A Fast Flow Tube Study of Gas Phase H/D Exchange of Multiply Protonated Ubiquitin

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An electrospray ionization (ESI)/fast-flow technique has been applied to the study of gas phase hydrogen/deuterium (H/D) exchange kinetics. Multiply charged ubiquitin ions [ubiquitin + nH]$^{n+}$, in charge states n = 7−13, were reacted with ND_3. The behavior of ND_3 as exchange reagent is different from that of the previously studied reagents, D_2O and CH_3OD. Contrary to those, the maximum number of exchanged hydrogen atoms and the overall exchange rate were observed to increase with increasing charge state of the ubiquitin ions. The results are reagent-dependent because the exchange mechanisms are different for the different reagents. This observation is in agreement with a recent conclusion by Beauchamp and co-workers that contrary to the assumption often expressed in earlier studies, H/D exchange kinetics may not directly reflect ion structures. The results for all three reagents are, however, consistent with observations of previous ion mobility experiments that with increasing charge state the conformers change from more compact, partially folded structures to elongated nearly linear ones. H/D exchange of (ubiquitin + 13H)$^{13+}$ with ND_3 leads to two separated ion populations reflecting the possible existence of two conformers with different exchange rates. The ions (ubiquitin + 8H)$^{8+}$ and (ubiquitin + 11H)$^{11+}$ represent a partially folded structure and an unfolded structure, respectively, and were studied in greater detail. The relative abundances of ions were measured in steps of 0.5 m/z (mass-to-charge ratio), as a function of the ND_3 flow rate. The experimental results were simulated by computer fitted curves based on a recently developed algorithm. The algorithm allows the extraction of sets of grouped rate constants. Eight rate constant groups were deduced for each of the two ions. These rate constants correspond to 32 and 44 H/D exchanges for the 8+ and 11+ charged ions, respectively. The results indicate higher individual rates for most of the exchanged atoms in the 11+ ion compared to the 8+ ion.

Introduction

There has been an increasing interest in recent years in anhydrous protein and peptide ions.[1] Evidence has been presented[2] that conformational properties of biomolecules in solution are preserved during the process of electrospray ionization (ESI) that is used in mass spectrometry (MS) to introduce these biomolecules into the gas phase. Conformational changes in proteins were probed by hydrogen-exchange ESI-MS.[2,3] The generally held idea has been that compact structures protect some labile hydrogen atoms from hydrogen/deuterium (H/D) exchange in the gas phase and open conformers are expected to reach higher levels of exchange than compact ones. ESI was combined with Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS), and correlations were drawn between specific H/D exchange levels observed in the gas phase and conformations that have been characterized in solution.[4] H/D exchange was studied for shape-resolved conformers preselected through their drift velocities in gas-phase ion mobility experiments.[5]

The 2004 Nobel Prize in Chemistry was awarded for the discovery of ubiquitin-mediated protein degradation, a regulated process by which proteins are cleaved into peptides inside cells. Ubiquitin is a small protein of 76 amino acids and 144 exchangeable hydrogens for the neutral protein. It has been found in the gas phase in a number of charge states, [ubiquitin + nH]$^{n+}$ (n = 5−13).[6] Various charge states, notably [ubiquitin + 12H]$^{12+}$, were observed to exist in more than one conformation that could be distinguished by gas-phase deprotonation reactions, H/D exchange reactions and collision-induced dissociation (CID).[7] The cation conformations were resolved by gas-phase H/D exchange, using FT-ICR.[6] Conformations were studied also by ion mobility/mass spectrometry techniques,[8−10] and by electron capture dissociation (ECD).[11,12] In some instances, e.g., the 12+ state, H/D exchange displays two conformers[6] whereas the ion mobility experiments display single peaks,[8] i.e., single conformers, because the two conformers have nearly identical collision cross sections.[13] In other cases, notably for the 8+ state, the ions contain under H/D exchange conditions primarily a single isotopic distribution, suggesting a single conformation,[6] whereas ion mobility experiments display multiple conformations.[8]

We reported recently[14] on our first results concerning H/D exchange kinetics with ND_3 of the well-known[15,16] gas phase multiply protonated cytochrome *c* ions in charge states 10+ to 17+. The experimental setup we are using is a unique electrospray ionization (ESI)/fast flow apparatus, described previously.[17] This apparatus allows the determination of rather accurate rate constants under truly thermal conditions. In addition, the branching ratios for consecutive exchanges can be determined as a function of the flow rate of the deuterating reagent.

The present paper applies the ESI/fast flow apparatus to the study of the H/D exchange kinetics of ubiquitin. The aim of the kinetics is the extraction of site-specific rate constants for the replacement of labile hydrogen atoms located differently in

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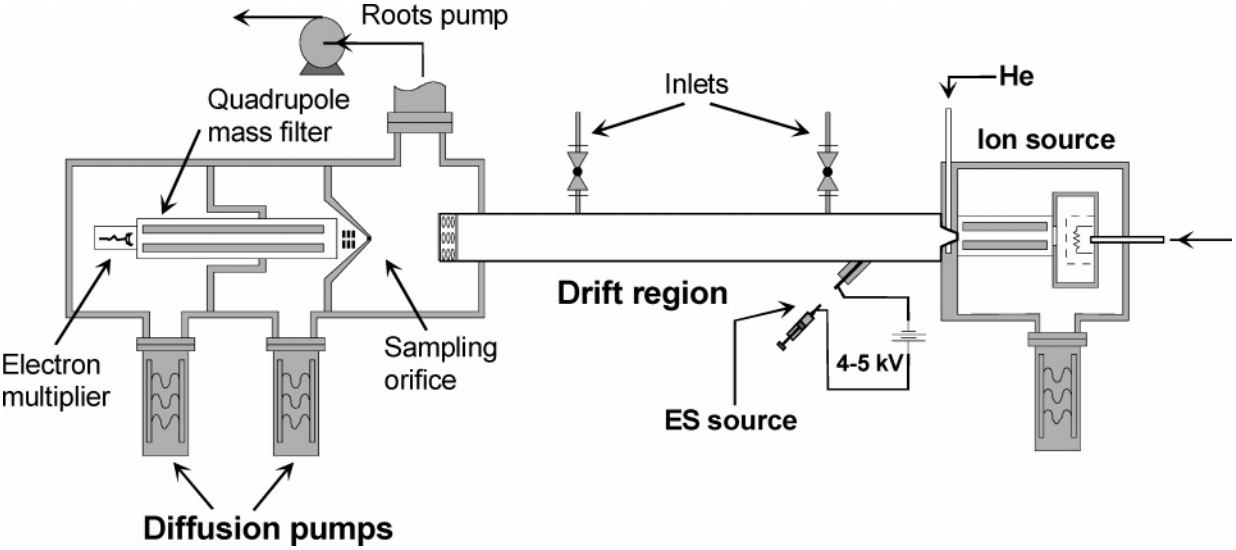

Figure 1. Schematic drawing of the experimental setup combining electrospray(ES) ionization with a fast flow technique.

the amino acid or peptide structure.[17d,18,19] The series of parallel and consecutive exchanges can be modeled using appropriate algorithms to deduce site-specific rate constants. We recently developed two new algorithms[20,21] for extracting site-specific rate constants for H/D exchange in gaseous protonated amino acids, their clusters and peptides. These were very successful in elucidating conformer structures for amino acid clusters. Our aim is to try to apply the algorithms to our data for ubiquitin in order to learn more about the conformers of the various charge states.

Experimental Section

The electrospray ionization/fast flow apparatus is shown schematically in Figure 1. It consists of an electrospray (ES) source connected directly to a flow tube. The apparatus is made of a flow reactor that is 123 cm in length and has an inner diameter of 74 mm. A neutral reagent is introduced into the flow tube through either one of two ring inlets. Tylan mass flow controllers define the flow rate of the neutral reactant into the flow tube. The quadrupole mass analyzer (652601 ABB EXTREL, Pittsburgh, PA) is housed in a differentially pumped chamber that is separated from the flow tube by a nose cone (NC) skimmer with a 1.0 mm sampling orifice. An NC voltage (5−20 V) is used for focusing ions into the analysis quadrupole. Helium buffer gas enters the flow tube at the upstream end through another Tylan flow controller. It is pumped through the tube by a Roots blower.

The electrospray ion source was designed as follows. A capillary tube serves as the interface between the electrospray and the helium flow reactor. Stainless steel tubes 15 cm in length and 0.05 cm i.d. are employed. The entire assembly is inserted into the flow tube at a distance of ∼96 cm from the sampling orifice, 135° to the direction of the helium flow, through an "O"-ring type vacuum fitting. A capillary tube of 0.05 cm i.d. introduces an air leak into the flow tube with a pressure of 0.065 Torr and a flow rate of 1 L/min (STP); these numbers have to be added to the helium pressure and helium flow rate when calculating rate constants. The experiments to be described were carried out at total flow velocities of ∼6000−7000 cm s^{-1}, leading to typical flow tube pressures of ∼0.1−0.35 Torr and reaction times of several ms.

Ions are electrosprayed ∼5 mm through ambient air into the grounded capillary tube from a nonconductive capillary made of fused silica tubing i.d. 50 μm biased at 5−6.5 kV DC. The contact between the power supply and the solution was provided by a metallic union some distance from the spray capillary tip. A large series resistor (∼10 GΩ) was placed between the power supply and the contact to the ESI to increase stability.[22] Dilute solutions (∼10^{-4}−10^{-5} M) of bovine ubiquitin from Sigma (Rehovot, Israel) in a polar solvent (methanol/water/acetic acid = 50/49/1 or 60/39/1) are delivered to the electrospray needle at flow rates of 1−5.5 μL min^{-1} from a 1 mL syringe mounted on a model 100 KD Scientific Syringe Pump(kdScientific, Holliston, MA). ND_3 was also from Sigma/Aldrich with a stated isotopic purity that exceeds 99.5 atom %D. Room temperature does not exceed 30 °C. The stainless steel capillary tube and the flow tube were heated and the temperature measured was 30−40 °C.

Results and Discussion

The first measurements involved mass spectra on the quadrupole mass spectrometer of the flow tube. The different charge states observed in the spectrum are due to different numbers of added protons. A typical spectrum containing ions 8+ through 13+ is shown in Figure 2. The ion 7+ was observed as well under some conditions. The mass spectrum we observe (Figure 2) is a low-resolution spectrum, whereas under high-resolution each of the peaks is observed to be made up of a large number of isotopic peaks.[4]

Upon introduction of ND_3 into the flow tube, each of the peaks in the mass spectrum is observed to shift upward by virtue of consecutive gas-phase H/D exchange reactions taking place in the flow tube. This shift can be converted to the number of hydrogen atoms exchanged through multiplication of *m*/*z* values by the corresponding charge. Figure 3 represents the percent deuterium incorporation for various charge states of ubiquitin plotted as a function of the concentration of ND_3 multiplied by the residence time of the ions in the flow tube. Similar plots were applied before[14a,23] in H/D exchange studies of cytochrome *c*. With increasing flow rate of the deuterating agent, the upward shift in the mass-to-charge ratio, *m*/*z*, increases and reaches saturation and a plateau value. The plateau region can be used to deduce the maximum number of hydrogen atoms exchanged. In previous measurements with D_2O as the exchange reagent, the maximum number of hydrogen atoms exchanged was observed in some instances to increase with increasing charge

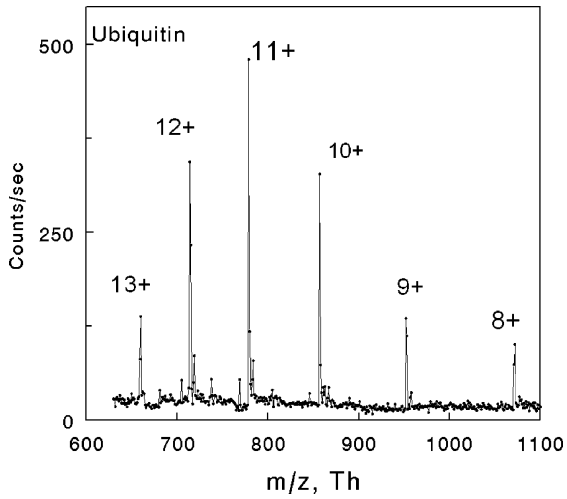

Figure 2. Mass spectrum for ubiquitin ions formed upon electrospraying a 50/49/1 (denatured) methanol/water/acetic acid solution; the relative abundance (in counts/ s) is plotted vs the mass to charge (*m*/*z*) ratio in Thomson units.

state,[4] and in other experiments, notably with ubiquitin ions,[6] it was observed to decrease with increasing charge state. A similar decrease was observed for ubiquitin ions when CH_3OD was used as the deuterating reagent.[24] D_2O and CH_3OD operate via the so-called "relay mechanism," in which the D_2O or CH_3OD molecule complexes with both the charge site and a neighboring basic site.[6] Although compact conformations possess fewer exposed hydrogens, the density of neighboring sites is greater. The situation is reversed for the elongated conformations. As the charge increases the conformations become more elongated because of Coulomb repulsion. The present data for ND_3 (Figure 3) demonstrate that the maximum number of hydrogen atoms exchanged increases with increasing charge state, as does the percent deuterium incorporation. This can be understood because ND_3 is a much stronger base than D_2O or CH_3OD, and exchange is not via the relay mechanism but rather by the so-called "onium mechanism".[25] The ND_3 molecule complexes with the charge site without involving neighboring basic sites. An endothermic proton transfer is rendered energetically feasible by simultaneous solvation of the resultant ammonium ion by the protein. In the higher charge states that have more elongated conformations as judged from their increased collision cross sections in ion mobility experiments,[8] more of the exchangeable hydrogens are exposed to ND_3, and the percent deuterium incorporation increases.

Kinetic data are normally extracted from flow tube experiments by measuring the decay of the reactant ion as a function of the flow rate of the neutral reagent (at a constant reaction time). Figure 4 presents the data for the 13+ ion of ubiquitin. The rate constants deduced on the basis of decay rates of the type shown in Figure 4 for the different charge states of ubiquitin are presented in Table 1. The rate constants are observed to increase with increasing charge state. In other words, the higher-charge states that have more elongated conformations undergo faster exchange with ND_3 than the lower-charge, more compact, states.

Beauchamp and co-workers have demonstrated recently[26] that in the process of H/D exchange, noncovalent complexation of the exchange reagent provides the energy required to access intermediates structurally distinct from the parent ion. They concluded contrary to the assumption often expressed in earlier studies, that H/D exchange kinetics may not directly reflect ion structures. The present ND_3 exchange rates and saturation data

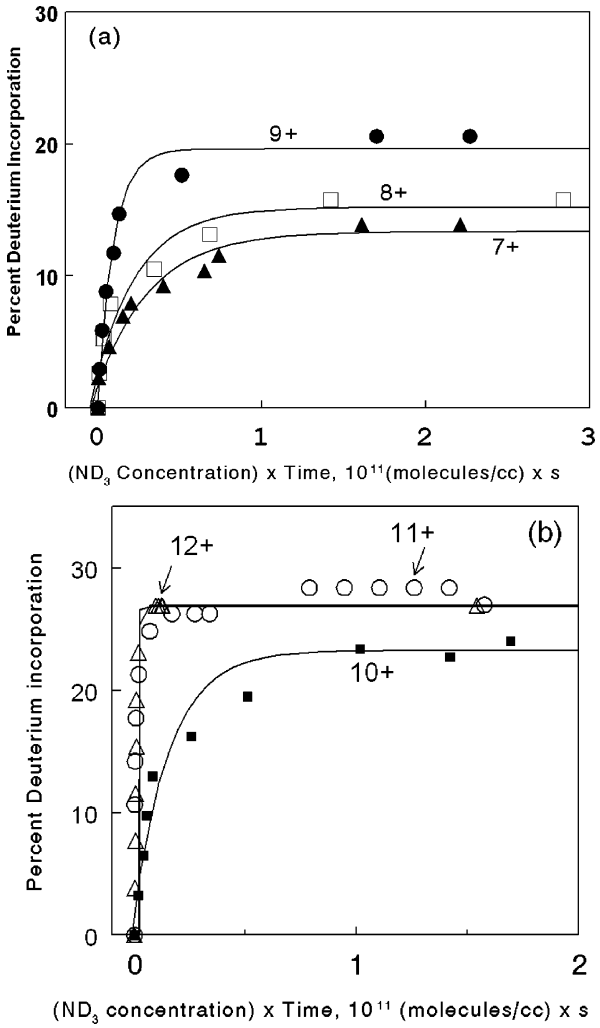

Figure 3. H/D exchange saturation profiles plotted against the ND_3 concentration (in molecules/cc) multiplied by the residence time in the flow tube (in seconds), for the various indicated ubiquitin charge states: (a) 7+, 8+, 9+; (b) 10+, 11+, 12+. The points are experimental whereas the curves are exponential fits. The exchange level is found from the *m*/*z* shift multiplied by the charge state; the percent deuterium incorporation is calculated from the number of H atoms exchanged and the total number of exchangeable labile hydrogens present. (It should be noted that the *m*/*z* shifts and the resulting plateau values were calculated from the positions of the maxima of peaks and each charge state demonstrates some additional higher exchanges albeit at lower abundances.)

for ubiquitin contradict the previous data for D_2O and CH_3OD because the exchange mechanisms are different. Since these mechanisms are known, the results can all be interpreted as indicating more elongated structures for the higher charge states and more compact structures for the lower charge states, in agreement with the ion mobility data.[8]

Ubiquitin has 13 basic sites (4 arginines, 7 lysines, 1 histidine, and the N-terminal amino group).[6] Exchange of the 13+ charge state with D_2O demonstrated a single isotopic distribution. This was interpreted as being due to the fact that all 13 basic sites are protonated and the protein must either adopt a single conformation or multiple conformations with similar exchange rates or multiple rapidly interconverting conformations.[6] However, exchange with ND_3 in the flow tube leads to separation into two isotopic distributions, see Figure 5. This is an indication for the existence of two conformations that have similar exchange rates with D_2O but different exchange rates with ND_3. As a result they separate out into two distributions in the present

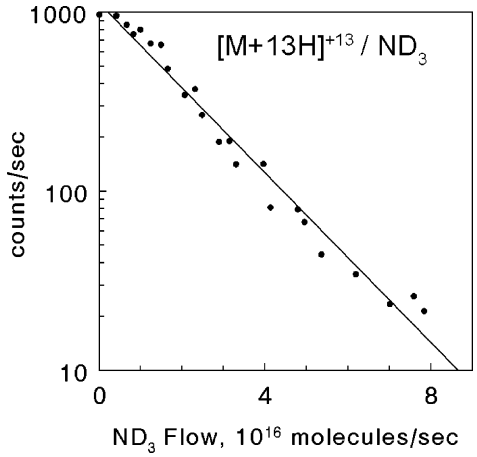

Figure 4. Semilogarithmic plot of the decay of the primary ion 13+ as a function of the neutral flow rate of ND_3.

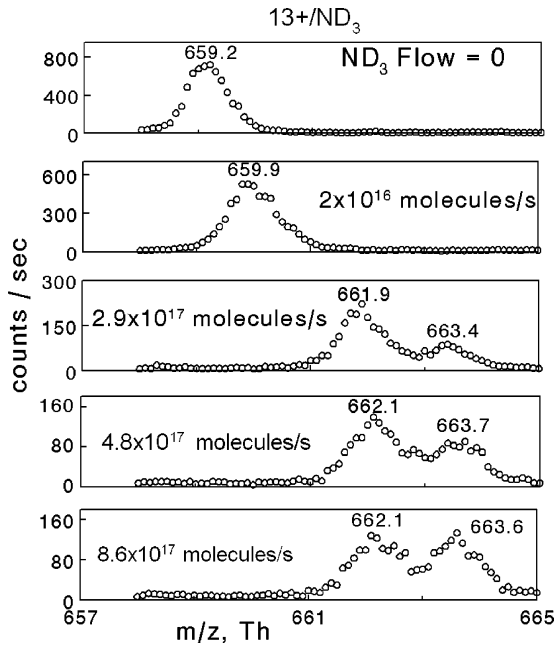

Figure 5. Mass spectra as a function of increasing flow rate of ND_3. Note the separation of the 13+ charge state into two peaks by virtue of different H/D exchange rates of two conformers. The *m*/*z* values for the two peaks correspond to 38 and 58 hydrogen atom exchanges, respectively, out of the 157 labile hydrogens present.

TABLE 1: Exchange Rate Constants with ND_3 (cc molecule^{-1} s^{-1}) for Various Ubiquitin Charge States

charge state	rate constant
7+	2.3×10^{-10}
8+	3.3×10^{-10}
9+	6.6×10^{-10}
10+	1.2×10^{-9}
11+	1.6×10^{-9}
12+	2.0×10^{-9}
13+	2.1×10^{-9}

experiment. This is another indication that H/D exchange kinetics may not directly reflect ion structures[26] and the outcome of the exchange experiment is reagent-dependent.

Each of the different charge states were subjected separately to H/D exchange with ND_3 and some of the data were fitted with our recently developed algorithms.[14b,20] To tackle the problem of 144 labile hydrogens (or more, depending on the extra number of protons), we have grouped several labile hydrogens together in the fit, so that the site-specific rate

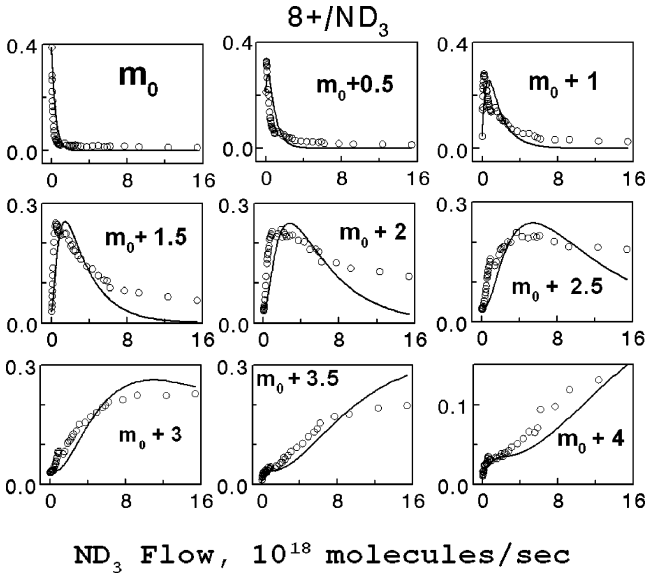

Figure 6. Relative abundance vs ND_3 flow rate in molecules/s for the unexchanged ion (ubiquitin + 8H)$^{8+}$, m_0 and for the various indicated cations for the $8 \times 4 = 32$ consecutive exchanges in the reaction of ubiquitin + $8H^+$ with ND_3. The reaction time is 4.8 ms; helium carrier gas flow, 1.2 L/min; flow tube pressure, 0.12 Torr. The open circles are experimental results; the continuous curves are the results of the simulation based on the algorithm described in this paper.[20] The mass-to-charge ratios *m*/*z* of the measured ions are presented as $m_0 + 0.5$, $m_0 + 1$, etc. The abundances contain contributions from ^{13}C, ^{15}N, and other natural isotopes in addition to the H and D isotopes. Convolutions with the natural isotopic abundance and the experimental peak shape were carried out for comparison of the simulated data with experiments.

constants are sum values for groups of labile hydrogen atoms. This approach is actually required for an additional reason, namely the quadrupole mass filter that we are employing in the flow tube experiment has a rather limited mass resolution.

Our ubiquitin mass spectrum (Figure 1) is characteristic for electrospraying denatured solutions.[27] We are presenting profiles of relative abundance data for H/D exchange, for the 8+ and the 11+ ions (Figures 6 and 7, respectively). According to ion mobility studies,[8,27] under our experimental denaturing conditions and low temperatures, the 8+ ion is a nearly single, partially folded, conformer. The 11+ ion was found to give a single isotopic distribution in H/D exchange experiments with D_2O[6] as well as ND_3 (present results). The 11+ ion is according to ion mobility studies, a single unfolded conformer with little tertiary structure.[8,27] Whereas the 9+ and 10+ ions demonstrate in their ion mobility spectra features corresponding to partially folded conformers in addition to unfolded conformers, the 11+ ion does not.[27] This shows up in our data (Figure 3b) in the sharp rise of the percent deuterium incorporation into the 11+ ion as a function of (ND_3 concentration) $\times$ (time), as well as in the high overall exchange rate constant (Table 1).

We denote in Figures 6 and 7 by m_0 the *m*/*z* value for the unexchanged ubiquitin ion. The relative abundances for *m*/*z* ions at m_0, $m_0 + 0.5$, $m_0 + 1$, ..., $m_0 + 4$ were measured as a function of ND_3 flow rate. The unit step of 0.5 *m*/*z* corresponds to a group of 4 exchanges in the case of the 8+ ion and 5.5 exchanges in the case of the 11+ ion.

The probabilistic algorithm used has been described in some detail previously.[20,21] Briefly, the probability that an H/D exchange of the *i*-th hydrogen atom (proceeding at a rate of k_i) has not yet taken place at time t is $-p_i(t) = \exp(-k_i t)$ whereas the probability that it has taken place is $p_i(t) = 1 - \exp(-k_i t)$. For molecules with more than one labile hydrogen atom, mass

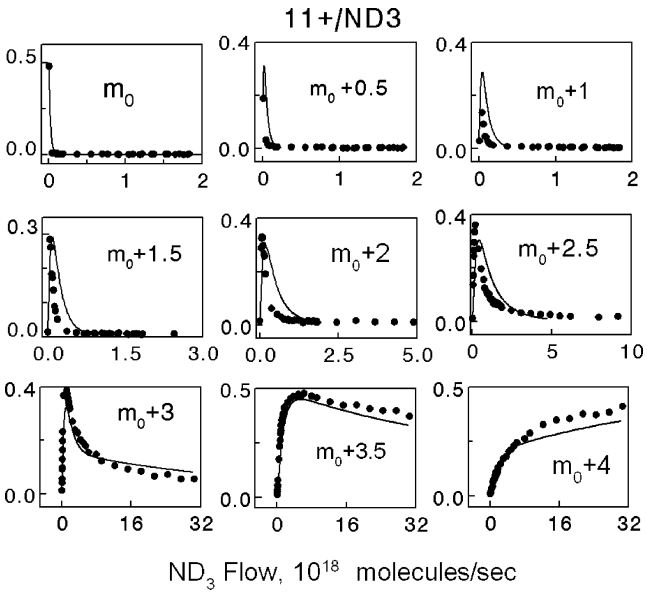

Figure 7. Relative abundance vs ND_3 flow rate in molecules/s for the unexchanged ion (ubiquitin + 11H)$^{11+}$, m_0 and for the various indicated cations for the $8 \times 5.5 = 44$ consecutive exchanges in the reaction of ubiquitin + $11H^+$ with ND_3. The reaction time is 12.2 ms; helium carrier gas flow, 4.1 L/min; flow tube pressure, 0.24 Torr. The filled circles are experimental results; the continuous curves are the results of the simulation (see caption to Figure 6).

TABLE 2: Grouped H/D Exchange Rate Constants (in units of cc molecule^{-1} s^{-1}) for the Reactions of (ubiquitin + 8H)$^{8+}$ and (ubiquitin + 11H)$^{11+}$ with ND_3[a]

group	(ubiquitin + 8H)8+	(ubiquitin + 11H)11+
1	1.0×10^{-12}	7.0×10^{-13}
2	8.6×10^{-12}	2.0×10^{-11}
3	8.8×10^{-12}	2.9×10^{-11}
4	8.8×10^{-12}	7.0×10^{-11}
5	1.8×10^{-11}	1.3×10^{-10}
6	4.4×10^{-11}	2.9×10^{-10}
7	1.3×10^{-10}	7.6×10^{-10}
8	1.3×10^{-10}	7.6×10^{-10}
sum	3.5×10^{-10}	2.1×10^{-9}

[a] Each group of rate constants contains 4 labile hydrogens in the case of the 8+ ion and 5.5 labile hydrogens in the case of the 11+ ion. The values given are sums of rate constants for individual members of the group (see text).

spectrometry reveals the number of accomplished H/D exchanges but not their position. Thus, *n* out of *N* H/D exchanges are experimentally observed, and the probability *P* that they have taken place is the sum of single-exchange probabilities. Minimizing the difference between the experimental data and the modeled probabilities $P_{n/N}(t)$, the site-specific H/D exchange rate constants k_i can be determined by nonlinear optimization.

The results of the simulations based on the algorithm we have used[20] are presented in Figures 6 and 7 for comparison with the experimental data. Since the experimental data presented are not isotopically deconvolved, for this comparison to be meaningful, the computed data are the results of convolutions with the natural isotopic abundances in the ions. The fits are not perfect but they allow us to deduce grouped H/D exchange rate constants that are presented in Table 2. The sums of the grouped rate constants (Table 2) are in quite good agreement with the corresponding overall rate constants for the 8+ and 11+ ions, respectively (Table 1). The average rate constant for a certain group of rate constants is obtained by dividing the numbers for the 8+ ion by 4 and for the 11+ ion by 5.5. Whereas the first two groups have similar low averages, groups 3−8 demonstrate increasingly higher averages for the 11+ ion compared to the 8+ ion. This is due to the fact that the 11+ ion has an unfolded, nearly linear, structure whose labile hydrogens are exposed to the ND_3 exchange reagent, whereas the 8+ ion is partially folded. Our data thus indicate not only a higher percentage of exchanged hydrogen atoms in the open conformer compared to the folded one, but also higher overall rates and higher individual rates for most of the exchanged atoms.

Conclusion

We have demonstrated, through H/D exchange kinetics of ubiquitin ions with ND_3, the agreement with the notion that compact structures protect some labile hydrogens from H/D exchange in the gas phase and open conformers reach higher levels of exchange than compact ones. This behavior is contrary to previous reports on H/D exchange with D_2O and CH_3OD. The use of a fast flow technique has allowed us to extract overall decay rate constants as well as sets of grouped rate constants for folded versus unfolded conformers. Future developments should lead to more detailed data and interpretations through the use of our more recently developed algorithm[21] in which hydrogens are grouped together according to their equivalent positions in the peptide or protein molecule. Further improvement would come from the use of high mass resolution Fourier transform-ion cyclotron resonance (FT-ICR) data. These developments should enable the extraction of site-specific rate constants, at least for small proteins of the size of ubiquitin.

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