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H/D exchange reactions of protonated diglycine; an electrospray ionization–flow tube reactor experiment

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Abstract

The H/D exchange reactions of protonated diglycine, GLY_2H^+, with ND_3 were studied under thermal conditions with a combination of an electrospray ion source and a flow tube reactor. Consecutive exchange of the five labile hydrogens is observed with increasing flow rate of ND_3. Collision complexes corresponding to the consecutive H/D exchanges are monitored for the first time. The role of multiple exchanges in a single collision event with ND_3 is probed. Results will be discussed in the light of previously suggested mechanisms of H/D exchange of GLY_2H^+ with deuterated ammonia. (Int J Mass Spectrom 182/183 (1999) 213–220) © 1999 Elsevier Science B.V.

Keywords: Peptides; ESI; SIFT; Ion complexes; H/D exchange

1. Introduction

There has been considerable interest in recent years in the analytical capabilities of mass spectrometry in the area of biomolecules and in the possibility of studying conformations of proteins by gas phase ion chemistry. As part of that effort, techniques have been developed for studying hydrogen-deuterium exchange between protonated peptides and deuterated solvent molecules such as ND_3, D_2O, and CH_3OD [1–7]. H/D exchange by mass spectrometry has complemented NMR to give insight into the populations and structures of folding intermediates [8]. The proposed H/D exchange mechanism invokes formation of an intermediate complex with multiple hydrogen bonding. Most of the work has been carried out under the very high vacuum conditions of Fourier transform ion cyclotron resonance (FTICR) ion sources and the complexes have not been observed directly. We have demonstrated recently [9] that an electrospray ion source combined to a flow tube reactor can be employed to study ion/molecule reactions of protonated diglycine under carrier gas pressures of several tenths of a Torr. We have furthermore observed the formation of collisionally stabilized complexes of GLY_2H^+ with NH_3, methanol and a series of amines and studied their formation kinetics. These are precisely the complexes which have been invoked as being instrumental for the H/D exchange mechanisms and the flow tube experiments are ideally suited for their study. The present experiment is a first effort in this direction, in which the reaction of protonated

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In memory of Ben S. Freiser who will always be remembered for his excellent science and good sense of humor.

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diglycine with ND_3 was studied and the H/D exchange of GLY_2H^+ was monitored in conjunction with the formation of the isotopically labeled collision complexes. ND_3 was chosen in this first study of H/D exchange carried out in the flow tube because it is known to be the most effective reagent for facile exchange of labile hydrogens in protonated peptides [2, 4–7].

The study of ion/molecule reactions of protonated peptides by means of a flow tube technique necessitates the introduction of these intact ions into the tube. The ability to combine electrospray ionization (ESI) with the flowing afterglow method has recently been demonstrated by Squires and his group [10]. We constructed a similar simple electrospray ionization source for our flow tube reactor and applied it to the study of reactions of GLY_2H^+ [9].

2. Experimental

We have constructed a selected ion flow tube (SIFT) apparatus some years ago, which has been described in detail elsewhere [11]. This apparatus has been modified to work with an electrospray ionization source connected directly to the flow tube, as in the work of Poutsma et al. [10] and not through the injector quadrupole; reactant ions were, thus, not mass selected. The system has been described in detail [9]. Briefly, the SIFT consists of a flow reactor that is 123 cm in length and 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 is housed in a differentially pumped chamber that is separated from the flow tube by a skimmer with a 1.0 mm sampling orifice. Helium buffer gas enters the flow tube at the upstream end near an electron impact ion source through another Tylan flow controller. It is pumped through the tube by a Roots blower with flow velocities of up to about 9000 cm s^{-1} with typical pressures of a few tenths of a Torr. The present experiments were run at pressures ranging from 0.1–0.4 Torr and at either one of the following reaction times: 2.3 or 6.6 ms.

The electrospray ion source was designed following [10]. A capillary tube serves as the interface between the electrospray and the helium flow reactor. Stainless-steel tubes 15–31 cm in length and 0.05 cm i.d. are employed. The entire assembly is inserted into the flow tube at a distance of $\sim$ 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 0.07 Torr and a flow rate of 1.3 L/min (STP); these numbers have to be added to the helium pressure and helium flow rate when calculating rate constants. Ions are electrosprayed $\sim$ 10 mm through ambient air into the grounded capillary tube from a stainless steel syringe needle biased at 4700 V dc. Dilute solutions of the analyte of interest in a polar solvent are delivered to the electrospray needle at flow rates of 3.3 μL min^{-1} from a 5000 μL syringe mounted on a model 100 KD Scientific Syringe Pump.

Second-order rate coefficients are obtained by monitoring the intensity of the primary ion decay as a function of the neutral gas B concentration introduced downstream. Product ion distributions are obtained by plotting the percentage of each product ion as a function of the gas B flow rate. Isotopic exchange is thus followed as a function of the concentration of ND_3 at a constant reaction time. Product ion distributions are corrected via the measured mass discrimination factors of the detector quadrupole mass filter and for ^{13}C contributions. Extrapolating product ion percentage curves to zero flow rate yields the percentage distribution of the primary products.

Diglycine was a sample from Sigma with a stated minimum purity of 99%. A 7.5×10^{-5} M solution of GLY_2 in 1% formic acid, 50% methanol and 49% water was employed to electrospray the protonated diglycine. ND_3 was from Cambridge Isotope Laboratories with a stated isotopic purity of $\geq$ 99 at % D.

3. Results and discussion

Protonated diglycine, GLY_2H^+ (or GGH^+), $^+H_3NCH_2CONHCH_2COOH$, was allowed to react

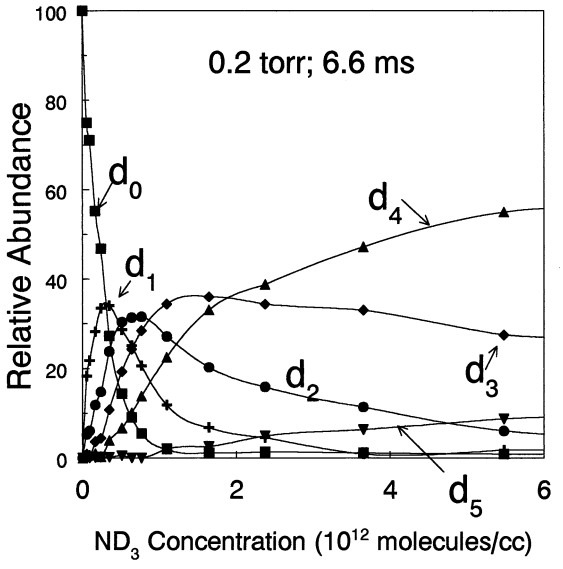

Fig. 1. H/D exchange of Gly_2H^+ with ND_3; The relative abundances of the Gly_2H^+ isotopomers are plotted as a function of the ND_3 concentration in the flow tube at a total (He plus air) flow tube pressure of 0.2 Torr and a reaction time of 6.6 ms. The symbol d_0 represents the unexchanged reactant ion while d_n, $n = 1 - 5$ are Gly_2H^+-d_n ions which have undergone 1–5 H/D exchanges.

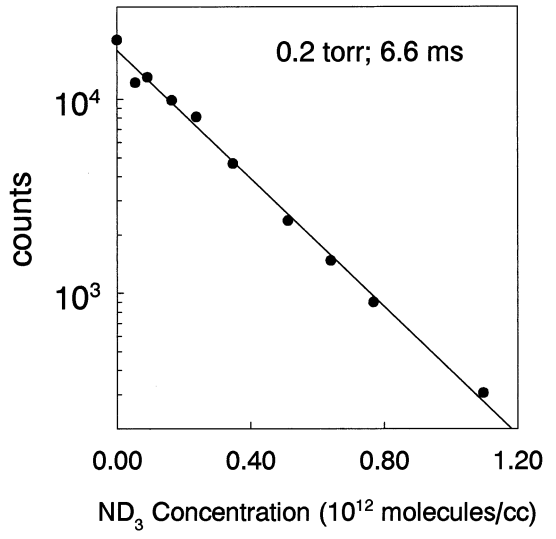

Fig. 2. Semilogarithmic plot of the decay of primary ions as a function of the neutral concentration for the reaction of protonated diglycine with ammonia-d_3 at a total flow tube pressure of 0.2 Torr.

with ND_3 and the incorporation of deuterium was monitored as a function of ND_3 flow rate at a constant reaction time. The ND_3 flow rate, $f(ND_3)$ in molecules/s, was converted to concentration, $[ND_3]$ in molecules/cc, through the relation:

$$[ND_3] = (\text{molecule/s})/(\text{cc/s}) = f(ND_3)/v\pi a^2 \quad (1)$$

where v is the average flow velocity of the carrier gas (in cm/s) and a is the radius of the flow tube. Typical results are plotted in Fig. 1 for a flow tube pressure of 0.2 Torr and a reaction time of 6.6 ms. The general kinetic behavior of the GGH^+/ND_3 exchange is very similar to that obtained previously by Beauchamp and co-workers [5] and Nibbering and co-workers [7] although these authors have employed FTICRs at very low pressures of $\sim 10^{-7}$ Torr and very long reaction times of up to 20 s [5] or even 120 s [7]. There are five labile sites in GGH^+, three N-terminus amino hydrogens, one amide hydrogen and one C-terminus carboxylic acid hydrogen, and they are all exchanged by ND_3 because it has a relatively high proton affinity of 204 kcal/mol [12].

Semilogarithmic plots of the decay of the GGH^+ as a function of the neutral flow rate were employed to deduce reaction rate constants. A typical result is shown in Fig. 2. The rate constant deduced from this experiment is 5.8×10^{-10} cm^3 molecule^{-1} s^{-1}. The average rate constant from a number of experiments is $(6.3 \pm 2)\ 10^{-10}$ cm^3 molecule^{-1} s^{-1} in excellent agreement with the results of [5] but lower than that of [7]. FTICR measurements of rate constants have several problems: (a) vibrationally and translationally hot populations which cool down with increasing reaction time [5]; (b) difficulty in accurate definition of the pressures [7]. Reactions in the flow tube are with thermal populations of ions and well defined pressures and reaction times. On the other hand, the flow tube experiments suffer, like the FTICR experiments or even more, from difficulties in determining the degree of isotopic exchange of the ND_3 reagent with the inlet system walls prior to reaction. Such H/D exchanges can lead to lowering of the apparent rate constants measured in the GGH^+/ND_3 reaction. Some measure of this exchange can be obtained from observation of ND_2H and NH_2D containing $[GGH^+/NX_3]$ (X = H or D) complexes. The present series of

experiments was run under conditions such that the contribution from such complexes as well as from water containing complexes was minimized. We estimate that our ammonia reactant contains more than 85 at % D.

ND_3 has the potential to participate in multiple deuterium exchanges. It was demonstrated [5] that ND_3 partakes in multiple exchanges during a single-collision event with Gly_2H^+ 28% of the time. This was achieved through observation of the reduction in higher mass exchange products upon continuous ejection of the d_1 species out of the cell in an FTICR experiment. We could check this point by determining the branching ratios between the primary products using extrapolation of product concentrations to zero flow rate. In these measurements we have also included the $[GGH^+/NX_3]$ complexes at several different flow tube pressures. At 0.38 Torr the primary products are the three exchange products: $d_1(m/z\ 134) = 53 \pm 5\%$; $d_2(m/z\ 135) = 17 \pm 2\%$; $d_3(m/z\ 136) = 6 \pm 2\%$ and the unexchanged ND_3 complex $[d_0/ND_3](m/z\ 153) = 24 \pm 4\%$. At 0.13 Torr these branching ratios change to: $d_1 = 72\%$; $d_2 = 16\%$; $d_3 = 9\%$ and $[d_0/ND_3] = 3\%$. (It should be emphasized that exchange inside the collisionally stabilized complex cannot be observed experimentally because there is no change in the mass-to-charge ratio value. As a result, whenever we refer to $[d_0/ND_3]$ it can very well include $[d_1/ND_2H]$, $[d_2/NDH_2]$, and $[d_3/NH_3]$.) Some experiments at low pressures yielded even lower branching ratios for $d_3(m/z\ 136)$, but $d_2(m/z\ 135)$ was observed as a primary product in all the experiments. These data demonstrate two significant results: (a) ND_3 does indeed partake in multiple exchanges in a single-collision event and (b) The formation of the collisionally stabilized unexchanged complex is strongly pressure dependent. We will see more evidence to this latter effect below.

The percentage abundances of the exchange products, as well as their collisionally stabilized ND_3 complexes, are plotted as a function of ND_3 concentration for two flow tube pressures, 0.16 and 0.36 Torr, in Figs. 3 and 4, respectively. Both of these experiments are for a 2.3 ms reaction time. The symbols 0, 1, 2, 3, 4, and 5 represent the unexchanged ion, the one which has undergone a single H/D exchange, with two atoms exchanged etc. Figs. 3(a) and 4(a) are for the uncomplexed ions while Figs. 3(b) and 4(b) are for the complexed ions (note the change of scale between Figs. 3(b) and 4(b). Two major results become apparent: (1) The relative importance of complexed ions increases with increasing flow tube pressure as expected. (2) The relative importance of complexed ions increases with the progress of the H/D exchange. We think that this latter point is of great significance. Collisions between protonated diglycine and ND_3 lead to chemically activated complexes which can in turn lead to two observable reaction products: Collisionally stabilized complexes and protonated diglycines which have undergone H/D exchange. This is exemplified in Eq. (2) for the protonated diglycine-d_n ion:

$$GLY_2H^+\text{-}d_n + ND_3 = [GLY_2H^+\text{-}d_n/ND_3]^* \quad (2a)$$

$$[GLY_2H^+\text{-}d_n/ND_3]^* + He \rightarrow [GLY_2H^+\text{-}d_n/ND_3] + He \quad (2b)$$

$$[GLY_2H^+\text{-}d_n/ND_3]^* \rightarrow GLY_2H^+\text{-}d_{n+1} + ND_2H \quad (2c)$$

The branching ratio between reaction channels (2b) and (2c) depends on the value of n in $GLY_2H^+\text{-}d_n$. For example at 0.36 Torr (Fig. 4), for $n = 0 - 2$, most of the reaction product is the exchange product, $d_1 - d_3$ (channel (2c)), while the reverse is true for $n = 3$ or 4 (leading to d_4 and d_5 exchange products, respectively) for which the collisionally stabilized complex (channel (2b)) is more abundant. (The value $n = 5$ can only lead to collisional stabilization). This result points to lower H/D exchange rates for the 4th and 5th deuterium exchanges in agreement with the FTICR results of Campbell et al. [5].

Fig. 5 gives an alternative presentation of the data (at 0.36 Torr). The collisionally stabilized complexes, $[GLY_2H^+\text{-}d_n/ND_3]$, are observed to track the corresponding uncomplexed $GLY_2H^+\text{-}d_n$ ions, having the same degree of isotopic exchange, n. Of special interest are those ions, e.g. $GLY_2H^+\text{-}d_2$ and its ND_3

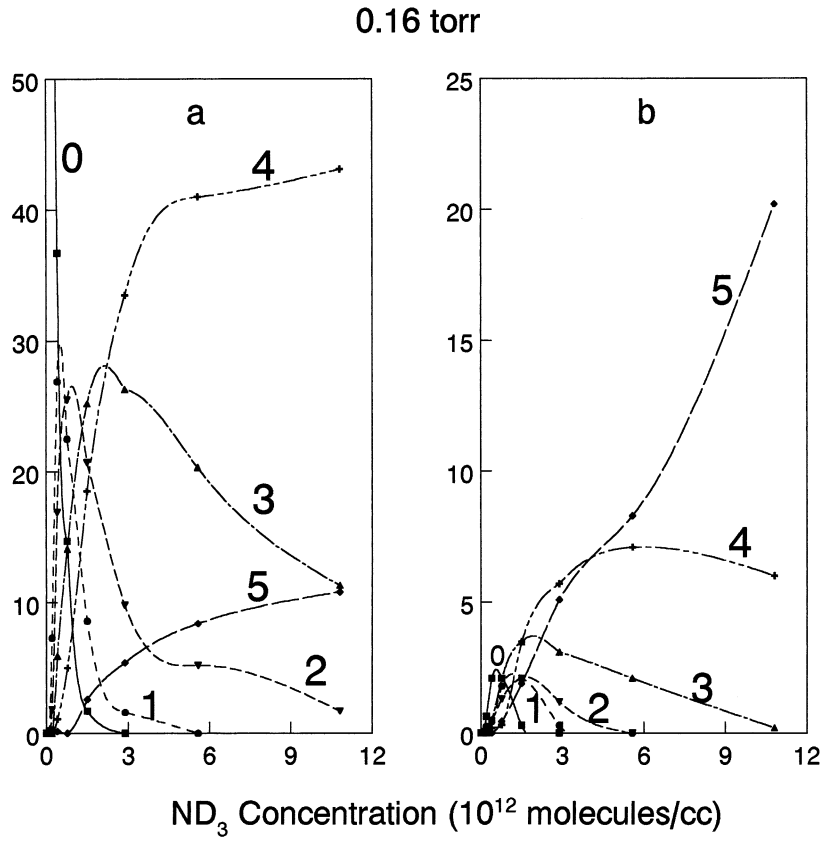

Fig. 3. Relative abundances of (a) Gly_2H^+-d_n, $n = 0 - 5$ ions and (b) collisionally stabilized [Gly_2H^+-d_n/ND_3] ion complexes, as a function of ND_3 concentration, at a total flow tube pressure of 0.16 Torr. Note the factor 2 expansion of the abundance scale in panel (b) compared to (a). The lines are drawn to lead the eye.

complex (Fig. 5(c)) which seem to indicate two separate chemically activated precursor complexes: (a) [GLY_2H^+-d_0/ND_3]* at low ND_3 concentrations and (b) [GLY_2H^+-d_1/ND_3]* at higher concentrations.

What is the nature of the collision complexes which lead to H/D isotope exchange? Beauchamp and co-workers [5] have suggested an "onium" mechanism for the H/D exchange of protonated diglycine. According to this mechanism an ion dipole complex is first formed between GLY_2H^+ and ND_3. This is followed by proton transfer from the N-terminus to ND_3 forming an ammonium ion. This proton transfer occurs in the complex although the proton affinity of ammonia is lower than that of diglycine, because it is assisted by simultaneous solvation of the ammonium ion by the carbonyl oxygens of the peptide. A multiply hydrogen bonded complex is, thus, formed in a relatively deep potential well. On the other hand, Nibbering and co-workers [7] have come to the conclusion that because of the high proton affinity of ND_3 compared to those of the alternative exchange reagents (D_2O or CH_3OD) reversible proton transfer has become energetically accessible and does not necessarily require specific multiple hydrogen-bonded stabilized intermediate complexes. According to their results—specifically H/D exchange rate constants which are equal to the collision rates for all five labile hydrogens—randomization within the reaction complex of the three ammonia and five active hydrogens leads to random incorporation of deuterium atoms. Our present results support the formation of multiply hydrogen-bonded complexes also in the case of ND_3,

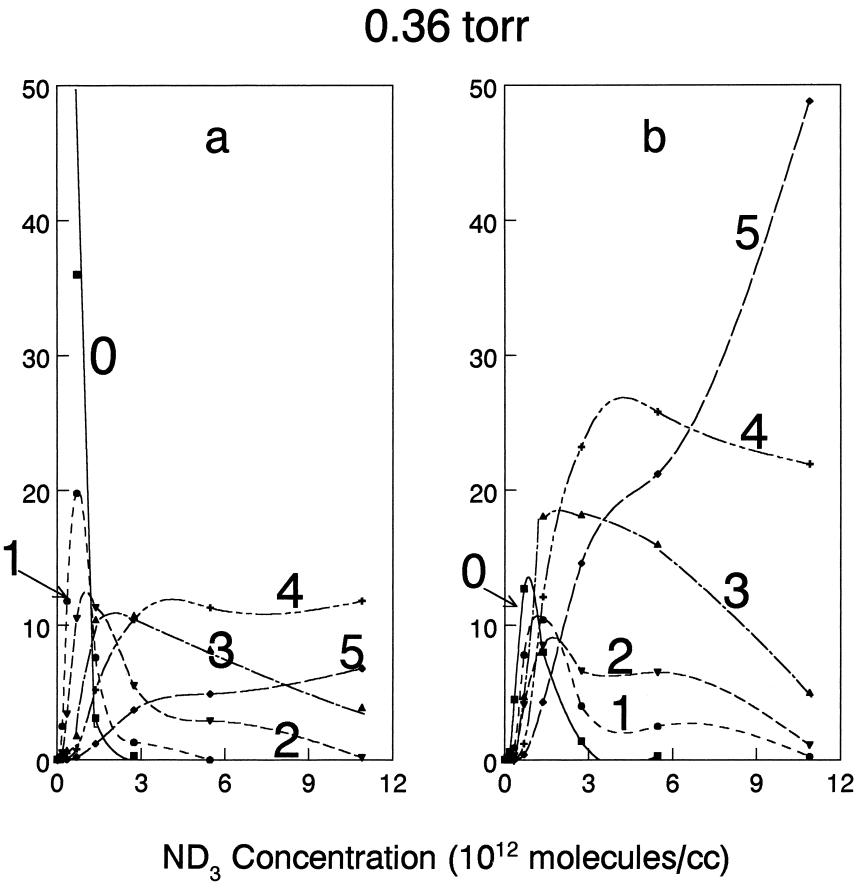

Fig. 4. Same as caption for Fig. 3 except for a total pressure of 0.36 Torr. Note that the abundance scales for panels (a) and (b) are equal.

because the exchange rates drop for the fourth and fifth exchange and all the rates are lower than collision rates. Furthermore, the difference between the proton affinities of ND_3 and diglycine is considerably higher than originally assumed [7] because the value for diglycine has been determined to be 223.4 kcal/mol [13]. This high value is in agreement with our recent bracketing experiments [9]. It leads to a calculated proton affinity difference in access of 19 kcal/mol between ammonia and diglycine. As a result, proton transfer to ammonia has to be assisted by hydrogen bonding to other sites such as the carbonyl groups. A tautomer mechanism was proposed for the exchange of the amide hydrogens with ND_3, while semiempirical calculations suggest that exchange of the C-terminus hydrogen proceeds via formation of a salt bridge with the ND_3, which deprotonates the C-terminus acid group, with the nearby N-terminus stabilizing the resultant ion pair [5]. The existence of salt bridge gas phase structures for oligoglycines [14] and betaine [15] has come under scrutiny, recently. As noted by Bowers and co-workers [14], the ion chromatography technique used for the oligoglycines monitors the ion ground states while the H/D exchange experiments probe transition structures while coordinated to the exchange reagent. Whatever the mechanisms for the exchange of the fourth and fifth hydrogen are, our results indicate higher exchange barriers for these hydrogens which result in preferential collisional stabilization of the precursor complexes.

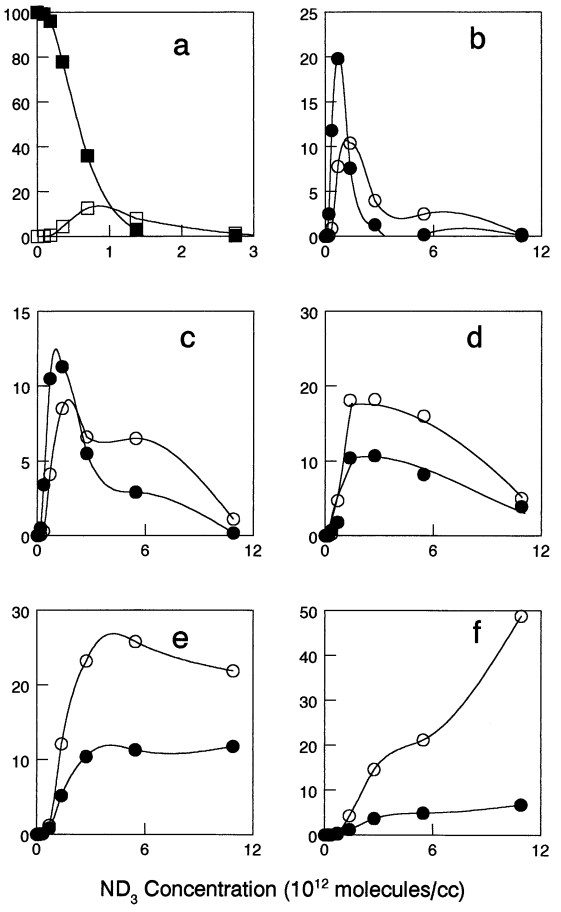

Fig. 5. Relative abundances at 0.36 Torr as a function of ND_3 concentration: Gly_2H^+-d_n (filled symbols); [Gly_2H^+-d_n/ND_3] (open symbols); (a) d_0; (b)-d_1; (c)-d_2; (d)-d_3; (e)-d_4; (f)-d_5.

4. Conclusions

Deuterium exchange reactions of protonated peptides can be carried out with a flow tube technique. Because of the relatively high pressures employed, the progress of H/D exchange can be monitored not only for the protonated peptides but also for their complexes with the exchange reagent used (ND_3 in the present experiments). All five labile hydrogens of protonated diglycine can be exchanged, however the reaction efficiency for the exchange of the first three is higher than for the last two, as judged by the competition with collisional stabilization of the corresponding precursor complexes. Further experimental studies are planned of larger protonated peptides including bradykinin, as are theoretical simulations of the experimental results and density functional theory (DFT) calculations of the structures and energetics of the complexes. The role of stabilized collision complexes may be crucial for the understanding of protein conformations through H/D isotopic exchange experiments.

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