Conformationally Driven Gas-Phase H/D Exchange of Dinucleotide Negative Ions

Dorothee Balbeur,^a Dominique Dehareng,^b and Edwin De Pauw^a

^a Laboratory of Mass Spectrometry, University of Liège, Liège, Belgium

^b Center for Protein Engineering, University of Liège, Liège, Belgium

Gas-phase hydrogen/deuterium exchange of six deprotonated dinucleotides with CD₃OD was performed in the second hexapole of a Fourier transform ion-cyclotron resonance (FTICR) mass spectrometer. To complete these experiments, dynamic simulations were carried out to investigate the different conformations adopted by the dinucleotides. In the experimental conditions and in integrating the experimental and theoretical results, H/D exchange was shown to be controlled by hydrogen accessibility and not by the chemical nature of the heteroatom bearing the exchangeable hydrogen. A model including simultaneous H/D exchanges at the experimental time scale was used to reproduce the dinucleotide H/D exchange kinetic plots. The relay mechanism was not relevant for dinucleotides. This allowed the H/D exchange rates to be directly linked to conformations. (J Am Soc Mass Spectrom 2007, 18, 1827–1834) © 2007 American Society for Mass Spectrometry

The conformations of biomolecules depend on intramolecular noncovalent interactions. These interactions determine, at a molecular level, a vast majority of biological processes (molecular recognition, regulation, transport, etc.) that define the function of the biomolecule. Hydrogen/deuterium (H/D) exchange combined with mass spectrometry is an efficient tool for studying the gas-phase structure of biomolecules [1–16]. The rate of gas-phase H/D exchange has been shown to be a function of the reagent used for exchange, the concentration of this reagent, the charge states of the biomolecule, the gas-phase basicity/acidity of exchangeable sites, and of the internal structure of the biomolecular ions [1–16]. So the determination of the exchanged hydrogen number and of the H/D exchange kinetics allows structural isomers of the biomolecule to be distinguished, and structural and thermochemical features to be deduced. H/D exchange reactions have been performed using a Fourier transform ion-cyclotron resonance (FTICR) mass spectrometer [1–11] or a quadrupole ion trap [10–16]. Though these two instruments show several differences, they allow the ions to be trapped for variable periods of time in the presence of the deuterating reagent held at a constant pressure.

Deoxyribose monophosphate nucleotides are the structural units of DNA. The gas-phase H/D exchange of these units have been carried out in positive [7, 8] or in negative [3–5, 8] ion mode with an FTICR-MS and, more recently, in negative ion mode with a quadrupole ion trap [16]. These different studies have shown that

the extents and rates of exchange depend on the nucleobase (identity, orientation, and gas-phase acidity of the exchangeable hydrogen) and on the position (3' or 5') and flexibility of the terminal phosphate group. They have also concluded that the most probable mechanism of exchange is the relay mechanism. This mechanism requires the formation of a hydrogen-bonded complex between a hydrogen donor site of the nucleotide, the deuterating reagent, and a deuterium acceptor site (the phosphate group) of the nucleotide, the deuterating reagent bridging the two sites of the nucleotide.

The present work focuses on the examination of the gas-phase H/D exchange behavior of six deprotonated dinucleotides. The gas-phase conformations of dinucleotides have already been investigated by molecular modeling calculations and ion mobility experiments [17]. The dinucleotides adopt distinctive conformations that interconvert at the experimental time scale of an FTICR-MS. To determine the lifetime of the distinctive structures, experimental conditions (the type and pressure of the exchange gas) were chosen such that the H/D exchange reaction was not the limiting step. The data presented here underline the importance of hydrogen accessibility to explain the rates of hydrogen exchange. The accessibility of the exchangeable hydrogen is controlled not only by the distinctive structures adopted by the dinucleotide but also by the protection extent of the hydrogen in these structures, which is linked to the mean time spent in the various isomeric structures. The relay mechanism is not relevant for dinucleotides as the distances between the hydrogen donor and deuterium acceptor sites are not compatible.

Address reprint requests to Dr. D. Balbeur, Laboratory of Mass Spectrometry, University of Liège, 3 Allée de la Chimie Bat. B6c, B-4000 Liège, Belgium. E-mail: dbalbeur@ulg.ac.be

Experimental

Sample Preparation

The six dinucleotides (dTt, dAt, dAA, dTG, dAG, and dGG) were obtained from Eurogentec (Seraing, Belgium). CD₃OD was purchased from Euriso-Top (Gif sur Yvette, France). All of these compounds were used without further purification. Stock solutions of the dinucleotides (400×10^{-6} M) were prepared in water. Working solutions (1.1765×10^{-5} M) were obtained by dilution in water. The injection solutions were prepared by adding the appropriate amount of MeOH to obtain a MeOH/H₂O ratio of 15/85 (vol/vol) and a dinucleotide concentration of 10^{-5} M.

Mass Spectrometry

Experiments were performed with a 9.4 tesla Apex-Qe FTICR mass spectrometer (Bruker Daltonics, Billerica, MA). The dinucleotide solutions were infused via an external Apollo electrospray ion source at a flow rate of 120 μ L/h with the assistance of N₂ nebulizing gas. The off axis sprayer was grounded, the end-plate was set to 3 kV and the inlet capillary was set to 3.5 kV for the generation of dinucleotide anions. N₂ heated drying gas (250 °C) was applied to assist desolvation of ESI droplets. Ions were accumulated in the first hexapole for 1.5 s and transferred non-mass selectively through the quadrupole into the second hexapole for 10 ms. They were trapped for variable periods of time in this second hexapole (h2) to carry out H/D exchange reactions, and were then transferred through high voltage ion optics and captured by static trapping in an ICR cell. All mass spectra were acquired with XMASS (version 7.0.8; Bruker Daltonics) in broadband mode from m/z 350 to 900 with 512 k data points and summed over 5 scans. A mass list, in which m/z values and peak heights are recorded, was created by DataAnalysis (version 3.4; Bruker Daltonics).

H/D Exchange

All of the H/D exchange reactions were conducted in the gas phase in the collision cell (second hexapole h2) of the 9.4 tesla Apex-Qe FTICR mass spectrometer. CD₃OD was used as the deuterating agent. Due to its vapor pressure at ~ 20 °C, it was introduced into the second hexapole through the nozzle valve usually used for the collision gas (Ar). Using this system, only CD₃OD was introduced into h2 with a measured pressure in the surrounding vacuum chamber of 3.75×10^{-6} torr, which corresponds to a pressure in the collision cell of $\sim 7.5 \times 10^{-4}$ torr. The extent of exchange was observed by monitoring the relative abundance of the precursor ion and of the deuterated species while the CD₃OD pressure was held at $\sim 7.5 \times 10^{-4}$ torr. Ions were accumulated in the collision cell for 10 ms and were then trapped for H/D exchange reactions from

10 ms to 600 s (the ions entered the hexapole in one group and no other ions entered during the exchange time). The six dinucleotides were analyzed individually on the same day under identical conditions. The experiments were repeated on other days for the same reaction times under conditions as similar as possible to assess reproducibility.

To analyze H/D exchange spectra, an in-house software was used. This software utilizes the known isotopic distribution (percentage of each peak) of the non-deuterated ion. Moreover, it is considered that the ion with a fixed number X , of exchanged hydrogens has the same isotopic distribution but shifted by X/z unit. The software determines, at each reaction time, the composition of each deuterated species that fits best to the experimental isotopic distribution. The plotting of these compositions to obtain the kinetic plots was done with SigmaPlot (version 9; Systat Software, Inc., San Jose, CA). Several methods for quantitation of peak magnitudes exist and are based on peak height or peak area [18]. In this work, isotopic distributions were described from relative peak heights as advised by A. G. Marshall et al. [19]. Peak area measurements can be problematic because of the presence of wiggles, known as "Gibbs oscillations," in FTICR mass spectra. These oscillations result from truncation of the time-domain ICR signal at time, t , before it has decayed to zero.

Molecular Modeling

Dynamic simulations were carried out with HyperChem (version 7; Hypercube, Inc., Gainesville, FL) to investigate the different conformations adopted by the dinucleotides. BIO+ (CHARMM) was used as the force field. Before dynamic simulations, the charges of different atoms were recalculated; atoms of the phosphate group, the first carbon and hydrogen atoms near the phosphate group, and the hydrogen atoms of 3' and 5' OH extremities. These charge recalculations were performed by single point calculation with ZINDO/1 (semi-empirical SCF method). The net atomic charges were recalculated to take into account the negative charge on the phosphate group. For each deprotonated dinucleotide, two molecular dynamics simulations of 10,000 ps at ~ 300 K were done. In these simulations, snapshots were saved every 0.05 ps and distances between key atoms were monitored. The two dynamic simulations differed by molecule conditioning. For the first simulation, the deprotonated dinucleotide was heated for 100 ps at 900 K and then cooled during 152 ps from 900 to 300 K. For the second simulation, the ion was geometrically optimized, heated during 150 ps from 0 to 300 K, and then kept for 50 ps at 300 K.

It has been shown via REMPI spectroscopy [20] and in modeling calculations [6, 21–23] that guanine favors a syn orientation in the gas phase. Syn and anti are terms that are associated with the orientation of the nucleobase with respect to the sugar. In the anti orientation, the six-membered pyrimidine ring in purines

and O₂ in pyrimidines are pointing away from the sugar, and in the syn orientation, it is over or toward the sugar [24]. To analyze the behavior of deprotonated dTG, dAG, and dGG in the previous molecular dynamics calculations, ab initio geometry optimizations with syn and anti conformations of G in the 3' position were performed with Gaussian 03 [25]. To compare the minimized energy obtained for the two conformations syn and anti, different levels of theory and different basis sets were used; RHF/6-31G-*aug*,

B3LYP/6-31G-*aug*, RHF/6-31*G-*aug*; *aug* means that the basis set is augmented with diffuse functions on the oxygens and nitrogens and polarization functions on phosphorus. The added basis functions were each represented by one Gaussian function. They corresponded, on the one hand, to s and p diffuse functions for oxygen and nitrogen atoms (with an exponent equal to 0.0845 and 0.0639 for oxygen and nitrogen atoms, respectively) and on the other hand to d polarization functions for the phosphorus atom

Figure 1. Representative mass spectra for the reaction of deprotonated dTG with CD₃OD in the second hexapole of an FTICR-MS. H/D exchange reaction times are (a) 0 s, (b) 30 s, (c) 60 s, (d) 150 s, and (e) 600 s.

(with an exponent equal to 0.55). Five starting points were used for each conformation.

Kinetic Simulations

To analyze the H/D exchange kinetics, the software Matlab (version 7.3.0; The MathWorks, Inc., Natick, MA) was used. This software allows personal sets of coupled differential equations and personal sets of rate constants to be introduced. The construction of kinetic plots was done to reproduce H/D exchange experimental plots.

Results and Discussion

H/D Exchange

The typical progression of the hydrogen/deuterium exchange reactions of the deprotonated dinucleotides with CD_3OD in the second hexapole of the FTICR-MS is illustrated in the case of dTG^- in Figure 1. As the reaction time increased from 0.01 to 600 s, the increase of exchanged hydrogen number [D(X) corresponds to X exchanged hydrogen atoms] was observed with a corresponding decrease of the abundance of the precursor ion, D(0). As described previously, an in-house software was used to analyze the H/D exchange mass spectra. Kinetic plots were obtained from the calculated composition of each deuterated species at each reaction time. The kinetic plots of dTT^- , dAA^- , and dTG^- are presented in Figure 2. From the kinetic plots, some features were underlined [the major species at given exchange times, the partial exchange, the simultaneous increase of the D(1) and D(2) species, the fast increase of species with a large number of exchanged hydrogens]. They are summarized for all of the six deprotonated dinucleotides in Table 1. These features could not be explained by only the hydrogen gas-phase acidities of 5' and 3' phosphate mononucleotides [16]. So, to explain the features, the three-dimensional gas-phase structure of the six deprotonated dinucleotides was investigated by molecular dynamics calculations.

Molecular Modeling

During molecular dynamics calculations, distances between exchangeable hydrogens and heteroatoms with which they could interact were monitored. Distinctive structures adopted by the dinucleotide were grouped according to these distances. For each structure or group of structures (in which structures interconverted rapidly), protection percentages were established for each exchangeable hydrogen. These protection percentages were defined as the time percentages (with regard to the "life" time of the structure) during which exchangeable hydrogens interacted with heteroatoms. The hydrogens were considered to be interacting when the distance between it and the heteroatom was between 1.5 and 4.5 Å. The previously mentioned concept

Figure 2. Kinetic plots of the H/D exchange reaction of deprotonated dTT⁻, dAA⁻, and dTG⁻.

of protection follows from the electrostatic approach. It illustrates the fact that when a hydrogen is involved in an interaction, it is blocked in a particular geometry for a given time during which it is made energetically nonexchangeable. For some hydrogen atoms, the protection extent was also estimated by the time percentage during which these atoms were protected in a cavity. This corresponds to a group of atoms that, surrounding a hydrogen, make it sterically inaccessible for H/D exchange. The distinctive structures and the protection extents are summarized for all of the six deprotonated dinucleotides in Table 2. A closed structure, unlike an

Table 1. Summary of underlined features for all of the six deprotonated dinucleotides

The deprotonated dinucleotide	The number of exchangeable hydrogens	The number of exchanged hydrogens after 600 s of reaction	Features	
			Major species at given reaction times	Kinetic progression
dTT [−]	4	2	D(2) was always the major species	A non-ordinary kinetic behavior was observed. D(2) appeared at the same time that D(1). The H/D exchange kinetics seemed to be non-consecutive
dAT [−]	5	2	D(2) was always the major species	
dAA [−]	6	6	D(2) was the major species until 475 s of reaction	
dTG [−]	6	5	D(1) was the major species from 0 to 90 s and D(5) became the major species after 90 s of reaction	A fast increase of species with a large number of exchanged hydrogens was observed
dAG [−]	7	7	D(1) was the major species from 0 to 150 s of reaction, D(5) was the major species from 150 s to 360 s	
dGG [−]	8	8	D(1) , D(2) and D(3) were the major species at first reaction times. D(8) became the major species after 200 s of reaction	

Table 2. Summary of the distinctive structures and of the protection extents for all of the six deprotonated dinucleotides

The deprotonated dinucleotide	The distinctive structures	The life time in percentage of the structure	The protection extent of each labile hydrogen	
			5'	3'
dTT [−]	The open structure	10.9%	OH: 0% NH: 0%	OH: 0% NH: 0%
	The closed structure	90.1%	OH: 0% NH: 85%	OH: 0% NH: 87%
dAT [−]	The open structure	14.9%	OH: 0% NH2: 0%–0%	OH: 0% NH: 0%
	The closed structure	85.1%	OH: 0% NH2: 76%–78%	OH: 0% NH: 75%
dAA [−]	The open structure	2.3%	OH: 0% NH2: 0%–0%	OH: 0% NH2: 0%–0%
	The group of closed structures	97.7%	OH: ~30% NH2: 53%–52%	OH: 0% NH2: 69%–68%
dTG [−]	The closed Structure A	50%	OH: 0% NH: 0%	OH: 0% NH: 0% NH2: 100%–0%
	The closed Structure B	50%	OH: 54% NH: 65%	OH: 0% NH: 64% NH2: 100%–62%
dAG [−]	The group of closed structures	100%	OH: ~25% NH2: 69%–70%	OH: 0% NH: 50% NH2: 65%–35%
dGG [−]	The closed structure with 3' G in syn orientation	50%	OH: 0% NH: 75% NH2: 94%–94%	OH: 0% NH: 0% NH2: 10%–90%
	The closed structure with 3' G in anti orientation	50%	OH: 100% NH: 75% NH2: 49%–45%	OH: 0% NH: 96% NH2: 92%–~25%

Figure 3. The distinctive structures of dGG[−]. On the left structure, the 3' nucleobase is in the syn orientation. One hydrogen from the NH₂ group of this nucleobase interacts with an oxygen of the phosphate group (the interacting atoms are painted in purple). On the right structure, the 3' nucleobase is in the anti orientation and the previously mentioned interaction is not present. The exchangeable hydrogens are painted in green.

open structure, corresponds to a structure where the two nucleobases are one above the other; a hydrogen of one nucleobase being able to interact with a heteroatom of the other nucleobase. In dTG[−] and dAG[−], the base G was always in the syn orientation. Because of this syn orientation, one hydrogen from the NH₂ group of the base G interacted with an oxygen of the phosphate group [6, 20–23]. For dTG[−], the interacting hydrogen was the same during the molecular dynamics calculations. For dAG[−], the interacting hydrogen changed once during the molecular modeling. This could explain the stopped H/D exchange to D(5) for dTG[−] and the complete exchange for dAG[−]. As dAG[−] was observed as a group of rapidly interconverted structures, these conformations were analyzed in more detail. In each of these structures, two hydrogens (not necessary the same ones) were more protected. This could explain that D(5) was the major species from 150 to 360 s of exchange. Taking into account data in Table 2 and according to the previous analysis, the vast majority of the observed features (Table 1) were justified. To confirm the major influence of the hydrogen accessibility on H/D exchange and to explain the observed nonconsecutive reaction kinetics, kinetic plots were simulated from the protection extents.

In dGG[−], the 3' base was in the syn orientation (one hydrogen from the NH₂ group interacts with an oxygen of the phosphate group) for one molecular dynamics calculation and in the anti orientation (the previously mentioned interaction is not present) for the second molecular modeling (Figure 3). To compare this different behavior

with respect to dTG[−] and dAG[−], ab initio geometry optimizations for the syn and anti conformations of the 3' nucleobase G were performed with Gaussian 03 for these three deprotonated dinucleotides. The obtained energies at different levels of theory and with different basis sets are presented in Table 3. For each conformation, all starting points resulted in identical local minimum. For dTG[−] and dAG[−] the more stable orientation of the 3' nucleobase G is the syn orientation. For dGG[−] the syn and anti orientations seem to have the same energy and so the same probability.

Table 3. The energy difference between the anti and syn orientations (kcal/mol)

The deprotonated dinucleotide	RHF/6-31G- <i>aug</i>	B3LYP/6-31G- <i>aug</i>	RHF/6-31*G- <i>aug</i>
dTG [−]	5.2256	6.4934	4.7621
dAG [−]	7.5325	11.3691	8.1387
dGG [−]	0.8655	0.3710	0.2215

The syn orientation is lower in energy.

• The consecutive H/D exchange

• The simultaneous H/D exchange

Figure 4. Illustration of the proposed kinetic mechanism that consists of consecutive and simultaneous H/D exchange reactions. M is the non-deuterated ion, a, b, c correspond to the three exchangeable hydrogens and k1, k2, and k3 are their rate constants.

Table 4. The protection extent and the corresponding rate constant of each exchangeable hydrogen of dAA[−] (the used set of coupled differential equations corresponds to the five most exchangeable hydrogens)

	The labile hydrogens	The protection extents	The rate constants (s ^{−1}) ^a	
5′	OH	30%	0.0012	b
	NH ₂	53%	0.00075	c
		52%	0.00075	d
3′	OH	0%	0.002	a
	NH ₂	69%	0.00045	e
		68%	0.00045	

^aAs CD₃OD is in excess, its concentration can be considered as constant and the reaction as having an apparent first-order kinetics.

Kinetic Simulations

As mentioned above, the H/D exchange itself was not the limiting step. The D(1) and D(2) species appeared at the same rate and species with a large number of exchanged hydrogens appeared rapidly. To explain these observations, a new kinetic mechanism was proposed. It is illustrated for three exchangeable hydrogens with the diagram in Figure 4. This kinetic mechanism indicates that if the number of collisions between deuterated methanol and dinucleotide ions was sufficient and if two hydrogens on the same deprotonated dinucleotide were accessible at the same time, these two hydrogens could be exchanged simultaneously at the experimental time scale. This mechanism was used to reproduce the H/D exchange experimental plots. To build these simulated kinetic plots, each exchangeable hydrogen was given an exchange rate constant according to its protection extent. Moreover, the model was based on the assumption that the rate constant of the simultaneous exchange reaction of two hydrogens corresponded to the rate constant of the slowest. The rate constants that were used for the simulation of dAA[−] kinetic plot are given in Tables 4 and 5. The simulated plots of dTT[−] and dAA[−] are presented in Figure 5. For dTG[−], the experimental plot was approached in adding the kinetic contributions of the two structures. This kinetic

Table 5. The set of rate constants (s^{−1})^a used to simulate the kinetic plot of dAA[−]

	The consecutive H/D exchange	The simultaneous H/D exchange
M	a = 0.002	ab = 0.0012
		ac = 0.00075
	b = 0.0012	ad = 0.00075
		ae = 0.00045
	c = 0.00075	bc = 0.00075
		bd = 0.00075
	d = 0.00075	be = 0.00045
		cd = 0.00075
	e = 0.00045	ce = 0.00045
		de = 0.00045

^aAs CD₃OD is in excess, its concentration can be considered as constant and the reaction as having an apparent first-order kinetics.

Figure 5. Kinetic simulated plots of dTT[−] and dAA[−] using MatLab. For dTT[−], the simultaneous exchange probability is twice the consecutive exchange probability. For dAA[−], these two probabilities are the same (for dAA[−], the used set of coupled differential equations corresponds to the five most exchangeable hydrogens).

mechanism was also applied to previously published mononucleotide H/D exchange [16]. A very good agreement with the kinetic plots was obtained. As previously mentioned, the relay mechanism requires the formation of a hydrogen-bonded complex between a hydrogen donor site of the nucleotide, the deuterating agent, and a deuterium acceptor site (the phosphate group) of the nucleotide, involving the restriction of the distance between the hydrogen donor and acceptor sites. The formation of such a three-partner complex is entropically unfavorable. Moreover, for dinucleotides, this relay mechanism is not appropriate as the distances between the hydrogen donor and deuterium acceptor sites are not compatible. A “direct” H/D exchange between the deuterating agent and the site bearing the exchangeable hydrogen is more relevant for dinucleotides. This “direct” exchange could be combined with the relay mechanism to explain the mononucleotides H/D exchange. In suitable experimental conditions, the simultaneous exchange should be taken into account for mononucleotides exchange. Possibly, this could be applied to the relay mechanism if it is sufficiently fast in comparison to the experimental time scale. For all

of the kinetic simulations, the value of the simultaneous exchange probability was either equal to or in the range of up to twice the value of the consecutive exchange probability. The fact that these two probabilities can differ is not excluded and must depend on the H/D exchange experimental conditions. To support the simultaneous exchange of two accessible hydrogens, the number of collisions between a dinucleotide ion and a CD₃OD gas was estimated. At the experimental pressure and according to [26], the mean free path should be such that the dinucleotide ions undergo at least 10⁴ collisions per second.

Conclusions

Studies have been performed on six dinucleotides with CD₃OD as the deuterating agent in the second hexapole of an FTICR-MS to assess the ability of H/D exchange to characterize coexisting conformers. In the experimental conditions, H/D exchange has been shown to be controlled by hydrogen accessibility. This hydrogen accessibility is in direct relationship with the biomolecular conformation and can be a function not only of interactions with heteroatoms but also of protection in a cavity. With hydrogen accessibility as the dominant factor, the H/D exchange study of biomolecular structures can be done without taking into account the chemical nature of the heteroatom bearing the hydrogen. This argument will be correct while the H/D exchange reaction is faster than the structure interconversion. Within this framework, a model including simultaneous H/D exchanges at the experimental time scale is proposed. This model allows dinucleotide H/D exchange kinetic plots and also mononucleotide H/D exchange kinetic plots to be reproduced. These conclusions are important for the study of the gas-phase conformations of larger biomolecules including noncovalent complexes. With hydrogen accessibility as the main factor for the exchange rates, structural modifications during ligand binding can be studied by direct counting of proton in groups associated with different structures.

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