Intrinsic Hydrogen Deuterium Exchange Rates in H_2O/D_2O Mixtures

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Abstract

Hydrogen–deuterium exchange (HDX) measurements are widely used to probe protein structural dynamics. Quantitative interpretation of HDX data relies on the concept of an intrinsic exchange rate, which is well characterized in isotopically pure H_2O or D_2O but does not explicitly account for the back exchange that necessarily occurs in H_2O/D_2O mixtures: in this case, both the approach-to-equilibrium rate and the equilibrium deuterium enrichment of amides depend nontrivially on solvent composition and acidity. A practical method is presented to predict intrinsic forward and reverse amide exchange rates in H_2O/D_2O mixtures. The approach combines known second-order reference rates measured in pure solvents with established empirical descriptions of H_2O/D_2O mixtures. The resulting framework yields explicit expressions for forward and back exchange rates as functions of solvent composition and acidity, and correctly recovers the known limits in pure H_2O and pure D_2O. The model predicts composition-dependent kinetic isotope effects and an equilibrium amide fractionation factor of $\phi = 1.20$ for unstructured peptides in base-catalyzed conditions, in close agreement with the experimental value 1.22 reported for poly-D,L-alanine. By providing a physically motivated description of exchange in mixed solvents, this method offers a practical starting point for quantitatively correcting back exchange in HDX–MS and HDX–NMR experiments.

Introduction

Biophysical techniques measuring amide hydrogen-deuterium exchange (HDX) are broadly used to fingerprint structural dynamics of proteins.[1–4] The Linderstrøm-Lang model describes HDX of protein backbone amides according to the reaction[5]

$$\mathrm{NH_{cl}} \underset{k_{\mathrm{cl}}}{\overset{k_{\mathrm{op}}}{\rightleftharpoons}} \mathrm{NH_{op}} \xrightarrow{k_{\mathrm{int}}} \text{exchanged}, \tag{1}$$

where the amide can assume either a closed ($\mathrm{NH_{cl}}$) or open ($\mathrm{NH_{op}}$) conformation, the former being exchange-incompetent. An amide in the open state exchanges with a pseudo-first-order rate k_{int}, called the intrinsic (chemical) exchange rate, which can be estimated as a function of sequence, temperature and pH (or pD) in pure H_2O (or D_2O).[6–8] The protection factor $P = k_{\mathrm{cl}}/k_{\mathrm{op}}$ is the equilibrium constant for site closing[3] that one aims to determine to characterize structural dynamics.[9–14] In EX2 conditions ($k_{\mathrm{cl}} \gg k_{\mathrm{op}}$, k_{int}), the protection factor is related to the observed exchange rate k_{obs} as $P = k_{\mathrm{int}}/k_{\mathrm{obs}}$.

HDX is typically detected using nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS). NMR experiments and the labeling step in HDX-MS are commonly conducted in buffers containing 80-95% D_2O.[15] While mixed-solvent equilibrium and kinetic isotope effects are well established,[16,17] they are often treated approximately or neglected in practical analyses, despite a body of work that used HDX in H_2O/D_2O mixtures to quantify amide fractionation and probe hydrogen-bonded structure.[18–21] Mixed H_2O/D_2O solvents are also deliberately used in NMR protocols that quantify amide–solvent exchange, including CLEANEX-PM[22] and SOLEXSY.[23]

In H_2O/D_2O mixtures, exchange is intrinsically bidirectional and cannot be described by a single rate constant. n this work, we make this explicit by decomposing intrinsic exchange into forward and reverse components, enabling a quantitative treatment of kinetic and equilibrium isotope effects in mixed solvents that is not accessible within standard intrinsic-rate models. This work presents a practical method to predict forward and reverse amide exchange rates in H_2O/D_2O mixtures as functions of the degree of deuteration and acidity. The approach combines published reference parameters measured in pure solvents with an operational acidity scale for mixtures, and a probabilistic treatment for reprotonation inserting H or D. The resulting expressions recover pure-solvent limits and provide explicit mixture-dependent rates.

Methods

Proton transfer theory

The chemistry of HDX reactions is described by proton transfer theory:[3,24]

$$\mathrm{XH} + \mathrm{Y} \rightleftharpoons \Big(\mathrm{XH} \cdots \mathrm{Y} \rightleftharpoons \mathrm{X} \cdots \mathrm{HY}\Big) \rightleftharpoons \mathrm{X} + \mathrm{YH}. \tag{2}$$

A proton donor XH collides with a proton acceptor Y, forming a short-lived encounter complex (bracketed in Eq. 2) in which proton redistribution is faster than dissociation. Here, XH and Y are generic acid/base labels and may be neutral or charged depending on the specific mechanism, the corresponding conjugate-acid pairs being XH/X and YH/Y. Complex formation occurs with a second-order (diffusion-limited) rate constant k_d estimated as $10^{10}\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$.[25] The complex is regarded as highly dynamical but always weakly populated. Focusing on the forward process $\mathrm{XH} + \mathrm{Y} \rightarrow \mathrm{X} + \mathrm{HY}$, the proton transfer rate constant k_t can be expressed as[3]

$$k_\mathrm{t} = \frac{k_\mathrm{d}}{1 + 10^{\Delta \mathrm{p}K_\mathrm{a}}}, \tag{3}$$

where $\Delta \mathrm{p}K_\mathrm{a} = \mathrm{p}K_\mathrm{a}(\mathrm{XH}) - \mathrm{p}K_\mathrm{a}(\mathrm{YH})$ is the difference between the acidity of the donor XH and that of the conjugate acid YH of the acceptor. The acidity constant of a generic acid HA is defined as

$$\mathrm{p}K_\mathrm{a}(\mathrm{HA}) = -\log K_\mathrm{a}(\mathrm{HA}) = -\log\left(\frac{[\mathrm{A}^-][\mathrm{H}^+]}{[\mathrm{HA}]}\right), \tag{4}$$

with $K_\mathrm{a}(\mathrm{HA})$ being the acid dissociation constant.

Intrinsic exchange rates

In 100% D_2O, amide HDX involves removal of a proton (H^+) from the amide group and the transfer of a deuteron (D^+) from bulk solvent to the amide. The reaction can be acid-, base-, or water-catalyzed, and the pseudo-first-order HDX intrinsic exchange rate of Eq. 1 can be written as

$$k_\mathrm{int} = k_\mathrm{acid}[\mathrm{D}^+] + k_\mathrm{base}[\mathrm{OD}^-] + k_\mathrm{water}[\mathrm{D_2O}], \tag{5}$$

where k_acid, k_base and k_water are the corresponding second-order rate constants. Temperature and pH dependence of HDX second-order rate constants were measured by Englander's group for poly-D,L-alanine (PDLA) and 3-alanine (3-Ala).[6–8]

Below, we dissect the base-catalyzed mechanism of HDX, which dominates by orders of magnitude at near-neutral conditions.[3] Extension to other mechanisms (acid- and water-catalyzed) is reported in the Appendix. For base-catalyzed HDX in pure D_2O, the kinetic

bottleneck is the first step, i.e. abstraction of the amide proton by OD^-. The amide is rapidly deuterated by a solvent D_2O molecule, and a new OD^- ion is produced. In this case, the intrinsic rate can be estimated as

$$k_{\mathrm{int}} = k_{\mathrm{B,ref}}(T)(B_\lambda \times B_\rho)[\mathrm{OD}^-]. \tag{6}$$

B_λ and B_ρ are tabulated factors that depend on amino acid type and position relative to the amide group (λ for left, ρ for right, see Table A1).[6] These account for steric and inductive effects from neighboring residues, and were demonstrated to be simply additive.[26] $k_{\mathrm{B,ref}}(T)$ is the reference rate (i.e. the exchange rate for PDLA or 3-Ala) at temperature T, which exhibits an Arrhenius-type dependence:

$$k_{\mathrm{B,ref}}(T) = k_{\mathrm{B,ref}}(T_{\mathrm{ref}}) \exp\left(-\frac{E_{\mathrm{B}}}{R}\left(\frac{1}{T} - \frac{1}{T_{\mathrm{ref}}}\right)\right), \tag{7}$$

where $E_{\mathrm{B}} = 17\,\mathrm{kcal\ mol^{-1}}$, R is the gas constant, and $k_{\mathrm{B,ref}}(T_{\mathrm{ref}})$ is the second-order rate constant measured. Values for $k_{\mathrm{B,ref}}(20°\mathrm{C})$ for H and D in H_2O and H in D_2O are given in Table 1.[6–8]

Table 1: Reference rates (PDLA) for the base-catalyzed exchange of amides in disordered peptides, at 20°C.[8] Reference rates are specified by two pedices, the first indicating the amide-bound isotope being removed, the second indicating the isotope in the catalytic base abstracting the proton, *cfr Forward and back exchange rates* Section. Rates for 3-Ala are obtained multiplying the reference rates by a factor 1.35.[8]

		$\log k_{\mathrm{B,ref}}$ ($\mathrm{M^{-1}\,min^{-1}}$)
NH in H_2O	k_{HH}	10.08
ND in H_2O	k_{DH}	10.00
NH in D_2O	k_{HD}	10.18

H_2O/D_2O mixtures

This section introduces an operational acidity scale for mixed H_2O/D_2O solvents and then analyzes the influence of solvent composition on hydroxide availability and reprotonation probabilities that dictate intrinsic exchange rates. Let L denote either protium (H) or deuterium (D) isotope. An isotopic exchange reaction in a solvent with D_2O mole fraction x (H_2O mole fraction $1 - x$) involving a solute XL is

$$\mathrm{XH} + \mathrm{DOL} \overset{\phi_{\mathrm{XL}}}{\rightleftharpoons} \mathrm{XD} + \mathrm{HOL}. \tag{8}$$

Here, XH and XD are the solute protiated and deuterated species, while HOL and DOL are solvent molecules containing at least one H or D atom, respectively. The equilibrium

constant ϕ_{XL} of the reaction (8) is called the fractionation factor of XL

$$\phi_{\mathrm{XL}} = \frac{(\mathrm{D/H})_{\mathrm{XL}}}{(\mathrm{D/H})_{\mathrm{L_2O}}} = \frac{[\mathrm{XD}]}{[\mathrm{XH}]} \times \frac{1-x}{x}, \tag{9}$$

which quantifies the enrichment in D of solute XL with respect to the solvent.[16]

The concentrations $[\mathrm{H}^+]$ and $[\mathrm{OH}^-]$ in pure $\mathrm{H_2O}$ follow from the pH and the ionic product, $K_{\mathrm{w,H_2O}}$, through

$$\mathrm{pH} = -\log[\mathrm{H}^+], \quad \mathrm{pOH} = \mathrm{p}K_{\mathrm{w,H_2O}} - \mathrm{pH} = -\log[\mathrm{OH}^-],$$

where $\mathrm{p}K_{\mathrm{w,H_2O}} = -\log K_{\mathrm{w,H_2O}}$. Analogous relations hold in pure $\mathrm{D_2O}$ defining $[\mathrm{D}^+]$ and $[\mathrm{OD}^-]$ in terms of pD, pOD and $K_{\mathrm{w,D_2O}}$.

In $\mathrm{H_2O/D_2O}$ mixtures, multiple isotopologue ionization equilibria coexist (involving $\mathrm{H_2O}$, HDO, $\mathrm{D_2O}$ and the corresponding ions). Rather than assigning separate equilibrium constants to each elementary process, it is convenient to work with an operational acidity scale based on the total concentration of $[\mathrm{L}^+] = [\mathrm{H}^+] + [\mathrm{D}^+]$, introducing the pL

$$\mathrm{pL} = -\log[\mathrm{L}^+], \tag{10}$$

as used in studies of mixed $\mathrm{H_2O/D_2O}$ solvents by glass electrodes.[27] Similarly one can define the pOL via

$$\mathrm{pOL} = -\log[\mathrm{OL}^-], \tag{11}$$

where $[\mathrm{OL}^-] = [\mathrm{OH}^-] + [\mathrm{OD}^-]$. At 25°C, the ionic product of $\mathrm{D_2O}$ ($\mathrm{p}K_{\mathrm{w,D_2O}} = 1.3 \times 10^{-15}$) is lower than that of $\mathrm{H_2O}$ ($\mathrm{p}K_{\mathrm{w,H_2O}} = 14$).[28,29] In mixed solvents, the corresponding effective ionic product $\mathrm{pK_w}(x) = \mathrm{pL}(x) + \mathrm{pOL}(x)$ varies nonlinearly with the deuterium mole fraction x, and can be described empirically as[30]

$$\Delta\mathrm{p}K_{\mathrm{w}}(x) = \mathrm{p}K_{\mathrm{w}}(x) - \mathrm{p}K_{\mathrm{w,H_2O}} = 0.7282x + 0.0512x^2 + 0.0826x^3. \tag{12}$$

To relate the mixture $\mathrm{pL}(x)$ to the glass electrode pH-meter reading, pH^*, the following relation can be used:[31]

$$\mathrm{pL}(x) = \mathrm{pH}^* \left(1 + \frac{\Delta\mathrm{p}K_{\mathrm{w}}(x)}{\mathrm{p}K_{\mathrm{w,H_2O}}}\right). \tag{13}$$

For $\mathrm{D_2O}$ at 25°C and $\mathrm{pH}^* = 7$, Eq. 13 yields $\mathrm{pD} = \mathrm{pL}(1) \simeq 7.43$, i.e., the traditional "+0.4" correction widely used by HDX practitioners.

The temperature dependence of $\mathrm{p}K_{\mathrm{w,H_2O}}$ has been empirically determined as[32]

$$\mathrm{p}K_{\mathrm{w,H_2O}}(T) = 670.86 - \frac{34691.6}{T} - 105.15\ln T + 0.10757\,T + \frac{2358000}{T^2}. \tag{14}$$

At given composition x, the p$K(x)$ can be computed via Eq. 12. Once the mixture pH* is measured, the pL(x) can be computed using Eq. 13 and the pOL(x) is given by subtraction of the two values as stated above. Reported values for the fractionation factor ϕ_{OL} (0.47[31] or 0.43[33]) can be used to compute $[\mathrm{OH}^-](x)$ and $[\mathrm{OD}^-](x)$, *cfr* Eq. A4. Examples are shown in the upper and middle panels of Figure 1.

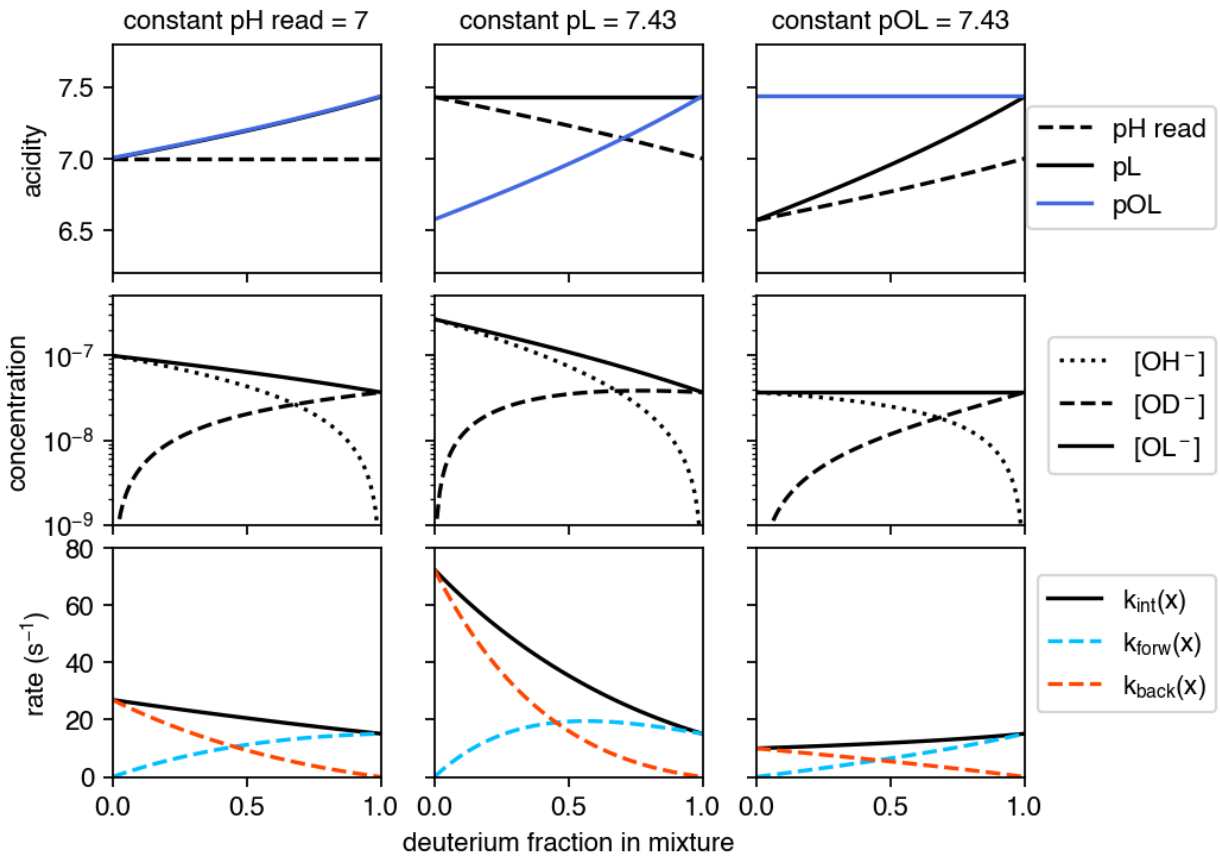

Figure 1: Acidity, concentration of ions and intrinsic rates in mixtures, expressed as a function of the deuterium fraction in the mixture, in three different scenarios: constant pH read (left), constant pL (center), constant pOL (right). Upper panels: mixture pH read, pL and pOL. Middle panels: concentrations of the ions relevant for base-catalyzed HDX: $[\mathrm{OH}^-]$, $[\mathrm{OD}^-]$, and $[\mathrm{OL}^-] = [\mathrm{OH}^-] + [\mathrm{OD}^-]$. Lower panels: intrinsic forward k_{forw}, back k_{back} and mixture intrinsic $k_{\mathrm{int}}(x) = k_{\mathrm{forw}}(x) + k_{\mathrm{back}}(x)$ exchange rates of PDLA. Magnitude and direction of kinetic isotope effects depend on the quantity fixed while exploring different H_2O/D_2O compositions. Data for 25°C.

Forward and back exchange rates

The reaction considered for HDX of an open amide in a H_2O/D_2O mixture is

$$\mathrm{NH} + \mathrm{DOL} \underset{\tilde{k}_{\mathrm{back}}}{\overset{\tilde{k}_{\mathrm{forw}}}{\rightleftharpoons}} \mathrm{ND} + \mathrm{HOL}, \tag{15}$$

where $\tilde{k}_{\mathrm{forw}}$ and $\tilde{k}_{\mathrm{back}}$ are the base-catalyzed reactions rate constants. Reference rates in pure solvents (Table 1) are denoted k_{HH}, k_{DH}, k_{HD}, k_{DD} respectively. Here, the first index indicates the isotope initially bound to the amide (NH or ND) and the second indicates the isotope of the catalytic base (OH^- or OD^-) performing proton abstraction. These correspond to the base-catalyzed rate removal of the amide-bound proton, followed by rapid reprotonation from the bulk solvent. The unmeasured rate k_{DD} was estimated as

$$k_{\mathrm{DD}} = \frac{k_{\mathrm{HD}} k_{\mathrm{DH}}}{k_{\mathrm{HH}}}, \tag{16}$$

that is, assuming that reaction rates are governed entirely by the zero-point energies of the participating species.[34]

The rates of reaction (15) are obtained as a sum of terms, *cfr* Eq. 5, each having the same structure as in Eq. 6:

$$\begin{aligned} \tilde{k}_{\mathrm{forw}}(x) &= k_{\mathrm{HH}}(B_\lambda \times B_\rho)_{\mathrm{HH}}[\mathrm{OH}^-](x) + k_{\mathrm{HD}}(B_\lambda \times B_\rho)_{\mathrm{HD}}[\mathrm{OD}^-](x) \\ \tilde{k}_{\mathrm{back}}(x) &= k_{\mathrm{DH}}(B_\lambda \times B_\rho)_{\mathrm{DH}}[\mathrm{OH}^-](x) + k_{\mathrm{DD}}(B_\lambda \times B_\rho)_{\mathrm{DD}}[\mathrm{OD}^-](x) \end{aligned}. \tag{17}$$

The coefficients of the products $(B_\lambda \times B_\rho)$ are tabulated, *cfr* Appendix.

The scheme (15) can be simplified to a pseudo-first order reaction

$$\mathrm{NH} \underset{k_{\mathrm{back}}}{\overset{k_{\mathrm{forw}}}{\rightleftharpoons}} \mathrm{ND}, \tag{18}$$

where the pseudo-first order forward and back exchange rates are weighted by the probability of encountering a reactive solvent molecule:

$$\begin{aligned} k_{\mathrm{forw}}(x) &= x\tilde{k}_{\mathrm{forw}}(x) \\ k_{\mathrm{back}}(x) &= (1-x)\tilde{k}_{\mathrm{back}}(x) \end{aligned}, \tag{19}$$

that is, upon removal of the proton, in the forward reaction reprotonation occurs by DOL with probability $x = [\mathrm{DOL}]/[\mathrm{L_2O}]$, and by HOL with probability $1 - x = [\mathrm{HOL}]/[\mathrm{L_2O}]$ in the reverse.

The solution to the kinetics in Eq. 18, with the constraint $\mathrm{d}\left([\mathrm{NH}] + [\mathrm{ND}]\right)/\mathrm{d}t = 0$ and calling $D(t)$ the normalized concentration of deuterated amides over time, is

$$D(t) = D_{\mathrm{eq}}(x) - (D_0 - D_{\mathrm{eq}}(x))\mathrm{e}^{-k_{\mathrm{int}}(x)t}, \tag{20}$$

where D_0 is the initial condition,

$$D_{\mathrm{eq}}(x) = \frac{k_{\mathrm{forw}}(x)}{k_{\mathrm{forw}}(x) + k_{\mathrm{back}}(x)} \tag{21}$$

is the fraction of deuterated amides at equilibrium, and the intrinsic rate in the mixture is defined as

$$k_{\mathrm{int}}(x) = k_{\mathrm{forw}}(x) + k_{\mathrm{back}}(x). \tag{22}$$

Results and discussion

The rates k_{forw} and k_{back} are generally different and depend on the fraction x of D_2O in the solvent. The stationary state (Eq. 21), as well as the approach-to-equilibrium rate (Eq. 22), are functions of x through $k_{\mathrm{forw}}(x)$ and $k_{\mathrm{back}}(x)$, *cfr* Eq. 19. In other words, kinetic and equilibrium isotope effects are present.

Kinetic isotope effects

Kinetic isotope effects cause the reaction rate $k_{\mathrm{int}}(x)$ to vary as a function of the solvent composition. These are due not only to the different second order rates of Table 1, but also to the solvent composition x, which influences the mixture ion product $\mathrm{p}K_{\mathrm{w}}(x)$ and acidity $\mathrm{pL}(x)$, *cfr* Eqs. 12 and 13.

By construction, the measured reference rates are recovered in pure H_2O and D_2O: by Eqs. 17 and 19, for $x = 0$ (pure H_2O),

$$\begin{aligned} k_{\mathrm{forw}}(0) &= 0, \\ k_{\mathrm{back}}(0) &= k_{\mathrm{DH}}(B_\lambda \times B_\rho)_{\mathrm{DH}}[\mathrm{OH}^-], \end{aligned}$$

while for $x = 1$ (pure D_2O),

$$\begin{aligned} k_{\mathrm{forw}}(1) &= k_{\mathrm{HD}}(B_\lambda \times B_\rho)_{\mathrm{HD}}[\mathrm{OD}^-], \\ k_{\mathrm{back}}(1) &= 0. \end{aligned}$$

The question "how does $k_{\mathrm{int}}(x)$ vary with x?" has no unique answer: it depends on the acidity measure held constant while varying x. At fixed temperature, the ionic product of the mixture $\mathrm{p}K_{\mathrm{w}}(x)$ is assumed to depend on x only, *cfr* Eq. 12. The observed exchange rate depends on the acidity of the solution through the concentration of ions OH^- and OD^-, *cfr* Eq. 17. However, different definitions of acidity are possible in a mixture, e.g. based on pH^*, pL or pOL. The behavior of $k_{\mathrm{int}}(x)$ in these scenarios is illustrated in the lower panels of Figure 1 and commented below. These results extend the observations made for pure solvents[7] to mixtures.

In the first column of Figure 1, mixtures of different compositions that yield same pH* are considered. Starting from neutral pure H_2O at 25°C (pH = 7), for $x > 0$ one finds $pK_w(x) > pK_{\mathbf{w},H_2O}$ (Eq. 12). For varying composition, the solution remains neutral, thus both pL and pOL increase. A higher pOL implies fewer available OL^- ions for base-catalyzed HDX, hence $k_{int}(x)$ decreases for increasing x. As a result, the intrinsic rate of H → D in pure D_2O is about 2-fold slower than D → H in pure H_2O.
In the second example, the effective acidity of the mixture, pL, was fixed to 7.43 (because pL(1) = 7.43 at pH* = 7 and T = 25°C). Here, decreasing x causes $pK_w(x)$ to decrease. Because pL is fixed, pOL decreases too, and a more substantial kinetic isotope effect is observed. D → H in H_2O results about 5-fold faster than H → D in D_2O on a pL scale.
The third scenario evaluates the effect of x at fixed pOL. In this case, $k_{int}(x)$ increases with x. This is because the concentration of catalysts is fixed hence the HDX rates depends on the rates of Table 1. Since $k_{HH} > k_{DH}$ and $k_{HD} > k_{DD}$, that is, it is easier to extract H than D from the amide group, the HDX rate is higher for increasing D_2O content. In this case, the extent of the isotope effect (moving from pure D_2O to H_2O) is smaller than the other cases (1.5 times faster in D_2O).

As an example, Figure 2 displays the approach-to-equilibrium rates $k_{int}(x)$ (Eq. 22) for two short sequences at fixed pH*, which is the experimentally controlled parameter, and varying D_2O content in the mixture. Kinetic isotope effects lead to a decrease of the reaction rate constant when the D_2O content is increased, as discussed above.

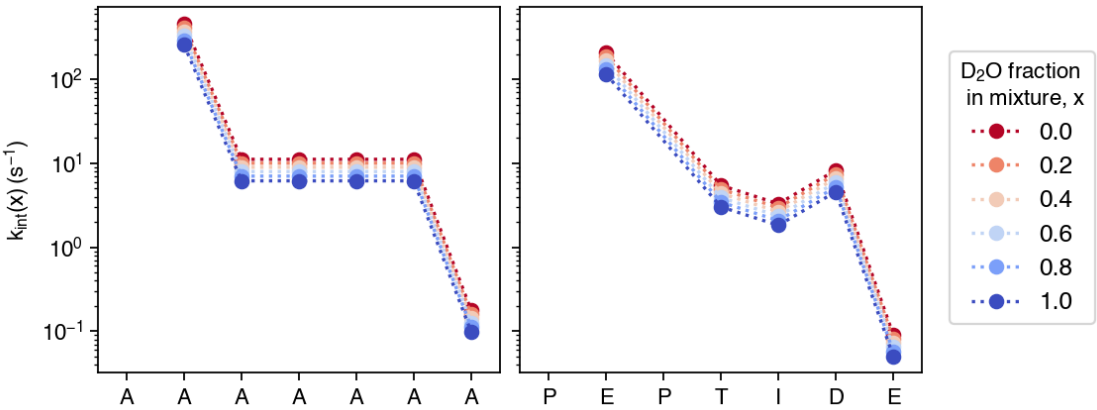

Figure 2: Intrinsic exchange rates $k_{int}(x)$ for short polypeptide sequences '`AAAAAAA`' (left) and '`PEPTIDE`' (right), calculated for varying D_2O fraction in the mixture x, T = 25°C, pH* = 7, and using PDLA reference rates.

Equilibrium isotope effects

An equilibrium isotope effect is described by the fractionation factor (Eq. 9), which for reaction (15) is simply $\phi = \tilde{k}_{forw}(x)/\tilde{k}_{back}(x)$. Within the assumptions of the base-catalyzed

model developed above, the fractionation factor reduces to

$$\phi = \frac{\tilde{k}_{\text{forw}}(x)}{\tilde{k}_{\text{back}}(x)} = \frac{k_{\text{HH}}}{k_{\text{DH}}} = 1.20, \tag{23}$$

with k_{HH} and k_{DH} from Table 1.

This value is unaffected by neighboring side chains, because the local sequence multiplicative factors ($B_\lambda \times B_\rho$) cancel in the ratio, *cfr* Appendix. A corollary of Eq. 23 is that, for random coil peptides in conditions where base catalysis dominates, the equilibrium deuterium enrichment of amides will exceed the bulk D_2O content, i.e. $D_{\text{eq}}(x) > x$. This is shown in Figure 3. A physical basis of this difference is given by the difference in the pK_{a} between amide deuterated (ND) and protiated (NH) forms: pK_{a}(ND) > pK_{a}(NH), thus D tends to be bound more tightly to the amide, and the amide is enriched with D at equilibrium.[7] The value $\phi = 1.20$, as well as the predicted deuterium enrichment, is insensitive to temperature and pH (provided that the base-catalyzed mechanism is the dominant one).

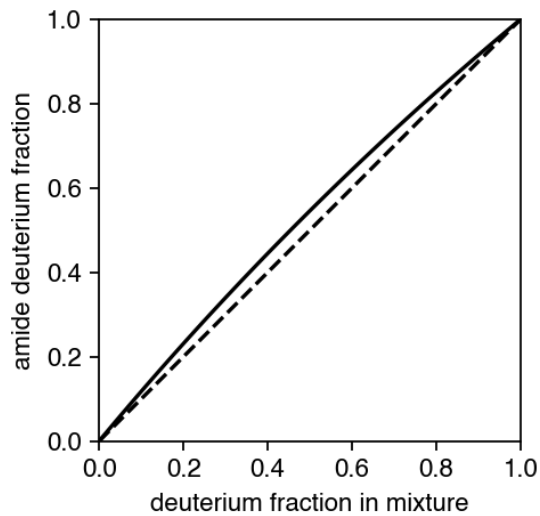

Figure 3: Equilibrium isotope effects. At equilibrium, the amide is enriched in D with respect to solvent ($D_{\text{eq}}(x) > x$, as $\phi = 1.20 > 1$).

The theoretical value $\phi = 1.20$ (Eq. 23) agrees closely with the measured one for PDLA ($\phi_{\text{PDLA}} = 1.22$).[19] The larger fractionation factor reported for poly-D,L-lysine (PDLK) within the same study[19] ($\phi_{\text{PDLK}} = 1.46$) may be attributed to a residual helical propensity of PDLA.[35] It is thus possible that the equilibrium fractionation expected for unstructured peptides is underestimated, since structured amides generally display lower fractionation (usually, $\phi \lesssim 1$) than unstructured ones.[18,19] Residual uncertainties remain also because acid- and water-catalyzed exchange, solvent–side-chain interactions, and buffer composition can all shift the apparent fractionation. Notably, the equilibrium fractionation factor emerges directly from the kinetic framework without introducing additional adjustable parameters or sequence-specific assumptions. This indicates that the observed fractionation is an inherent

consequence of intrinsic exchange chemistry in mixed solvents rather than an independent thermodynamic input.

Conclusions

This work provides a practical method to estimate intrinsic exchange rates in H_2O/D_2O mixtures as a function of solvent composition and acidity, recovering known limits for pure solvents.[6–8] The model yields closed expressions for forward and back exchange rates, that for base-catalyzed exchange incorporate mixture-dependent catalyst availability and probabilistic insertion of H or D. For completeness, the corresponding acid- and water-catalyzed contributions are described in the Appendix.

Kinetic and equilibrium isotope effects emerge as natural consequences of the chemistry of the mixture and are not determined by composition alone: both the magnitude and the direction of the kinetic isotope effect depend on how acidity is specified or experimentally controlled (e.g. fixed pH^* versus fixed operational acidity). Under base-catalyzed conditions, the framework predicts equilibrium amide enrichment in D, in good agreement with measurements performed on PDLA ($\phi \simeq 1.20$).[19] Mismatch with PDLK ($\phi \simeq 1.46$)[19] suggests that residual structure or other unmodeled factors can affect fractionation and should be assessed by systematic benchmarking against random-coil standards.

Beyond providing explicit intrinsic exchange rates in mixtures, this framework emphasizes that HDX is bidirectional and that should be described by both forward and back exchange rates to capture kinetic and equilibrium isotope effects. The same framework can be applied to HDX-NMR data, where mixture-dependent kinetics and final enrichment must be accounted for to obtain unbiased estimates of local stability (in terms of protection factors).[36] This is also relevant to HDX-MS workflows, where back exchange can occur both during labeling (due to incomplete buffer deuteration) and in subsequent handling steps (e.g. quenching, usually performed at intermediate D_2O content, low temperature and pH).[15] A consistent treatment of exchange in mixture is the first step towards physically-grounded back exchange corrections. In this case, the chemistry of the mixture defines baseline effects, that must be integrated with the environment (e.g. salts, co-solvents, denaturants, and whatever additive that can alter exchange kinetics).

Code availability

A Python[37] implementation of the method presented, allowing to compute k_{forw} and k_{back} for arbitrary sequences as a function of solvent temperature, glass electrode pH read, and composition, is available at `https://github.com/pacilab/hdx-rates-mixtures`.

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Appendix

Multiplicative factors for intrinsic exchange rates

Table A1: Multiplicative factors for intrinsic exchange rates.[6–8]

	$\log A_\lambda$	$\log A_\rho$	$\log B_\lambda$	$\log B_\rho$
A	0.00	0.00	0.00	0.00
C	−0.54	−0.46	0.62	0.55
C2	−0.74	−0.58	0.55	0.46
D0	0.90	0.58	0.10	−0.18
D+	−0.90	−0.12	0.69	0.60
E0	−0.90	0.31	−0.11	−0.15
E+	−0.60	−0.27	0.24	0.39
F	−0.52	−0.43	−0.24	0.06
G	−0.22	0.22	−0.03	0.17
H0	—	—	−0.10	0.14
H+	−0.80	−0.51	0.80	0.83
I	−0.91	−0.59	−0.73	−0.23
K	−0.56	−0.29	−0.04	0.12
L	−0.57	−0.13	−0.58	−0.21
M	−0.64	−0.28	−0.01	0.11
N	−0.58	−0.13	0.49	0.32
P	—	−0.19	—	−0.24
Pc	—	−0.85	—	0.60
Q	−0.47	−0.27	0.06	0.20
R	−0.59	−0.32	0.08	0.22
S	−0.44	−0.39	0.37	0.30
T	−0.79	−0.47	−0.07	0.20
V	−0.74	−0.30	−0.70	−0.14
W	−0.40	−0.44	−0.41	−0.11
Y	−0.41	−0.37	−0.27	0.05
NT	—	−1.32	—	1.62
CT	0.96	—	−1.80	—

Coefficients A_λ, A_ρ, B_λ, B_ρ are given in Table A1 in both H_2O and D_2O for all residues except Asp (D), Glu (E) and His (H).[8] In these cases, the coefficients $A_{\zeta,\mathrm{L}}$, $B_{\zeta,\mathrm{L}}(\mathtt{X})$ of residue X, where ζ is λ or ρ, and L is H (for D → H exchange) or D (H → D), are given by

$$\begin{aligned} A_{\zeta,\mathrm{L}}(\mathtt{X}) &= \frac{10^{A_\zeta(\mathtt{X+})-\mathrm{p}K_{\mathrm{c,L}}(\mathtt{X})} + 10^{A_\zeta(\mathtt{X0})-\mathrm{pL}(x)}}{10^{-\mathrm{p}K_{\mathrm{c,L}}(\mathtt{X})} + 10^{-\mathrm{pL}(x)}}, \\ B_{\zeta,\mathrm{L}}(\mathtt{X}) &= \frac{10^{B_\zeta(\mathtt{X+})-\mathrm{p}K_{\mathrm{c,L}}(\mathtt{X})} + 10^{B_\zeta(\mathtt{X0})-\mathrm{pL}(x)}}{10^{-\mathrm{p}K_{\mathrm{c,L}}(\mathtt{X})} + 10^{-\mathrm{pL}(x)}}, \end{aligned} \tag{A1}$$

where

$$\mathrm{p}K_{\mathrm{c,L}}(\mathtt{X}) = -\log\left(10^{C_{\mathrm{L}}(\mathtt{X})}\exp\left(-\frac{E_{\mathrm{a,L}}(\mathtt{X})}{R}\left(\frac{1}{T}-\frac{1}{T_{\mathrm{ref}}}\right)\right)\right), \tag{A2}$$

and parameters $C_{\mathrm{L}}(\mathtt{X})$, $E_{\mathrm{a,L}}(\mathtt{X})$, T_{ref} given in Table A2.

Table A2: Parameters for the computation of B_λ and B_ρ coefficients for Asp, Glu, His.[8]

residue	$E_{\mathrm{a,H}}$ (kcal/mol)	C_{H}	$E_{\mathrm{a,D}}$ (kcal/mol)	C_{D}	T_{ref}(K)
D	0.96	-3.87	1	-4.48	278
E	1.083	-4.33	1.083	-4.93	278
H	7.5	-7	7.5	-7.42	278

Calculation of ϕ

From definitions of Eqs. A1 and A2, one has that $(B_\lambda \times B_\rho)_{\mathrm{HH}} = (B_\lambda \times B_\rho)_{\mathrm{DH}}$ and $(B_\lambda \times B_\rho)_{\mathrm{DD}} = (B_\lambda \times B_\rho)_{\mathrm{HD}}$. Calling $\xi = (B_\lambda \times B_\rho)_{\mathrm{HD}}/(B_\lambda \times B_\rho)_{\mathrm{HH}}$ ($\xi = 1$ if Asp, Glu or His are not neighboring to the amide group), the rates $\tilde{k}_{\mathrm{forw}}$ and $\tilde{k}_{\mathrm{back}}$ of Eq. 17 are written

$$\begin{aligned}\tilde{k}_{\mathrm{forw}}(x) &= (B_\lambda \times B_\rho)_{\mathrm{HH}}\left(k_{\mathrm{HH}}[\mathrm{OH}^-](x) + \xi k_{\mathrm{HD}}[\mathrm{OD}^-](x)\right)\\ \tilde{k}_{\mathrm{back}}(x) &= (B_\lambda \times B_\rho)_{\mathrm{HH}}\left(k_{\mathrm{DH}}[\mathrm{OH}^-](x) + \xi k_{\mathrm{DD}}[\mathrm{OD}^-](x)\right)\end{aligned}. \tag{A3}$$

$[\mathrm{OH}^-](x)$ and $[\mathrm{OD}^-](x)$ can be written in terms of $[\mathrm{OL}^-](x)$ using the definition of fractional abundance of OD^- in OL^-:[16]

$$f_{\mathrm{OL}}(x) = \frac{[\mathrm{OD}^-](x)}{[\mathrm{OL}^-](x)} = \frac{x\phi_{\mathrm{OL}}}{1 - x + x\phi_{\mathrm{OL}}}. \tag{A4}$$

Hence Eq. A3 becomes

$$\begin{aligned}\tilde{k}_{\mathrm{forw}}(x) &= (B_\lambda \times B_\rho)\left(k_{\mathrm{HH}}(1-f_{\mathrm{OL}}) + \xi k_{\mathrm{HD}} f_{\mathrm{OL}}\right)[\mathrm{OL}^-](x)\\ \tilde{k}_{\mathrm{back}}(x) &= (B_\lambda \times B_\rho)\left(k_{\mathrm{DH}}(1-f_{\mathrm{OL}}) + \xi k_{\mathrm{DD}} f_{\mathrm{OL}}\right)[\mathrm{OL}^-](x)\end{aligned}, \tag{A5}$$

where the subscript "HH" of $(B_\lambda \times B_\rho)$ has been dropped to ease the notation. Recalling Eq. 16, and defining $\psi = k_{\mathrm{HD}}/k_{\mathrm{HH}} = k_{\mathrm{DD}}/k_{\mathrm{DH}}$,

$$\begin{aligned}\tilde{k}_{\mathrm{forw}}(x) &= k_{\mathrm{HH}}(B_\lambda \times B_\rho)\left(1 - f_{\mathrm{OL}} + \xi\psi f_{\mathrm{OL}}\right)[\mathrm{OL}^-](x)\\ \tilde{k}_{\mathrm{back}}(x) &= k_{\mathrm{DH}}(B_\lambda \times B_\rho)\left(1 - f_{\mathrm{OL}} + \xi\psi f_{\mathrm{OL}}\right)[\mathrm{OL}^-](x)\end{aligned}, \tag{A6}$$

from which one has

$$\frac{\tilde{k}_{\mathrm{forw}}}{\tilde{k}_{\mathrm{back}}} = \frac{k_{\mathrm{HH}}}{k_{\mathrm{DH}}}(= 1.20). \tag{A7}$$

Extension to other mechanisms

In H_2O/D_2O mixtures (D_2O mole fraction x), the intrinsic exchange rates $k_{\text{forw}}(x)$, $k_{\text{back}}(x)$ are given as a sum of contributions (of acid-, base-, and water-catalyzed exchange):

$$\begin{aligned} k_{\text{forw}}(x) =& k_{\text{A,forw}}(x) + k_{\text{B,forw}}(x) + k_{\text{W,forw}}(x), \\ k_{\text{back}}(x) =& k_{\text{A,back}}(x) + k_{\text{B,back}}(x) + k_{\text{W,back}}(x). \end{aligned}$$

In the main text, the derivation focused on the base-catalyzed contribution, which dominates near-neutral conditions and therefore governs most HDX measurements. For completeness, this appendix outlines how the other two pathways can be incorporated into the same framework as presented in details in the main text for the case of the base-catalyzed pathway.

Table A3: Reference rates for acid- and water-catalyzed exchange of at 20°C.[8]

		$\log k_{\text{A,ref}}$ ($M^{-1}\,min^{-1}$)		$\log k_{\text{W,ref}}$ ($M^{-1}\,min^{-1}$)
NH in D_2O	$k_{\text{A}}^{\text{H}\to\text{D}}$	1.62	$k_{\text{W}}^{\text{H}\to\text{D}}$	-1.50
ND in H_2O	$k_{\text{A}}^{\text{D}\to\text{H}}$	1.40	$k_{\text{W}}^{\text{D}\to\text{H}}$	-1.60

Water-catalyzed exchange

In the water-catalyzed pathway, a solvent water molecule acts as a base, abstracting the amide H or D. This contribution is essentially pH-independent and usually negligible compared to the other terms, except in the region of the minimum. Sequence effects are addressed by the coefficients of Table A1, as $k_{\text{W}} = k_{\text{W,ref}}(B_\lambda \times B_\rho)$, with reference rates given in Table A3. Literature tabulates the rates of H $\to$ D exchange in D_2O ($k_{\text{W}}^{\text{H}\to\text{D}}$) and the rate of D $\to$ H in H_2O ($k_{\text{W}}^{\text{D}\to\text{H}}$). In a mixed solvent with deuterium mole fraction x, it is assumed that the rate of abstraction depends only on the isotope being removed, while the isotope incorporated upon reprotonation is determined solely by the solvent composition. In this case, the forward and back water-catalyzed intrinsic exchange rates are

$$k_{\text{W,forw}}(x) = x k_{\text{W}}^{\text{H}\to\text{D}}, \quad k_{\text{W,back}}(x) = (1-x) k_{\text{W}}^{\text{D}\to\text{H}}. \tag{A8}$$

Acid-catalyzed exchange

In the acid-catalyzed pathway, exchange proceeds through protonation of the amide by solvated hydronium species before loss of the bound isotope. In this case, the isotopic identity of the proton-donating species, i.e. D^+ or H^+, determines whether the event contributes to H $\to$ D or D $\to$ H exchange. Acid-catalyzed second-order rates $k_{\text{A}}^{\text{H}\to\text{D}}$, $k_{\text{A}}^{\text{D}\to\text{H}}$ can be calculated from reference values (Table A3) and accounting for the sequence dependence with the corresponding multiplicative factors A_λ, A_ρ from Table A1, e.g. $k_{\text{A}}^{\text{H}\to\text{D}} = k_{\text{A,ref}}^{\text{H}\to\text{D}}(A_\lambda \times$

$A_\rho)_{\mathrm{HD}}$. Since the key isotopic selectivity lies in the isotope donated to the amide, it is assumed that the intrinsic rate remains the same as in pure solvent, but the relevant concentrations $[\mathrm{H}^+](x)$ and $[\mathrm{D}^+](x)$ should be used:

$$k_{\mathrm{A,forw}}(x) = k_{\mathrm{A}}^{\mathrm{H}\to\mathrm{D}}[\mathrm{D}^+](x), \quad k_{\mathrm{A,back}}(x) = k_{\mathrm{A}}^{\mathrm{D}\to\mathrm{H}}[\mathrm{H}^+](x). \tag{A9}$$

The relative abundance of $[\mathrm{D}^+]$ is given, analogously to Eq. A4, as

$$f_{\mathrm{L}}(x) = \frac{[\mathrm{D}^+](x)}{[\mathrm{L}^+](x)} = \frac{x\ell}{1 + x - x\ell}, \tag{A10}$$

where $\ell = 0.69$[16] is the fractionation parameter for $\mathrm{L_3O^+}$ and $[\mathrm{L}^+](x) = [\mathrm{H}^+](x) + [\mathrm{D}^+](x)$, *cfr* main text.