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# Applying a new algorithm to H/D exchange of multiply protonated cytochrome *c*

Orit Geller[a,b], Chava Lifshitz[a,b,*]

[a] *Department for Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*
[b] *The Farkas Center for Light Induced Processes, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

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In celebration of Tilmann Märk's 60th birthday, in appreciation of his unique contributions to the physics of ionization phenomena and cluster science, and in thanks for our collaborations.

**Abstract**

The experimental technique involving electrospray ionization (ESI) combined with a fast flow method has been applied to gas phase H/D exchange with $ND_3$ of two multiply protonated ions of cytochrome *c*: (cytochrome $c + 10H^+)^{10+}$ and (cytochrome $c + 12H^+)^{12+}$, respectively. The relative abundances of ions were measured in steps of 0.5 *m*/*z* (mass to charge ratio) units, 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 minimizes the mutual entropy or the Kullback–Leibler information divergence between the observed concentrations and the model and allows the extraction of site-specific rate constants. Ten rate constants were deduced for each of the two ions. These rate constants correspond to 50 and 60 H/D exchanges for the +10 and +12 charged ions, respectively. The results are discussed in terms of a compact +10 ion and a more elongated +12 conformer and the related more general issue of cooperativity in protein folding.


*Keywords:* ESI; Fast flow; H/D exchange; Multiply protonated cyt *c*; Compact and elongated conformers; Site-specific rate constants

## 1. Introduction

The study of hydrogen/deuterium (H/D) exchange in the gas phase has developed into a powerful technique for probing the structure of gas phase proteins [1]. Studies of cytochrome *c* (cyt *c*) with Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry provided evidence of multiple conformations in the gas phase. Low levels of exchange were correlated with compact tertiary structures and higher levels, observed for higher charge states, with more elongated conformations [2]. This is because some hydrogen atoms are protected on interior sites as is known from H/D exchange of proteins in solution. Gaseous $D_2O$ replaces 30–70% of the ions' exchangeable hydrogen atoms in distinct values indicative of at least six conformational states [3]. H/D exchange coupled with ion mobility studies confirmed that the compact conformers of cyt *c* exchange fewer sites than elongated structures at room temperature [4]. Raising the temperature resulted in greater maximum exchange for the lower charge (more compact) states [5]. Studies of H/D exchange kinetics with $D_2O$ have demonstrated [5] that the reactions are slow at all temperatures with rate constants in the range from ~1 to $50 \times 10^{-14}\,cm^3\,s^{-1}$ molecule$^{-1}$.

We reported recently [6] on our first results concerning H/D exchange kinetics with $ND_3$ of the well-known [7,8] gas phase multiply protonated cyt *c* ions in charge states +10 to +17. The experimental set-up we are using is a unique electrospray ionization/fast flow apparatus, described previously [9]. 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 deuterating agent employed, $ND_3$ is considerably more efficient than $D_2O$ because of the much higher proton affinity of ammonia: $PA(NH_3) = 204$ kcal/mol versus $PA(H_2O) = 165.2$ kcal/mol [10]. This allowed the observation of exchanges in the flow tube experiment [6] that would otherwise be impossibly slow to observe.

* Corresponding author. Tel.: +972-2-6585866; fax: +972-2-6522472.
*E-mail address:* chavalu@vms.huji.ac.il (C. Lifshitz).

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The aim of the kinetics is the extraction of site-specific rate constants for the replacement of labile hydrogen atoms differently located in the amino acid or peptide structure [9d,11,12]. The series of parallel and consecutive exchanges can be modeled using appropriate algorithms to deduce site-specific rate constants. We recently developed a new algorithm [13] for extracting site-specific rate constants for H/D exchange in gas phase protonated amino acids, their clusters and peptides. The algorithm minimizes the mutual entropy or the Kullback–Leibler [14] information divergence between the observed concentrations and an appropriate model. Electrospray ionization-mass spectrometry (ESI-MS) results from fast flow tube and FT-ICR experiments, respectively were modeled. This algorithm has now been applied to some of the experimental data for cyt *c* and the results will be presented here.

## 2. Experimental

The electrospray ionization/fast flow apparatus is shown schematically in Fig. 1. It consists of a SIFT apparatus that we constructed several years ago and modified to work with an electrospray (ES) source connected directly to the flow tube. The apparatus is made 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 (652601 ABB EXTREL) 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. A 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 near an electron impact ion source 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 non-conductive 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 [15]. Dilute solutions of cytochrome *c* (horse heart, Sigma >99%) in a polar solvent 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. Room temperature does not exceed 30°. The stainless steel capillary tube and the flow tube were heated and the temperature measured was 30–40°.

## 3. The algorithm

The algorithm has been described in some detail recently [13] and only the major ideas behind it will be presented here. Consider a species (amino acid, peptide or cluster) with *N* replaceable hydrogens. Each molecule is represented by an *N* bit binary number, where 1 represents deuterium and 0 represents hydrogen. In the hydrogen–deuterium exchange system, it is assumed that each of these protons is replaced independently in a first order process with rate constants $\theta_1$, $\theta_2,\ldots,\theta_n,\ldots,\theta_N$. The probability $p_{n,t}$ that the *n*th hydrogen will have been replaced by deuterium after time *t* is given by:

$$p(\mathrm{D})_{n,t} = 1 - \exp(-\theta_n t)$$

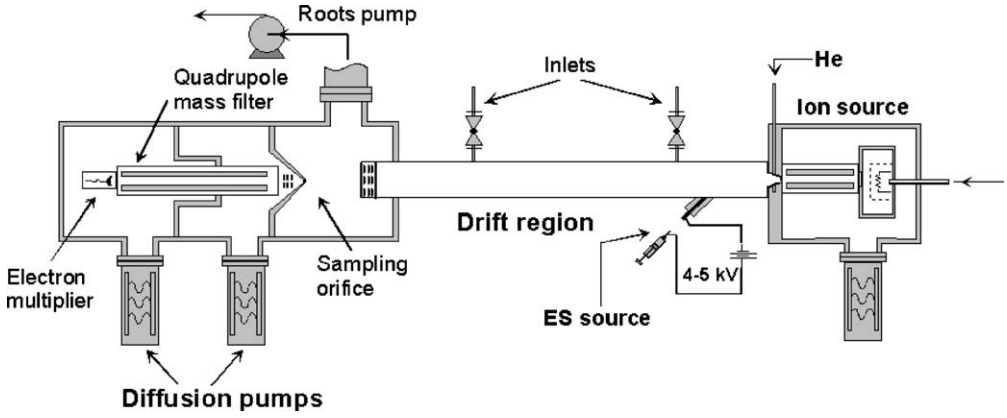


Fig. 1. Schematic drawing of the experimental setup combining electrospray (ES) ionization with a fast flow technique.

The probability that the $n$th hydrogen will not have been replaced is then

$$p(\mathrm{H})_{n,t} = \exp(-\theta_n t)$$

The overall probability of any structure occurring is the product of the individual probabilities, $p(\mathrm{D})_{n,t}$ and $p(\mathrm{H})_{n,t}$ selected according to whether the digit in the corresponding binary number is 1 or 0. Summation of the digits of the binary number gives the number of deuteriums in the intermediate, and the probabilities of structures containing the same numbers of deuteriums can be summed to give an overall probability for that number of replacements.

The experimental profiles for the abundance of species containing 0, 1, 2,. . . $n$. . . $N$ deuterium atoms at various flow rates are then normalized and compared with the above calculation of the predicted probabilities at the same flow rates. Fitting the experimental curves by a theoretical model is done by minimization of the distance between the theoretical and experimental profiles. The algorithm minimizes the mutual entropy or the Kullback–Leibler [14] information divergence between the observed concentrations and the model.

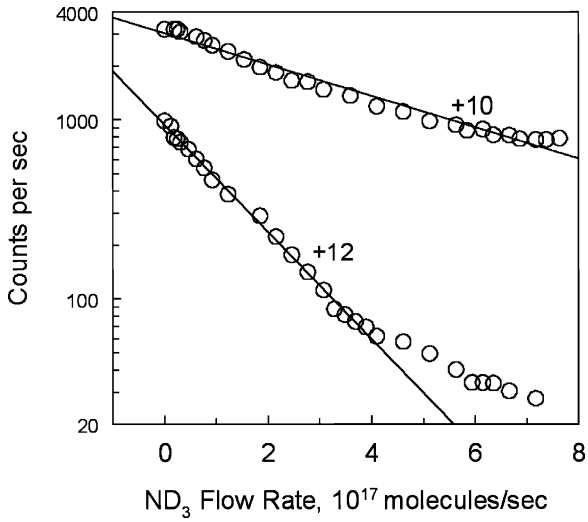


Fig. 2. Semilogarithmic plots of the decay of primary ions as a function of the neutral flow rate for the reaction of multiply protonated (+10 and +12) cytochrome $c$ ions with $ND_3$. The open circles are experimental and the solid lines are the best linear fits to semilogarithmic (exponential) decays. The decay of the +12 ion is multi-exponential and the fit obtained is for the flow rates range 0–4 × $10^{17}$ molecules/s.

## 4. Results and discussion

We have applied the new algorithm in our previous study [13] to two experimental systems: protonated glycine that has four labile hydrogens and the proton bound dimer of arginine that has 15 labile hydrogens. We were able to deduce the corresponding 4 and 15 site-specific rate constants, respectively. Cyt $c$ has 198 labile hydrogens plus the protons added in the ESI process. In order to tackle this problem, we have grouped several labile hydrogens together in the fit, so that the site-specific rate constants are sum values for groups of labile hydrogen atoms. This approach has an added necessary experimental advantage, because the quadrupole mass filter we are employing in the flow tube experiment has a rather limited mass resolution.

Our recent flow-tube study of cyt $c$ [6] has demonstrated a sharp change in behavior between the +10 ion, and the ions with charges +11, +12 and higher. The average decay rates and the overall decay rate constants were quite different. Fig. 2 presents typical data for the decay of the reactant ion as a function of the flow rate of the neutral reagent (at a constant reaction time) for the charge states +10, (cytochrome $c + 10\mathrm{H}^+)^{10+}$ and +12, (cytochrome $c + 12\mathrm{H}^+)^{12+}$. The decay of the +10 charged ion is the slowest among the unexchanged reagent ions. The behavior of the +10 ion is that of a compact conformer whereas the higher charged ions have elongated structures that react faster and undergo higher degrees of overall exchange. In the present application of the algorithm we have therefore undertaken the comparison of the +10 compact ion with the +12 ion as a representative of the elongated ions.

We denote by $m_0$ the $m/z$ value for the unexchanged cyt $c$ ion. The relative abundances for $m/z$ ions at $m_0$, $m_0 + 0.5$, $m_0 + 1, \ldots, m_0 + 5$ were measured as a function of $ND_3$ flow rate. The unit step of 0.5 $m/z$ corresponds to a group of five exchanges in the case of the +10 ion and to six exchanges in the +12 ion. The results are presented for the +12 ion in Fig. 3. The results of the simulations based on the algorithm are presented in Fig. 3 as well.

The simulated fits allow derivation of 10 rate constants for the 60 H/D exchanges undergone by the +12 ion, where the rate constants are sums of values for groups of six exchanges, respectively. The rate constants are summarized in Table 1. Ten rate constants derived for the 50 H/D exchanges undergone by the +10 ion are similarly included in Table 1; the rate constants are in this case

Table 1
Grouped H/D exchange rate constants (in units of $cm^3$/molecule s) for the reaction of (cytochrome $C + 10\mathrm{H}^+)^{10+}$ and (cytochrome $C + 12\mathrm{H}^+)^{12+}$, respectively with $ND_3$[#]

| Group | (Cytochrome $c + 10\mathrm{H}^+)^{10+}$ | (Cytochrome $c + 12\mathrm{H}^+)^{12+}$ |
|---|---|---|
| 1 | $2.2 \times 10^{-14}$ | $1.3 \times 10^{-11}$ |
| 2 | $1.1 \times 10^{-11}$ | $1.3 \times 10^{-11}$ |
| 3 | $1.1 \times 10^{-11}$ | $1.3 \times 10^{-11}$ |
| 4 | $1.1 \times 10^{-11}$ | $1.4 \times 10^{-11}$ |
| 5 | $1.1 \times 10^{-11}$ | $2.8 \times 10^{-11}$ |
| 6 | $1.1 \times 10^{-11}$ | $5.6 \times 10^{-11}$ |
| 7 | $1.1 \times 10^{-11}$ | $9.0 \times 10^{-11}$ |
| 8 | $7.5 \times 10^{-11}$ | $1.2 \times 10^{-10}$ |
| 9 | $8 \times 10^{-11}$ | $6.8 \times 10^{-10}$ |
| 10 | $8 \times 10^{-11}$ | $1.1 \times 10^{-9}$ |

[#] Each group of rate constants contains five labile hydrogens in the case of the +10 ion and six labile hydrogens in the case of the +12 ion. The values given are sums of rate constants for individual members of the group (see text).

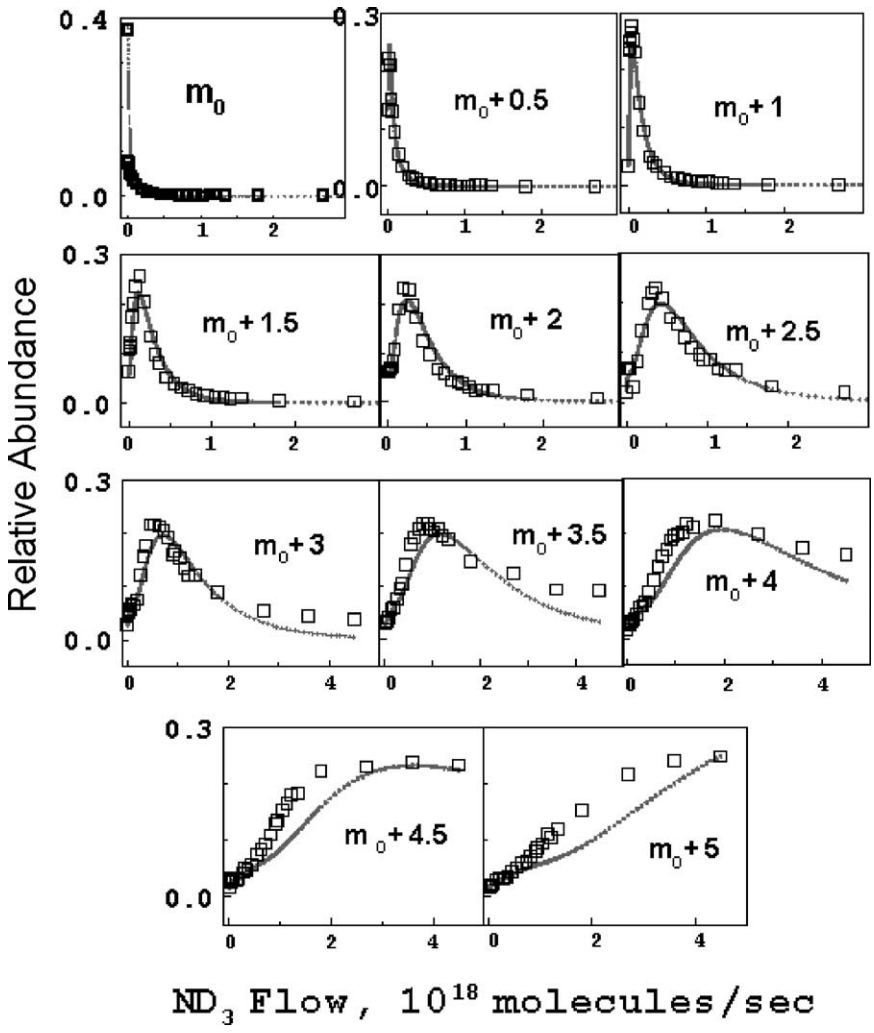


Fig. 3. Relative abundance vs. $ND_3$ flow rate in molecules/s for the unexchanged ion (cytochrome $c + 12H^+)^{12+}$, $m_0$ and for the various indicated cations for the 10 consecutive exchanges in the reaction of cytochrome $c + 12H^+$ with $ND_3$. The reaction time is 13.37 ms; helium carrier gas flow: 1.7 l/min; flow tube pressure: 0.143 Torr. The open squares are experimental results; the continuous dotted curves are the results of the simulation based on the algorithm described in this paper that minimizes the KL divergence (see text). 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.

sums of values for groups of five exchanges respectively. Both sets of rate constants demonstrate similar values for several site-specific H/D exchanges. There are, for example, 30 rate constants (groups 2–7, Table 1) equal to $\sim 1.1 \times 10^{-11}/5 = 2.2 \times 10^{-12}$ $cm^3$/molecule s in the case of the +10 ion and 24 rate constants (groups 1–4, Table 1) equal $\sim 2.2 \times 10^{-12}$ $cm^3$/molecule s in the case of the +12 ion. Similarly, there are 15 and 18 rate constants $\sim 1.5 \times 10^{-11}$ $cm^3$/molecule s each for the +10 ion (groups 8–10) and +12 ion (groups 6–8), respectively. However, there is one set of five very low rate constants in the +10 ion (group 1) that is absent in the +12 ion fit and there are two sets of six quite high rate constants, $6.8 \times 10^{-10}/6 \cong 1.1 \times 10^{-10}$ $cm^3$/molecule s (group 9) and $1.1 \times 10^{-9}/6 \cong 1.8 \times 10^{-10}$ $cm^3$/molecule s (group 10) in the +12 fit.

The sums of the 10 grouped rate constants of Table 1 are $\sim 3 \times 10^{-10}$ (sum of 50 individual H/D exchanges) and $\sim 2.1 \times 10^{-9}$ $cm^3$/molecule s (sum of 60 individual exchanges) for (cytochrome $c + 10H^+)^{10+}$ and (cytochrome $c + 12H^+)^{12+}$, respectively, in fairly good agreement with overall rate constants deduced from semilogarithmic decay plots of the corresponding reactant ions, $m_0$.

Pulsed hydrogen exchange/mass spectrometry has been employed in a kinetics study of cyt *c* folding [16]. This study demonstrated segments of the cyt *c* that were either unfolded or completely folded, a behavior consistent with cooperative localized folding. The results were interpreted to show that the N/C-terminal regions folded cooperatively. The near equality of H/D exchange rate constants of a fairly large number of labile hydrogens that we observe for ions of different degrees of charging are in agreement with cooperative

folding and unfolding. The group of 12 high rate constants that is present in the +12 ion but absent in the +10 ion are interpreted as localized unfolding in the +12 ion. We plan in the future to improve the kind of study whose results have been presented here, to better understand the various cooperative effects in the protein folding process.

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