of gas- and solution-phase conformations would provide insight into the nature of the folding process, particularly vis-a-vis the role of the solvent. It should be noted that it has not yet been determined to what degree gas- and solution-phase structures correspond; indeed, it is to be expected that some differences will exist (Wolynes, 1995; Wood et al., 1995), given the key role that hydrophobic effects and hydrogen bonding to the solvent plays in protein conformation. A recent ESI-MS study (Robinson et al., 1996), comparing gas and solution phase abundances of complexes between acyl CoA binding protein and a series of acyl CoA derivatives, suggests that binding effects that are due mainly to hydrophobic forces in solution are lost in the gas phase, whereas those effects due to nonpolar stacking and hydrogen bonding are retained. Intuitively, one would expect that, as the size of a molecule increases and intramolecular interactions play an increasingly important role, the gas- and solution-phase structures would more closely resemble each other.

Gas-phase H/D exchange of proteins was first reported by Smith and co-workers (Winger et al., 1992), who reacted electrosprayed proteins with D_2O in a heated capillary and observed the *m/z* shifts caused by deuteration. They observed qualitative differences in the exchange reactivity for the multiply protonated native and reduced forms of bovine proinsulin and α-lactalbumin. However, the results were contrary to what was expected, based on solution experience. The more compact, native forms of the proteins showed a higher exchange reactivity in this study, whereas more compact forms generally show less exchange in solution. This difference was interpreted as a case of coulombic effects dominating over steric effects. For the same charge state, folding will bring the charges closer together, resulting in higher coulombic repulsions between the protons and, hence, effectively lowering the PA of the ion. As discussed above, a lower PA difference between substrate and reagent is expected to enhance reactivity.

A later study on cytochrome c ions by McLafferty and co-workers (Suckau et al., 1993), however, found reactivity trends more in line with the solution-phase behavior. In that work, protein ions produced by ESI were allowed to react with D_2O in the ICR cell of an FTMS instrument, and the *m/z* shift was monitored as a function of time. The quality of the data was sufficient to allow an extrapolation of the observed shifts to infinite time, and, hence, to an estimation of the number of exchangeable hydrogens. These authors reported that reduced and alkylated RNase exchanged ca. four times as many hydrogens as native RNase in the gas phase. Furthermore, cytochrome *c* ions formed by ESI appeared to exist in different forms that could be distinguished by the number of exchangeable hydrogens. Forms exchanging 53, 113, and 74 hydrogens, possibly corresponding to solution conformers III (native), II (denatured, unfolded), and I (denatured, folded), respectively, were observed. The 53-exchangeable

hydrogen form, associated with low charge states, was produced from neutral solutions, in which conformer III is stable. The 113- and 74-hydrogen forms, associated with higher charge states, were the only species produced from acidic (denaturing) solutions. It should be noted that the production of higher charge state protein ions by electrospray of solutions containing denatured forms is well-established. Interestingly, unlike the gas-phase results for small peptides and solution-phase results for proteins, kinetic analysis showed, in all but one case, only a single rate constant for exchange. The presence of a single rate constant indicates that the ability to distinguish different sites by their reactivity was apparently lost.

From the published exchange rate data for peptides and proteins, it is clear that exchange rates are generally much lower for the large species; for example, Beauchamp and co-workers (Campbell et al., 1995; Campbell et al., 1994) reported that, for the singly charged glycine oligomers, $(gly)_n$, the exchange rates for D_2O, CD_3OD, and CD_3COOD were maximum for $n = 2$ and decreased as the number of gly residues increased. Thus, for D_2O, apparent rates for the first exchange were 3.1×10^{-10} and 2×10^{-12} cm^3 molec^{-1} sec^{-1} for $n = 2$ and $n = 5$, respectively. If the rates are normalized to rate/reactive hydrogen, then the difference is even more striking: 6×10^{-11} cm^3 molec^{-1} sec^{-1} for the dimer and 2×10^{-13} cm^3 molec^{-1} sec^{-1} for the pentamer. The drop in rates for peptides has been attributed to an internal solvation of the proton, which results in an effective increase in the basicity of the protonation site (Campbell et al., 1995; Schnier et al., 1995). The effect of solvation is also apparent in the complexes of protonated amino acids and sugars. The exchange rates for protonated amino acids are decreased by at least a factor of five upon complexation to sugars (Green et al., 1995) because of solvation of the protonated amine by the sugar. In the case of ND_3, Beauchamp and co-workers (Campbell et al., 1995) found that exchange rates did not decrease with an increasing size of the oligomer. Apparently, the PA of ND_3 is sufficiently high for the 'onium' mechanism to still operate.

Suckau et al. (Suckau et al., 1993) reported an exchange rate of 6×10^{-13} cm^3 molec^{-1} sec^{-1} (effectively normalized to the number of exchanges observed) for the +10 charge state of cytochrome c. The ADO collision rate (Su et al., 1973) for a +10 charge state ion should be an order of magnitude higher than for a singly charged species; the large difference between observed and theoretical rate constants implies a very low reaction efficiency. However, collision theory for large, multiply charged ions is not well-developed. The above-mentioned inability to discern different rate constants for cytochrome c suggests that some process is blurring rates that are distinguishable in small peptides and in solution-phase proteins. One possibility is that the nondistinct rates reflect an increased importance of intramolecular exchange, with slow exchange at a few active sites being followed by relatively rapid intramolecular exchanges that allow the deuterons to effectively diffuse from the original exchange site.

A follow-up study by the same group using improved instrumentation (Wood et al., 1995) found at least six gas-phase states of cytochrome c distinguishable by different levels of hydrogen exchange. Only one of the forms (113 exchangeable H's) closely coincided with a level of exchange found in the earlier study, perhaps because of the large number of changes made to the experimental protocol. In the absence of any perturbation, the different forms do not appear to interconvert in the gas phase. However, in this work the authors were able to induce the selected forms to unfold to other forms by adding energy (either collisionally or radiatively) or to fold up by deprotonating to reduce coulombic repulsion. The observation of a larger number of forms in the gas phase can be rationalized. Forms that interconvert rapidly in the solution phase may not do so in the gas phase, and, hence, become distinguishable. Alternatively, gas-phase forms may exist that have no corresponding solution-phase counterparts.

In a related study, Cassady and Carr (Cassady et al., 1996) used proton transfer, CID, and H/D exchange to investigate two distinct forms of the +12 charge state of ubiquitin produced by electrospray. It was possible to remove the most reactive form from the gas-phase mixture by proton transfer to an amine. They were able to demonstrate that the form that was most reactive to proton transfer was also most reactive with respect to H/D exchange. By comparing CID results for the mixture and for the pure slow-reacting form, they concluded that the two forms differed in the location of the 12th proton as well as in conformation. If, indeed, different forms of the same charge state differ in protonation site, then the relative importance of protonation site and conformation on H/D exchange rate must be considered. Intuitively, one might expect that, for a large multiply charged protein ion, a global conformational change would have a much larger effect on H/D exchange reactivity than the shift of a proton between two sites of presumably similar proton affinity. However, clarification of this difference requires a determination of just how many of the protonation sites of a multiply charged species are involved in reaction.

Recently, McLafferty and co-workers (Guan et al., 1996) investigated the location of H/D exchange sites on gas-phase cytochrome c. Cytochrome c was reacted with D_2O and the deuterated species subjected to CID. The fragment masses indicated that most of the exchange had occurred in the termini of the sequence. This exchange pattern is different from that observed in solution phase studies (Bai et al., 1995; Wand et al., 1986), in which it was shown by H/D exchange/NMR that the termini are among the most stable regions of cytochrome c in solution.

This difference in regions of fast exchange could be a consequence of either significant differences in gas-phase and solution structures or the differences in the solution and gas-phase exchange processes.

There is some evidence that even relatively small peptides can exhibit dual gas-phase reactivity. Zhang et al. (Zhang et al., 1996) reported that an undecapeptide showed two distinctly different exchange rates in reaction with D_2O. Distinguishing between conformational and protonation site effects should be easier in a relatively small peptide, because such species are simpler than proteins and are more accessible to theoretical modeling.

V. CONCLUSION

Ion-molecule reactions will continue to be an important probe of gas-phase ion structures. Their application to peptides and proteins, though recent, has already provided important information regarding the structure and intrinsic thermochemistry of these compounds. This information will, in turn, yield a better understanding of intramolecular interactions in proteins. The studies on small peptides will continue to complement the studies on more complex systems, with all studies being driven by the continuing intense interest in biological compounds.

It is likely that gas-phase probes will soon be applied to help unravel the details of complicated systems of molecular aggregates, including noncovalently bound complexes and solvated complexes. It is only a matter of time before relatively simple reactions of biological significance can be studied in the gas phase. A gas-phase enzymatic reaction would be a good example of such a system.

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