Kinetic isotope effects for fast deuterium and proton exchange rates

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By monitoring the effect of deuterium decoupling on the decay of transverse $^{15}$N magnetization in D–$^{15}$N spin pairs during multiple-refocusing echo sequences, we have determined fast D–D exchange rates $k_D$ and compared them with fast H–H exchange rates $k_H$ in tryptophan to determine the kinetic isotope effect as a function of pH and temperature.

Introduction

In the parlance of magnetic resonance, chemical exchange is a process where a nucleus undergoes a change of its environment.1 The determination of the exchange rates of labile protons can provide valuable insight into both structural and dynamic aspects of a wide range of molecules,2–4 such as the opening of base-pairs in nucleic acids and protection factors in protein–ligand complexes.5,6 In this paper, we shall focus on measurements of D–D exchange rates $k_D$ and their comparison with H–H exchange rates $k_H$ in tryptophan.7 The knowledge of kinetic isotope effects, i.e., of the ratio $k_D/k_H$ that expresses the reduction of D–D exchange rates $k_D$ compared to H–H exchange rates $k_H$, may contribute to the characterization of reaction mechanisms.8,9 The kinetic isotope effect can give insight into the stability of hydrogen-bonded secondary structures in biomolecules.10 In this work, we shall consider exchange processes involving labile D$^\text{N}$ deuterons and H$^\text{N}$ protons that are covalently bound to the nitrogen atom in the indole ring of tryptophan.†

Experimental section

We have adapted to the case of deuterium (spin $S = 1$) a scheme that was originally designed to determine fast exchange rates of protons7,11,12 (spin $S = 1/2$) by monitoring the effect of deuterium decoupling on the decay of transverse $^{15}$N magnetization during multiple-refocusing sequences (CPMG).13,14 The modified pulse sequence is shown in Fig. 1. The scheme requires isotopic enrichment with $^{15}$N and $^{13}$C, since the $^{15}$N coherence is excited by transfer from neighboring protons through two successive INEPT transfer steps via $j_{13}(13C,1H)$ and $j_{15}(15N,13C)$. The decay of the $^{15}$N coherence is monitored indirectly after transferring the coherence back to the proton of origin. The $^{15}$N,$^{13}$C-labelled isomers of tryptophan are dissolved in either D$_2$O or H$_2$O to determine the kinetic isotope effect $k_D/k_H$ of the following reactions:

$$N-D + D^+ \rightarrow N-D' + D^+ \text{ rate } k_D$$ (1)
$$N-H + H^+ \rightarrow N-H' + H^+ \text{ rate } k_H$$ (2)

where $k_D$ and $k_H$ are the pseudo-first order rate constants since the concentration of the solvent D$_2$O or H$_2$O, which is the source of the incoming D$^+$ or H$^+$ ions, is constant and much higher than the concentration of the solute.§

The first and last parts of the pulse sequence in Fig. 1 lead to a transfer of the magnetization from the blue non-exchanging ‘spy’ proton to $^{15}$N and back, via the adjacent $^{13}$C nuclei, by two successive pulse sequences for Insensitive Nuclei Enhanced by Polarization Transfer (INEPT).15 The first INEPT sequence transforms longitudinal proton magnetization $H_z$ into two-spin order $2H_zC_z$. The second INEPT sequence converts $2H_zC_z$ into $2C_zN_z$. WALTZ-16 proton decoupling16 is used to suppress the evolution under $j_{13}(1H,13C)$ during the intervals of the INEPT sequences.

† We use the symbol $^2$D when referring to isotopes as in the expressions $j_{13}(^{1H,^{15}N})$ or $j_{13}(^{2D,^{15}N})$.
§ We shall refer to H or D for atoms that appear in molecular formulae and to H$^\text{N}$ or D$^\text{N}$ in N-H and N-D groups. For the Cartesian components of angular momentum operators, we have used $H_x, H_y, H_z, D_x, D_y, D_z, N_x, N_y, N_z, C_x, C_y, C_z$ rather than the common notation $I_x, R_x, S_x$ etc.
where the coherence is transferred from $^{13}$C to $^{15}$N. The anti-phase coherence $2N_yC_z$ excited at the beginning of the multiple-refocusing CPMG interval decays in the course of this pulse train. At this point, two variants (A and B) of the experiments must be performed. In experiment B, continuous wave (CW) deuterium decoupling is applied during the CPMG pulse train, while in experiment A the deuterium irradiation is applied for the same duration but prior to the CPMG pulse train in order to avoid differences in temperature.

The remaining coherence $2N_yC_z$ is transferred back to the 'sp3' proton for detection. The intensity of the resulting peak near 7.22 ppm in the proton spectra is proportional to the magnitude of the nitrogen $2N_y$ coherence that remains at the end of the CPMG interval. In order to extract $k_D$, one can determine the ratio $I_B/I_A$ of the peak intensities recorded without decoupling during the CPMG pulse train (experiment A) and with deuterium decoupling (experiment B). The delay $\tau$ is defined as one-half of the interval between consecutive nitrogen $\pi$-pulses. The $\tau$ delays need to be long enough to ensure that the ratio $I_B/I_A$ is significantly different from 1. Typically, values of $\tau = 10.6$ or 21.2 ms have been used. The scalar coupling is $|J(15N,2D)| = 15.4$ Hz, smaller than $|J(15N,1H)| = 98.6$ Hz by the factor $\gamma(D)/\gamma(H) \approx 0.15$, but $|J(15N,2D)|$ is still large enough to act as an efficient vehicle of scalar relaxation.

We can construct the matrix representations of the $4 \times 9 = 36$ Cartesian operators that span a complete basis set for a system comprising a $^{15}$N nucleus with spin $I = 1/2$ and a $^2$D nucleus with spin $S = 1/2$. When a CPMG multiple echo sequence is applied to the $^{15}$N spins with an off-resonance rf field at the chemical shift of $^{15}$N, while deuterium decoupling is applied with an amplitude $\omega_D^0$ at an offset $Q^D$ with respect to the chemical shift of $^2$D. The rf pulses applied to the $^{15}$N spins are considered to be ideal. Starting from an operator $N_x$, coherent evolution leads to the following terms:

$$
N_x \xrightarrow{\omega_D^0} N_y \xrightarrow{J_x} N_z \xrightarrow{\omega_D^0} N_y \xrightarrow{(3D^2 - 2E)} \xrightarrow{\omega_D^0} N_x \xrightarrow{(D_2 + D_3)} \xrightarrow{\omega_D^0} N_x \xrightarrow{(D_2 + D_3)} \xrightarrow{\omega_D^0} N_x \xrightarrow{(D_2 + D_3)} \xrightarrow{\omega_D^0} N_x \xrightarrow{(D_2 + D_3)} \xrightarrow{\omega_D^0} N_x \xrightarrow{(D_2 + D_3)} \xrightarrow{\omega_D^0} N_x \xrightarrow{(D_2 + D_3)}
$$

Therefore the dimension of the basis set can be reduced from 36, leaving only 9 terms:

$$
\left\{ 2 \sqrt{2} N_x, 2N_y, 2N_z, 2\sqrt{2} N_x (3D^2 - 2E), 2N_y (3D^2 - 2E), 2\sqrt{2} N_z (3D^2 - 2E), 2\sqrt{2} N_y (3D^2 - 2E), 2\sqrt{2} N_z (3D^2 - 2E), 2\sqrt{2} N_y (3D^2 - 2E) \right\}
$$
Note that in the experiments of Fig. 1, the single-quantum coherence at the beginning of the CPMG period is an antiphase operator $2N_cC_r$. Since the presence of the $C_r$ term affects the signal intensities in experiments A and B equally, this $C_r$ term can be omitted without loss of generality. The solution of the Liouville-von Neumann equation \(^19\) up to the $n^{th}$ echo is:

$$\sigma(t = 2\pi) = \left[\exp(-L\tau)R_N\exp(-L\tau)\right]^n\sigma(0) \quad (5)$$

The matrix representation of the Liouvillian $L$ in the basis of eqn (4) is:

$$L = \begin{bmatrix}
0 & 2\sqrt{\frac{2}{3}}J\pi & 0 & 0 & 0 & 0 & 0 & 0 \\
-2\sqrt{\frac{2}{3}}J\pi & k & -2\sqrt{\frac{2}{3}}J\pi & \omega_l^D & 0 & 0 & 0 & 0 \\
0 & \frac{2}{\sqrt{3}}J\pi & k & 0 & \sqrt{3}\omega_l^D & 0 & 0 & 0 \\
0 & -\omega_l^D & 0 & k & -J\pi & 0 & \Omega_D & 0 \\
0 & 0 & -\sqrt{3}\omega_l^D & J\pi & k & -\omega_l^D & 0 & \Omega_D \\
0 & 0 & 0 & \omega_l^D & k & 0 & 0 & 2\Omega_D \\
0 & 0 & 0 & -\Omega_D & 0 & 0 & k & -J\pi \\
0 & 0 & 0 & 0 & -\Omega_D & 0 & J\pi & k & -\omega_l^D \\
0 & 0 & 0 & 0 & 0 & -2\Omega_D & 0 & \omega_l^D & k
\end{bmatrix} \quad (6)$$

The matrix representation of $R_N$ represents a $\pi_y$ pulse applied to the $^{15}$N spins:

$$R_N = \begin{bmatrix}
1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1
\end{bmatrix} \quad (7)$$

If the $rf$ field for deuterium decoupling is applied on resonance, the evolution of the density operator can be described in a simplified base comprising only 6 product operators:

$$\left\{2\sqrt{\frac{2}{3}}N_y, 2N_xD_z, 2\sqrt{2}N_y(3D_z^2 - 2E), 2N_xD_y, 2\sqrt{2}N_y(D_zD_y + D_yD_z), 2\sqrt{2}N_y(D_x^2 - D_y^2)\right\} \quad (8)$$

For the $\pi_y$ pulse applied to the $^{15}$N spins one obtains in this reduced base:

$$R_N = \begin{bmatrix}
1 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & -1 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 1 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 1 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 1 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1
\end{bmatrix} \quad (10)$$

In this reduced base, the matrix representation of the Liouvillian is:

$$L = \begin{bmatrix}
0 & 2\sqrt{\frac{2}{3}}J\pi & 0 & 0 & 0 & 0 & 0 \\
-2\sqrt{\frac{2}{3}}J\pi & k & -2\sqrt{\frac{2}{3}}J\pi & \omega_l^D & 0 & 0 & 0 \\
0 & \frac{2}{\sqrt{3}}J\pi & k & 0 & \sqrt{3}\omega_l^D & 0 & 0 \\
0 & -\omega_l^D & 0 & k & -J\pi & 0 & \Omega_D \\
0 & 0 & -\sqrt{3}\omega_l^D & J\pi & k & -\omega_l^D & 0 & \Omega_D \\
0 & 0 & 0 & \omega_l^D & k & 0 & 0 & 2\Omega_D \\
0 & 0 & 0 & -\Omega_D & 0 & 0 & k & -J\pi \\
0 & 0 & 0 & 0 & -\Omega_D & 0 & J\pi & k & -\omega_l^D \\
0 & 0 & 0 & 0 & 0 & -2\Omega_D & 0 & \omega_l^D & k
\end{bmatrix} \quad (9)$$

In the experiment of Fig. 1, the amplitude $\nu_{rf}^1$ of the continuous-wave $rf$ field applied to the deuterium spins should be chosen carefully. The higher the $rf$ amplitude $\nu_{rf}^1$, the more efficient the decoupling, although one should avoid excessive heating. On the other hand, if the $rf$ amplitude is too low, the ratio $I_A/I_B$ is affected in a manner that can lead to erroneous measurements of the exchange rates. By way of illustration, at $pD \approx 7.7$ and $T = 300$ K, where the exchange rate is very low (see Table 1), the ratio $I_A/I_B$ has been determined as a function of the $rf$ amplitude for $\tau = 10.6$ ms and $n_{CPMG} = 2$. For these experimental conditions, the amplitude can be attenuated as low as $\nu_{rf}^1 = \omega_l^D(2\pi) = 100$ Hz without affecting significantly the ratio $I_A/I_B$. For lower amplitudes the ratio is very sensitive to the exact amplitude. An $rf$ field with an
amplitude $\nu_1^D = 3$ kHz seems to be a safe value regardless of the exchange rates and can be used for all experiments. The ratio $I_A/I_B$ also depends on the offset $\Omega^D$ of the rf carrier with respect to the exchanging $^2$D spins, since decoupling becomes less efficient when the carrier is off-resonance. The ratio $I_A/I_B$ has the smallest value when the carrier coincides with the chemical shift of the exchanging $^2$D spins, i.e., when $\Omega^D = 0$ (Fig. 2). The heteronuclear scalar coupling constant $\gamma(\text{C}^1\text{H}) = 15.4$ Hz at $pD = 7.7$ was determined experimentally from the doublet in the $^2$H spectrum and corresponds to the expected value $\gamma(\text{C}^1\text{H}) = \gamma(\text{C}^{13}\text{N}, \text{H}) + \gamma(\text{H})/\gamma(\text{H})$ with $\gamma(\text{C}^{13}\text{N}, \text{H}) = 98.6$ Hz.

All experiments were performed at 14.1 T (600 MHz for $^1$H, 151 MHz for $^{13}$C, 92 MHz for $^2$H, and −61 MHz for $^{15}$N) using a Bruker Avance III spectrometer equipped with a cryogenically cooled TXI probe. The samples were prepared by dissolving 20 mM tryptophan (fully $^{13}$Ca and $^{15}$N enriched) in 100% D$_2$O buffered with 20 mM citrate, acetate, Tris or phosphate buffer depending on the pH range. We determined the $k_D$ in our earlier work$^7$ using 97% H$_2$O and 3% D$_2$O; the indicated pH values include corrections to take into account that the pH was measured in D$_2$O with an electrode calibrated for H$_2$O according to the following equation$^{10}$

$$pD = \text{pH}_{\text{apparent}} + 0.4 \quad (11)$$

Results and discussion

For each pH and temperature, the exchange rates $k_D$ have been determined from three to seven ratios $I_A/I_B$ of the signal intensities corresponding to six to fourteen experiments performed with variable numbers of $\pi$-pulses $2 \leq n \leq 8$ in the CPMG trains, and different intervals, $\tau = 2.6, 5.3, 10.6$ and 21.2 ms, but with the same total relaxation time $2\tau_{\text{CPMG}}$. A minimum of two ratios $I_A/I_B$ at different delays are required for an unambiguous determination of $k_D$, since two rates can be compatible with a single $I_A/I_B$ ratio. Fig. 3 shows how this ambiguity is lifted by changing the inter-pulse delay $2\tau$ in the CPMG pulse train. The pseudo first-order exchange rate constants were found to lie in a range $0 < k_D < 40 000$ s$^{-1}$, depending on pH and temperature (Table 1). At each temperature, the exchange rate $k_D$ was found to be slowest for $pD_{\text{min}} 4.8$. When the exchange rate $k_D$ is very low, one cannot neglect contributions due to the difference in relaxation rates of the in-phase $^{15}$N coherence and other rates in the relaxation matrix.

Table 1  Pseudo first-order exchange rate constants $k_D$ [s$^{-1}$] without corrections for contributions due to quadrupolar relaxation as a function of temperature and pD

<table>
<thead>
<tr>
<th>$pD$</th>
<th>290 K</th>
<th>300 K</th>
<th>310 K</th>
<th>320 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.05</td>
<td>273 ± 21</td>
<td>491 ± 63</td>
<td>697 ± 110</td>
<td>1670 ± 250</td>
</tr>
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<td>1.49</td>
<td>56.9 ± 11</td>
<td>65.9 ± 13</td>
<td>81.6 ± 17</td>
<td>91.6 ± 19</td>
</tr>
<tr>
<td>2.18</td>
<td>49.7 ± 4.8</td>
<td>47.9 ± 4.2</td>
<td>57.6 ± 11</td>
<td>61.8 ± 8.7</td>
</tr>
<tr>
<td>3.29</td>
<td>39.1 ± 1.9</td>
<td>30.2 ± 1.8</td>
<td>26.3 ± 1.5</td>
<td>23.6 ± 0.7</td>
</tr>
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<td>4.78</td>
<td>36.2 ± 3.0</td>
<td>26.2 ± 3.3</td>
<td>20.9 ± 2.9</td>
<td>16.5 ± 3.2</td>
</tr>
<tr>
<td>5.98</td>
<td>37.1 ± 3.1</td>
<td>27.1 ± 1.9</td>
<td>22.9 ± 1.9</td>
<td>18.7 ± 1.4</td>
</tr>
<tr>
<td>6.43</td>
<td>38.7 ± 5.7</td>
<td>28.6 ± 4.2</td>
<td>23.7 ± 3.1</td>
<td>19.4 ± 2.6</td>
</tr>
<tr>
<td>7.97</td>
<td>41.4 ± 6.4</td>
<td>31.6 ± 4.3</td>
<td>27.2 ± 3.4</td>
<td>23.8 ± 3.1</td>
</tr>
<tr>
<td>9.11</td>
<td>82.4 ± 15</td>
<td>88.1 ± 8.2</td>
<td>110 ± 11</td>
<td>182 ± 31</td>
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<tr>
<td>9.68</td>
<td>121 ± 13</td>
<td>328 ± 17</td>
<td>507 ± 24</td>
<td>884 ± 49</td>
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<tr>
<td>10.8</td>
<td>831 ± 42</td>
<td>1546 ± 90</td>
<td>2670 ± 330</td>
<td>4330 ± 380</td>
</tr>
<tr>
<td>12.1</td>
<td>3220 ± 840</td>
<td>8060 ± 2200</td>
<td>11800 ± 2600</td>
<td>18800 ± 1700</td>
</tr>
<tr>
<td>12.97</td>
<td>14000 ± 3400</td>
<td>17600 ± 6300</td>
<td>40400 ± 11000</td>
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</table>
of eqn (6). From an earlier study of the exchange of indole protons,\textsuperscript{7} we know that the exchange rate $k_D$ almost vanishes near pH$_{\text{min}}$. On the other hand, as can be seen in Table 1, the exchange rates $k_D$ do not vanish near pH$_{\text{min}}$. Moreover, if one neglects relaxation of deuterium, some apparent exchange rates increase at lower temperatures, which is physically impossible. Hence, we incorporated a temperature-dependent quadrupolar relaxation rate $R_Q$ in eqn (12) and subtracted it from the apparent exchange rates at all pH. The use of a single constant $R_Q$ to describe the effects of deuterium relaxation is rather naive. In particular for weak rf fields or large deuterium offsets, this assumption may lead to errors. We can calculate the relaxation rates of operator products containing terms such as $D_2$ (2D$^2$ – 2E), $D_2D_O$ ($D_2^x$ – $D_2^y$), ($D_2D_z$ + $D_2D_z$) and ($D_2D_z$ + $D_zD_z$).\textsuperscript{21} However, we have verified that under the conditions for which the rates of Fig. 4 were obtained, i.e., for strong rf fields and vanishing deuterium offsets, the exchange rates are barely affected if we assume that all deuterium terms have a common relaxation rate. The errors in the experimental ratios $I_D/I_O$ were determined from standard deviations. The error propagation was further simulated by the Monte Carlo technique. The errors in the exchange rates $k_D$ were estimated from the curvature around the minima of $\chi^2$ and found to lie in a range between 3 and 28%.

If the exchange rate constants $k_D$ are plotted as a function of pH on a logarithmic scale, one obtains a V-shaped curve that is characteristic of acid catalysis by $D^+$ ions and basic catalysis by OD$^-$ ions, the latter being more efficient (Fig. 4). In the cationic, zwitterionic and anionic forms of tryptophan, the exchange rates result from sums of acidic and basic contributions. The overall exchange rate constant $k_D$ can be written as:\textsuperscript{2,22}

$$k_D = k_{D_o}[D^+]_c + k_{D_o}[D^+]_a + k_{OD_o}[OD^-]_c + k_{OD_o}[OD^-]_a + R_Q$$  

(12)

where the rate $R_Q$ expresses contributions due to the quadrupolar deuterium relaxation to the decay of antiphase $^{15}$N coherences. The indices D and OD represent the contributions of acidic and basic mechanisms (see below) for the cationic, zwitterionic and anionic forms of tryptophan, abbreviated by c, z, and a in Fig. 5.

The mole fractions $f_c$, $f_z$ and $f_a$ of the cationic, zwitterionic and anionic forms of tryptophan are:

$$f_c = (1 + 10^{pD-pK_{a1}} + 10^{2pD-pK_{a1}} - pK_{a2})^{-1}$$
$$f_z = (1 + 10^{-pD-pK_{a1}} + 10^{pD-pK_{a1}} - pK_{a2})^{-1}$$
$$f_a = (1 + 10^{-pD-pK_{a2}} + 10^{-2pD+pK_{a2}} - pK_{a1})^{-1}$$  

(13)

Where [D$^+$] = 10$^{-pD}$, [OD$^-$] = $K_W$10$^{pD}$. The auto-ionization constant $K_W$ of D$_2$O depends on the temperature.\textsuperscript{23} In H$_2$O at 25 °C, pK$_{a1}$ = 2.46 for the protonation of the carboxyl group, while pK$_{a2}$ = 9.41 corresponds to the protonation of the amine group. In D$_2$O at 25 °C, we have determined that pK$_{a1}$ = 2.60 and pK$_{a2}$ = 10.05.\textsuperscript{24} The variation of pK$_a$ with temperature\textsuperscript{23} has been taken into account. Fig. 4 and Table 2 show the results of the fitting of the exchange rate constants $k_D$ to eqn (12), which allows one to obtain the catalytic rate constants for the contributions of acid and basic mechanisms for each of the three forms c, z, and a. The basic contribution of the cationic form and the acidic contribution of the anionic form are masked by other terms and can be neglected.

The activation energy $E_a$ of the transition state provides a measure of the strength of N–D or N–H bonds.\textsuperscript{25} The activation energy $E_a$ is defined by the Arrhenius equation

$$k = Ae^{-E_a/RT}$$  

(14)

where $A$ is an empirical pre-exponential “frequency factor”, $R$ the universal gas constant, $T$ the temperature and $k$ the exchange rate. The dependence of $E_a$ on pH or pD for H–H and D–D exchange processes and the activation energies and pre-exponential frequency factors are shown in Table 3 for protons and in Table 4 for deuterium.

One can speak of a kinetic isotope effect when the exchange rate is affected by isotopic substitution.\textsuperscript{26} In the present case, we compare the exchange rates of indole protons in tryptophan with H$_2$O on the one hand, and analogous exchange rates of indole deuterons with D$_2$O on the other. The kinetic isotope effect is defined as the ratio of the rate constants $k_D/k_o$. The change in exchange rates results from differences in the vibrational frequencies of the N–H or N–D bonds formed between $^{15}$N and $^1$H or $^2$D.\textsuperscript{27–29} Deuterium will
Table 2 Exchange rate constants $k_0$ and $k_i$ [s$^{-1}$] derived by fitting to eqn (12)

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$k_0$</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_3$</th>
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<td>26.51</td>
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<td>3.70</td>
<td>8.28</td>
<td>8.13</td>
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<td>320</td>
<td>4.14</td>
<td>8.41</td>
<td>10.01</td>
<td>8.29</td>
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Table 3 Activation energies $E_a$ and pre-exponential frequency factors $A$ for the indole proton $H^+$ in tryptophan

<table>
<thead>
<tr>
<th>pH</th>
<th>$E_a$ [kJ mol$^{-1}$]</th>
<th>$A$</th>
</tr>
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<tbody>
<tr>
<td>6.3</td>
<td>88 ± 2</td>
<td>37 ± 1</td>
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<td>7.41</td>
<td>84 ± 2</td>
<td>37 ± 1</td>
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<td>8.31</td>
<td>83 ± 4</td>
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<td>9.08</td>
<td>82 ± 6</td>
<td>40 ± 2</td>
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<td>10.01</td>
<td>86 ± 14</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>10.6</td>
<td>94 ± 12</td>
<td>47 ± 4</td>
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Table 4 Activation energies $E_a$ and pre-exponential frequency factors $A$ for the indole deuterium $D^+$ in tryptophan. The activation energies and the pre-exponential factors are strongly correlated.

<table>
<thead>
<tr>
<th>pH</th>
<th>$E_a$ [kJ mol$^{-1}$]</th>
<th>$A$</th>
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<tbody>
<tr>
<td>1.0</td>
<td>38 ± 17</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>1.5</td>
<td>40 ± 12</td>
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<td>37 ± 3</td>
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<td>82 ± 9</td>
<td>38 ± 3</td>
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<td>36 ± 3</td>
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<td>44 ± 9</td>
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</tbody>
</table>

Deuterium and proton exchange rates $k_D$ (blue) and $k_H$ (red) as a function of pH or pD at 300 K. The pD scale has been corrected according to eqn (11) to take into account the use in D$_2$O of a glass electrode designed for H$_2$O.

Fig. 6 shows exchange rates $k_D$ and $k_H$ at 300 K. For acid catalyzed exchange, $k_D/k_H > 2.5$ because D$_2$O$^+$ is a stronger acid than H$_2$O$^-$. For base catalyzed exchange, $k_D/k_H < 1$. However, to compare the difference between catalysis by OH$^-$ and OD$^-$, we need to take into account the difference of the ionization constants: $pK_w(D_2O) = 14.95$ and $pK_w(H_2O) = 13.99$ at 25 °C.

Fig. 7 shows the base-catalyzed exchange rate constants $k_D$ and $k_H$ as a function of pOH or pOD. The exchange rates $k_D$ are slightly lower than $k_H$, giving the approximate kinetic isotope effects: $k_D/k_H = 2.2 ± 0.3, 2.3 ± 0.3$ and 2.1 ± 0.3 at 300 K, 310 K and 320 K respectively (Fig. 7.) These values result from averages of the exchange rate constants for the zwitterionic and anionic forms (Table 5).

In Table 5 the KIE is defined as $k_i/k_D$ for acid catalysis or as $k_i/k_H$ for base catalysis, where $i = c, z$, and $a$ stand for the cationic, zwitterionic, and anionic forms of tryptophan in solution, with the heaviest isotope always in the denominator. If tunneling can be neglected, the KIE depends on the nature of the transition state. The maximum isotope effect for N–H bonds is $k_{H}/k_{D} \approx 9$, assuming that the bond is completely broken in the

lead to a lower vibrational frequency because of its heavier mass (lower zero-point energy). If the zero-point energy is lower, more energy is needed to break an N–D bond than to break an N–H bond, so that the rate of the exchange will be slower. Moreover, one expects $E_a$ to be larger for deuterium. The results in Tables 3 and 4 do not support this expectation, but if one assumes the same pre-exponential frequency factor for H and D, $E_a$ is indeed larger for the heavier isotope.

Fig. 5 Tryptophan exists in three forms c (cationic), z (zwitterionic) and a (anionic), with mole fractions $f_c, f_z$ and $f_a$ that depend on pD.
transition state (TS). The KIE can be reduced if the bonds are not completely broken in the TS. The KIE can be close to 1 if the TS is very similar to the reactant (N–D bond nearly unaffected) or very similar to the product (N–D bond almost completely broken).

The experimental ratio $k_{\text{OH}}/k_{\text{OD}}$ is near its maximum when $p\text{H} > pK_a$, which suggests that the N–D bond is broken in the rate-limiting step and that the deuteron is half-way between the donor and the acceptor. However the ratio $k_{\text{OH}}/k_{\text{OD}}$ suggests that the N–D bond is either only slightly or almost completely broken in the TS. The protonation of the amine withdraws electron density and increases the acidity of the HN group which favors the formation of the anionic form. This explains why $k_{\text{OH}}/k_{\text{OD}} > 1$ and $k_{\text{OD}}/k_{\text{OD}} > 1$. For the acid-catalyzed exchange constants, we observe an inverse kinetic isotope effect. This can happen when the degree of hybridization of the reactant is lower than that of the reaction center in the TS during the rate-limiting step.

The mechanisms for proton or deuteron exchange have been thoroughly reviewed.30–32 Englander30 and his collaborators pointed out that the rate of the exchange of protons attached to nitrogen depends on the ability to form hydrogen-bonded complexes in the transition state involving the donor (tryptophan) and the acceptor (D$_2$O or OD$^-$). This occurs in three steps: (i) encounter of the donor and the acceptor, (ii) formation of the transition state involving the donor and acceptor, and (iii) cleavage of the N–D bond. The mechanism of acid-catalyzed exchange consists of the addition onto the nitrogen of a D$^+$ ion from the solvent, followed by removal of D$^+$ by D$_2$O (Fig. 8). The mechanism of the base-catalyzed reaction involves removing the indole deuteron to create the conjugate base, which then abstracts a D$^+$ from D$_2$O to regenerate the indole (Fig. 9).

Altogether we can say that the rate-limiting step in the base-catalyzed mechanism is the removal of the proton or deuteron from the nitrogen. On the other hand, for the acid-catalyzed mechanism, is the donation of a proton or deuteron by H$_3$O$^+$ respectively D$_3$O$^+$. Finally, the curves of log $k_{\text{D}}$ vs. pD and of log $k_{\text{H}}$ vs. pH show a combination of specific base catalysis at high pH, and a specific acid catalysis at low pH, which becomes more important at higher temperatures.

Conclusions

We have adapted our method that was originally designed for measuring fast H–H exchange rates $k_{\text{H}}$ to the study of D–D exchange rates $k_{\text{D}}$. In tryptophan in aqueous solution over a range of pH, respectively pD, the kinetic isotope effect, defined as the ratio $k_{\text{H}}/k_{\text{D}}$ between the H–H and D–D exchange rates, was determined at several temperatures. The dependence of the activation energies on pH provides new insight into the mechanisms of the exchange processes. The results agree with the mechanisms discussed by Englander et al.30

Abbreviations

CPMG  Carr Purcell Meiboom Gill
KIE  Kinetic isotope effect
TS  transition state

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**Table 5** Kinetic isotope effects (KIE) $k_{\text{H}}/k_{\text{D}}$ for the exchange rate constants of each of the three forms of tryptophan in solution: c (cationic), z (zwitterionic), and a (anionic)

<table>
<thead>
<tr>
<th></th>
<th>300 K</th>
<th>310 K</th>
<th>320 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{c}}/k_{\text{D}}$</td>
<td>$0.40 \pm 0.04$</td>
<td>$0.40 \pm 0.04$</td>
<td>$0.40 \pm 0.04$</td>
</tr>
<tr>
<td>$k_{\text{z}}/k_{\text{D}}$</td>
<td>$0.37 \pm 0.09$</td>
<td>$0.37 \pm 0.09$</td>
<td>$0.37 \pm 0.09$</td>
</tr>
<tr>
<td>$k_{\text{OH}}/k_{\text{OD}}$</td>
<td>$1.1 \pm 0.1$</td>
<td>$1.0 \pm 0.3$</td>
<td>$1.1 \pm 0.5$</td>
</tr>
<tr>
<td>$k_{\text{OD}}/k_{\text{OD}}$</td>
<td>$7 \pm 2$</td>
<td>$7 \pm 2$</td>
<td>$6 \pm 3$</td>
</tr>
</tbody>
</table>

* Proton exchange rates were not measured at these temperatures.
Acknowledgements

The authors thank Dr Akansha Ashvani Sehgal, Dr Fatiha Kateb and Dr Roberto Buratto for valuable assistance. This work was supported by the Swiss National Science Foundation (SNSF), the Ecole Polytechnique Fédérale de Lausanne (EPFL), the Swiss Commission for Technology and Innovation (CTI), the French CNRS, and the European Research Council (ERC contract ‘Dilute para-water’).

Notes and references

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