Method to estimate amide base-catalyzed back exchange rates in H_2O/D_2O mixtures

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Abstract

Measurements of hydrogen deuterium exchange (HDX) in proteins provide means to characterize protein structural dynamics. The Linderstrøm-Lang describes exchange in isotopically pure solvents, but does not account for the back exchange occurring in H_2O/D_2O mixtures used in experiments. "Intrinsic" exchange rates of amides maximally exposed to solvent have been measured in pure solvents, but systematic investigations in mixtures are lacking. Here, a method to predict intrinsic forward and back HDX rates in mixtures is described for the base-catalyzed pathway, which dominates near neutral conditions. The model combines literature second-order reference rates in pure solvents with the empirical dependence of the mixture ionic product of and with the probability of reprotonation by H versus D solvent species. It recovers the known rates for pure solvents, predicts composition-dependent kinetic isotope effects, and yields an equilibrium fractionation factor $\phi = 1.20$ for unstructured peptides (in close agreement with the reported PDLA value 1.22). Because it provides explicit forward and back rates as functions of solvent composition and acidity, the method offers a practical starting point for quantitatively correcting back exchange in HDX–MS and HDX–NMR workflows.

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 exchange rate, which can be estimated as a function of sequence, temperature and pH in pure H_2O or D_2O.[6–8] The protection factor $P = k_{\mathrm{cl}}/k_{\mathrm{op}}$ is the physical quantity one wishes to determine to characterize structural dynamics.[9–14]

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. Downstream HDX-MS procedures, including quenching and chromatographic separation, are performed at lower deuterium concentrations. As a result, due to the use of H_2O/D_2O mixtures, amides are susceptible to back-exchange once deuteration has occurred.[3,4,15] Equilibrium and kinetic isotope effects that are present in mixtures[16,17] are overlooked, set aside a body of literature that measured fractionation by HDX in mixtures to probe amide hydrogen-bonded structure.[18–21] To explicitly account for back exchange, one needs to introduce forward and back exchange rates (k_{forw} and k_{back}), which have not been systematically characterized for mixtures.

Here, a method to determine forward and back exchange rates in H_2O/D_2O mixtures as a function of D_2O content is described for base-catalyzed exchange. Such a mechanism is dominant at slightly or non acidic conditions.[3,4] The derivation is based on the reaction mechanism considered as well as the literature on proton transfer processes in H_2O/D_2O mixtures. The measured reference rates[6–8] are recovered for the limit cases of pure H_2O or D_2O. Agreement with previously reported results[19] is shown.

Methods

Proton transfer theory

The chemistry of HDX reactions is described by proton transfer theory:[3,22]

$$\mathrm{XH^+ + Y} \rightleftharpoons \left(\mathrm{XH^+ \cdots Y} \rightleftharpoons \mathrm{X \cdots H^+Y}\right) \rightarrow \mathrm{X + H^+Y}. \tag{2}$$

A proton donor XH^+ collides with a proton acceptor Y forming a complex (denoted in brackets in Eq. 2) in which redistribution can occur before dissociation. Complex formation occurs with a second-order (diffusion-limited) rate constant k_{D} estimated as $10^{10}\,\mathrm{M^{-1}\,s^{-1}}$.[23] Complexes are highly dynamical but always lowly populated; in this case, the rate of proton

transfer k_{T} for the reaction $\mathrm{XH}^+ + \mathrm{Y} \xrightarrow{k_{\mathrm{T}}} \mathrm{X} + \mathrm{H}^+\mathrm{Y}$ is

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

where $\Delta \mathrm{p}K_{\mathrm{a}} = \mathrm{p}K_{\mathrm{a}}(\mathrm{donor}) - \mathrm{p}K_{\mathrm{a}}(\mathrm{acceptor}) = \mathrm{p}K_{\mathrm{a}}(\mathrm{X}) - \mathrm{p}K_{\mathrm{a}}(\mathrm{Y})$,

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

is a measure of acidity and $K_{\mathrm{a}}(\mathrm{A})$ is the acid dissociation product of HA.

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 occur by different pathways (acid-, base-, or water-catalyzed), and the pseudo-first-order HDX intrinsic exchange rate of Eq. 1 can be written as

$$k_{\mathrm{int}} = \sum_{\mathrm{cat}} k_{\mathrm{cat}}[\mathrm{cat}], \tag{5}$$

where k_{cat} is the second-order rate constant for a specific pathway and [cat] is the concentration of the correspondent catalyst. Temperature and pH dependence of HDX intrinsic rates were measured by Englander's group for poly-D,L-alanine (PDLA) and 3-alanine (3-Ala).[6–8]

At near-neutral conditions, the base-catalyzed mechanism of HDX represents the dominant contribution by orders of magnitude,[3] and will be the only discussed in this paper. In base-catalyzed HDX in pure D_2O, the amide proton is first removed 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, *cfr* Table A1).[6] These account for steric and inductive effects from neighboring residues, and were demonstrated to be simply additive.[24] $k_{\mathrm{B,ref}}(T)$ is the reference rate (i.e. the exchange rate for PDLA or 3-Ala) at temperature T, which exhibits an Arrhenius-type dependence:

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

where $E_{\mathrm{B}} = 17\,\mathrm{kcal\ mol^{-1}}$, R is the gas constant, and $k_{\mathrm{B,ref}}$ is the reference measured at $T_{\mathrm{ref}} = 20°\mathrm{C}$. Values for $k_{\mathrm{B,ref}}$ 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 unfolded proteins, at 20°C.[8] k_{L^*L} is the rate of base-catalyzed exchange for $L^* \to L$ exchange in pure L_2O, where $L, L^* = H, D$, *cfr Forward and back exchange rates* Section. The rate for ND in pure D_2O, which was not experimentally measured, was estimated as $k_{DD} = k_{HD}k_{DH}/k_{HH}$, *cfr Forward and back exchange rates* Section. Rates for 3-Ala are obtained multiplying the reference rates by a factor 1.35.[8]

	k_{L^*L}	$\log k_{B,ref}$ ($M^{-1}\,min^{-1}$)
NH in H_2O	k_{HH}	10.08
ND in H_2O	k_{DH}	10.00
NH in D_2O	k_{HD}	10.18
ND in D_2O	k_{DD}	10.10

H_2O/D_2O mixtures

In this section, definitions that are used to calculate the ionic product and the acidity of H_2O/D_2O mixtures are introduced. 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 $[H^+]$ and $[OH^-]$ in H_2O can be computed if the pH and the ionic product, K_{w,H_2O}, are known: $\mathrm{pH} = -\log[H^+]$ and $\mathrm{pOH} = \mathrm{p}K_{w,H_2O} - \mathrm{pH} = -\log[OH^-]$, where $\mathrm{p}K_{w,H_2O} = -\log K_{w,H_2O}$. Analogous relations can be written for $[D^+]$ and $[OD^-]$ in D_2O in terms of pD, pOD and K_{w,D_2O}. The acidity of a mixture can be effectively described by the pL, which is defined as

$$\mathrm{pL} = -\log[L^+] = -\log([H^+] + [D^+]). \tag{10}$$

The ionic product of D_2O is lower than that of H_2O;[25] experiments in mixtures showed that it varies nonlinearly with the deuterium content x, and an empirical relation was given:[26]

$$\Delta\mathrm{p}K_w(x) = \mathrm{p}K_w(x) - \mathrm{p}K_{w,H_2O} = 0.7282x + 0.0512x^2 + 0.0826x^3. \tag{11}$$

To relate the mixture $\mathrm{pL}(x)$ to the glass electrode pH-meter reading, pH^*, the following

relation can be used:[27]

$$\mathrm{pL}(x) = \mathrm{pH}^* \left(1 + \frac{\Delta \mathrm{p}K_{\mathrm{w}}(x)}{\mathrm{p}K_{\mathrm{w,H_2O}}}\right). \tag{12}$$

The temperature dependence of $\mathrm{p}K_{\mathrm{w,H_2O}}$ has been empirically determined as[28]

$$\mathrm{p}K_{\mathrm{w,H_2O}}(T) = 670.86 - \frac{34691.6}{T} - 105.15 \ln T + 0.10757\, T + \frac{2358000}{T^2}. \tag{13}$$

Once the mixture $\mathrm{p}K(x)$ is known, $\mathrm{pL}(x)$ and $\mathrm{pOL}(x)$ can be computed. Reported values for ϕ_{OL} (0.47[27] or 0.43[29]) can be used to compute $[\mathrm{OH}^-](x)$ and $[\mathrm{OD}^-](x)$. Examples are shown in Figure 1.

Forward and back exchange rates

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

$$\mathrm{NH} + \mathrm{DOL} \underset{\tilde{k}_{\mathrm{back}}}{\overset{\tilde{k}_{\mathrm{forw}}}{\rightleftharpoons}} \mathrm{ND} + \mathrm{HOL}, \tag{14}$$

where $\tilde{k}_{\mathrm{forw}}$ and $\tilde{k}_{\mathrm{back}}$ are the base-catalyzed reactions rate constants.

Reference rates for base-catalyzed $\mathrm{L}^* \rightarrow \mathrm{L}$ exchange in pure L_2O that were reported in Table 1 are $k_{\mathrm{L^*L}}$ (with $\mathrm{L}, \mathrm{L}^* = \mathrm{H}, \mathrm{D}$). The rate $k_{\mathrm{L^*L}}$ should correspond to the rate of removal of L^* from the amide group by OL^-, assuming fast reprotonation. The unmeasured rate k_{DD}, was estimated as

$$k_{\mathrm{DD}} = \frac{k_{\mathrm{HD}} k_{\mathrm{DH}}}{k_{\mathrm{HH}}}, \tag{15}$$

that is, assuming that reaction rates are governed entirely by the zero-point energies of the participating species.[30]

The rates of reaction (14) are obtained as a sum of terms, *cfr* Eq. 5, each having the same structure as in Eq. (6):

$$\begin{aligned} \tilde{k}_{\mathrm{forw}}(x) &= k_{\mathrm{HH}}(B_\lambda \times B_\rho)_{\mathrm{HH}}[\mathrm{OH}^-](x) + k_{\mathrm{HD}}(B_\lambda \times B_\rho)_{\mathrm{HD}}[\mathrm{OD}^-](x) \\ \tilde{k}_{\mathrm{back}}(x) &= k_{\mathrm{DH}}(B_\lambda \times B_\rho)_{\mathrm{DH}}[\mathrm{OH}^-](x) + k_{\mathrm{DD}}(B_\lambda \times B_\rho)_{\mathrm{DD}}[\mathrm{OD}^-](x) \end{aligned}. \tag{16}$$

The coefficients of the products $(B_\lambda \times B_\rho)_{\mathrm{L^*L}}$ are tabulated, *cfr* Appendix.

The scheme (14) can be simplified to a pseudo-first order reaction

$$\mathrm{NH} \underset{k_{\mathrm{back}}}{\overset{k_{\mathrm{forw}}}{\rightleftharpoons}} \mathrm{ND}, \tag{17}$$

where the pseudo-first order forward and back exchange rates are weighted by the probability

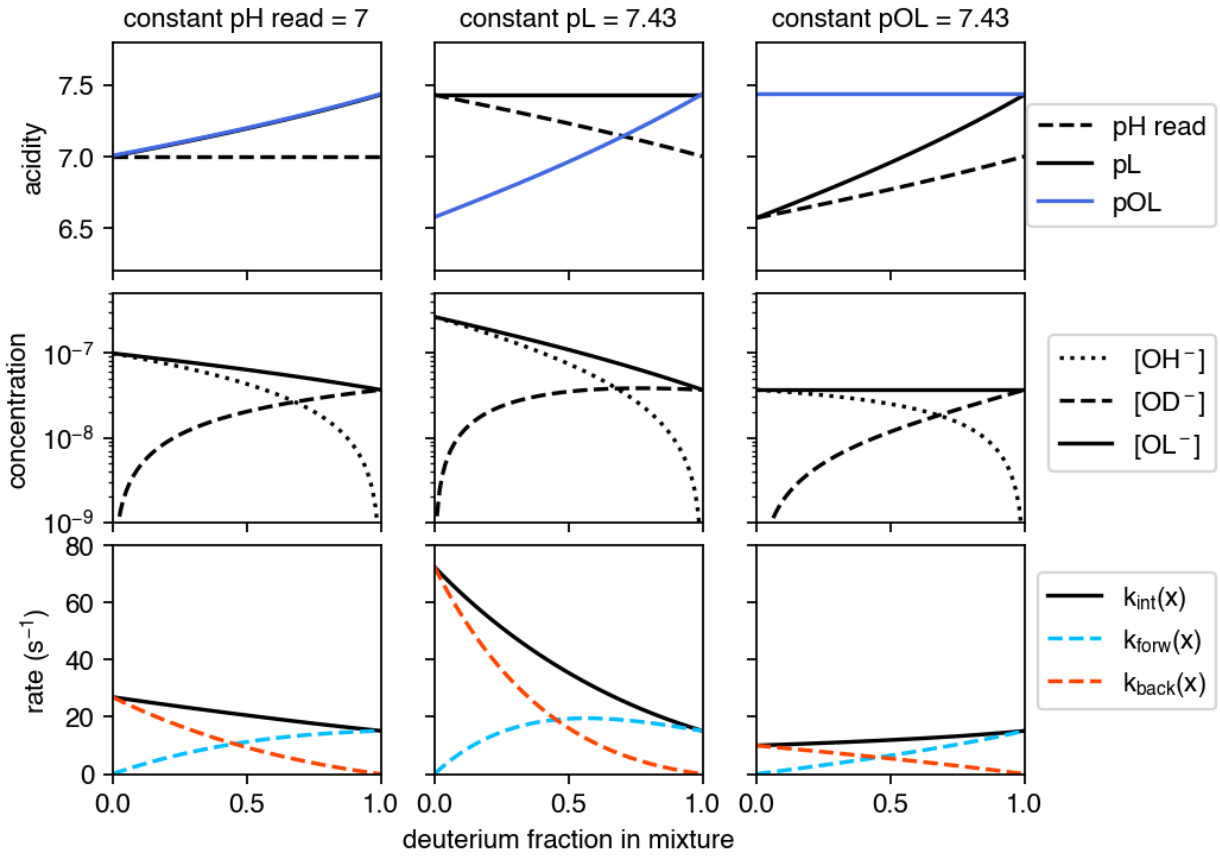

Figure 1: Acidity, concentration of ions and intrinsic rates in mixtures, expressed as a function of the deuterium fraction in the mixture, in three different scenarios: constant pH read (left), constant pL (center), constant pOL (right). Upper panels: mixture pH read, pL and pOL. Central panels: concentrations of ions $[OH^-]$, $[OD^-]$, and $[OL^-] = [OH^-] + [OD^-]$. Lower panels: intrinsic forward k_{forw}, back k_{back} and mixture intrinsic $k_{\text{int}}(x) = k_{\text{forw}}(x) + k_{\text{back}}(x)$ 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.

of encountering a reactive solvent molecule:

$$\begin{aligned} k_{\text{forw}}(x) &= x\tilde{k}_{\text{forw}}(x) \\ k_{\text{back}}(x) &= (1-x)\tilde{k}_{\text{back}}(x) \end{aligned}, \tag{18}$$

that is, upon removal of the proton, in the forward reaction reprotonation occurs by DOL with probability $x = [\text{DOL}]/[\text{L}_2\text{O}]$, and by HOL with probability $1 - x = [\text{HOL}]/[\text{L}_2\text{O}]$) in the reverse.

The solution to the kinetics of Eq. 17, with the constraint $\text{d}\left([\text{NH}] + [\text{ND}]\right)/\text{d}t = 0$ and calling $D(t)$ the normalized concentration of deuterated amides over time, is

$$D(t) = D_{\text{eq}}(x) - (D_0 - D_{\text{eq}}(x))\text{e}^{-k_{\text{int}}(x)t}, \tag{19}$$

where D_0 is the initial condition,

$$D_{\text{eq}}(x) = \frac{k_{\text{forw}}(x)}{k_{\text{forw}}(x) + k_{\text{back}}(x)} \tag{20}$$

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

$$k_{\text{int}}(x) = k_{\text{forw}}(x) + k_{\text{back}}(x). \tag{21}$$

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. 20) as well as the approach-to-equilibrium rate (Eq. 21) are functions of x through $k_{\text{forw}}(x)$ and $k_{\text{back}}(x)$, *cfr* Eq. 18. In other words, kinetic and equilibrium isotope effects are present.

Kinetic isotope effects

Kinetic isotope effects cause the reaction rate $k_{\text{int}}(x)$ 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 $\text{p}K_{\text{w}}(x)$ and acidity $\text{pL}(x)$, *cfr* Eqs. 11 and 12.

By construction, the measured reference rates are recovered in pure H_2O and D_2O: by Eqs. 16 and 18, for $x = 0$ (pure H_2O),

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

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

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

The question "how does $k_{\text{int}}(x)$ vary with x?" has no unique answer: it depends on the quantity that is fixed while varying x. At fixed temperature, the ionic product of the mixture $\text{p}K_{\text{w}}(x)$ is assumed to depend on x only, *cfr* Eq. 11. The observed exchange rate depends on the acidity of the solution through the concentration of ions OH^- and OD^-, *cfr* Eq. 16. However, different definitions of acidity are possible in a mixture, e.g. based on pH^*, pL or pOL. The behavior of $k_{\text{int}}(x)$ 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 $\text{p}K_{\text{w}}(x) > \text{p}K_{\mathbf{w},H_2O}$ (Eq. 11). 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 $k_{\text{int}}(x)$ decreases for increasing x. As a result, the intrinsic rate of $\text{H} \rightarrow \text{D}$ in pure D_2O is about 2-fold slower than $\text{D} \rightarrow \text{H}$ in pure H_2O.

In the second example, the effective acidity of the mixture, pL, was fixed to 7.43 (because $\text{pL}(1) = 7.43$ at $\text{pH}^* = 7$ and $T = 25°\text{C}$). Here decreasing x causes $\text{p}K_{\text{w}}(x)$ to decrease. Because pL is fixed, pOL decreases too, and a more substantial kinetic isotope effect is observed. $\text{D} \rightarrow \text{H}$ in H_2O results about 5-fold faster than $\text{H} \rightarrow \text{D}$ in D_2O on a pL scale.

The third scenario evaluates the effect of x at fixed pOL, i.e. $[\text{OL}^-](x) = [\text{OH}^-](x) + [\text{OD}^-](x) = \text{constant}$. In this case, $k_{\text{int}}(x)$ increases with x. This is because the concentration of catalysts are fixed hence the HDX rates depends on the rates of Table 1. Since $k_{\text{HH}} > k_{\text{DH}}$ and $k_{\text{HD}} > k_{\text{DD}}$, that is, it is easier to extract H than D from the amide group, the HDX rate results 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 $k_{\text{int}}(x)$ (Eq. 21) 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.

Equilibrium isotope effects

An equilibrium isotope effect is described by the fractionation factor (Eq. 9), which for reaction (14) is simply $\phi = \tilde{k}_{\text{forw}}(x)/\tilde{k}_{\text{back}}(x)$. Within the assumptions of the base-catalyzed

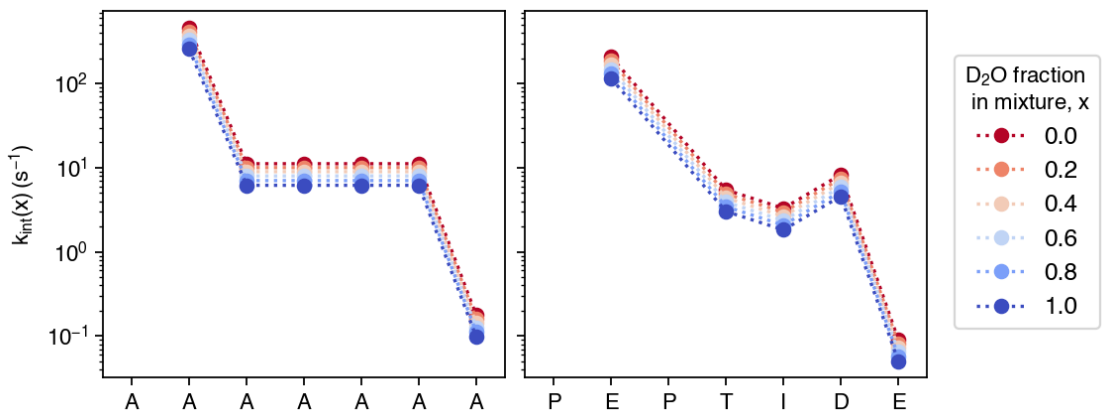

Figure 2: Intrinsic exchange rates $k_{\text{int}}(x)$ for short polypeptide sequences '`AAAAAAA`' (left) and '`PEPTIDE`' (right), calculated for varying D_2O fraction in the mixture x, $T = 25°C$, $pH^* = 7$, and using PDLA reference rates.

model developed above, the fractionation factor reduces to

$$\phi = \frac{\tilde{k}_{\text{forw}}(x)}{\tilde{k}_{\text{back}}(x)} = \frac{k_{\text{HH}}}{k_{\text{DH}}} = 1.20, \tag{22}$$

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. 22 is that, for random coil peptides in conditions where base catalysis dominates, the equilibriun 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: $pK_a(\text{ND}) > pK_a(\text{NH})$, 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).

The theoretical value $\phi = 1.20$ (Eq. 22) 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.[31] It is thus possible that the equilibrium fractionation expected for unstructured peptides is underestimated, since structured amides generally display lower fractionarion (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.

Using an incorrect fractionation factor, or neglecting equilibrium enrichment, can bias inference of protection factors and distort thermodynamic interpretations of how local stability is affected by H/D exchange. A working value of $\phi = 1.20$ is a reasonable starting

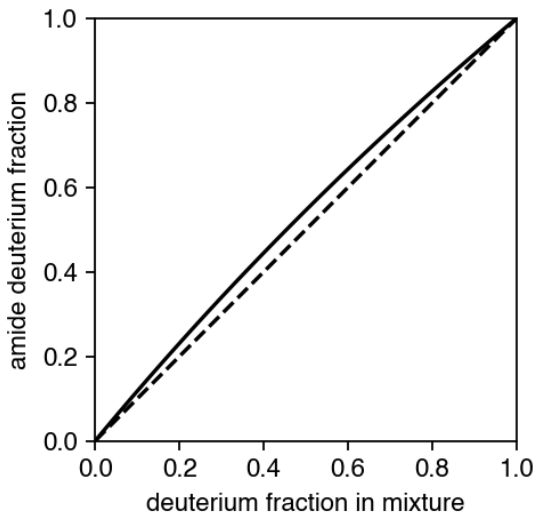

Figure 3: Equilibrium isotope effects. At equilibrium, the amide is enriched in D with respect to solvent ($D_{\mathrm{eq}}(x) > x$, as $\phi = 1.20 > 1$).

point for unstructured peptides, but it should be confirmed on appropriate calibrants.

Conclusions

In this work a method to estimate base-catalyzed exchange rates of unstructured peptides in H_2O/D_2O mixtures from rates measured in pure H_2O and D_2O[6–8] was described. This was done assuming that, in a mixture, amide deprotonation can occur by both OH^- and OD^-, that the probability of reprotonation by D (H) is proportional to x $(1 - x)$, and that the ionic product of H_2O/D_2O mixtures depends on the relative abundance of H and D isotopes in solvent. The model yields explicit $k_{\mathrm{forw}}(x)$ and $k_{\mathrm{back}}(x)$ as functions of composition and acidity, and recovers the pure-solvent limits.

Isotope effects that affect base-catalyzed exchange have been discussed. Kinetic isotope effects are different in magnitude and direction depending on how the acidity of the mixture is controlled while varying the composition. In particular, the approach-to-equilibrium rate decreases with increasing D_2O at fixed pH^*, but increases at fixed pOL; at fixed pL the effect is largest. A slight preference of the amide for D at equilibrium ($\phi = 1.20$) is found, implying $D_{\mathrm{eq}}(x) > x$ under base-catalyzed conditions. A good agreement is found with the measured fractionation for PDLA ($\phi_{\mathrm{PDLA}} = 1.22$). However, this agreement should be interpreted cautiously (see above).

A clear grasp of exchange in mixtures is a necessary starting point for developing back-exchange models, a cornerstone to accurate quantitative HDX/MS data analysis.[15,32] The limitations of the present treatment include its focus on the base-catalyzed mechanism (acid- and water-catalyzed pathways become relevant at low pH), as well as dissolved salts, chaotropic agents or other substances that may influence the local pK_a and affect exchange

kinetics. Systematic benchmarking against dedicated random-coil calibrants (in addition to PDLA/PDLK) will further refine the accuracy and scope of the method for HDX–MS and HDX–NMR workflows.

Code availability

A Python[33] 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 base-catalyzed exchange rates

Table A1: Multiplicative factors for base-catalyzed exchange rates.[6–8]

	$\log B_\lambda$	$\log B_\rho$
A	0.00	0.00
C	0.62	0.55
C2	0.55	0.46
D0	0.10	−0.18
D+	0.69	0.60
E0	−0.11	−0.15
E+	0.24	0.39
F	−0.24	0.06
G	−0.03	0.17
H0	−0.10	0.14
H+	0.80	0.83
I	−0.73	−0.23
K	−0.04	0.12
L	−0.58	−0.21
M	−0.01	0.11
N	0.49	0.32
P	—	−0.24
Pc	—	0.60
Q	0.06	0.20
R	0.08	0.22
S	0.37	0.30
T	−0.07	0.20
V	−0.70	−0.14
W	−0.41	−0.11
Y	−0.27	0.05
NT	—	1.62
CT	−1.80	—

The coefficients B_λ and 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 coefficient $B_{\zeta,\mathrm{L}}(\mathtt{X})$ of residue X (where ζ is λ or ρ, and L is H or D indicating removal by OH^- or OD^-), is given by

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

where

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

The parameters $C_{\mathrm{L}}(\mathtt{X})$, $E_{\mathrm{a,L}}(\mathtt{X})$, T_{ref} are 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, it follows $(B_\lambda \times B_\rho)_{\mathrm{L^*L}} = (B_\lambda \times B_\rho)_{\mathrm{LL}}$, with L* and L being H or D. 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. 16 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 fractionational 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. 15, 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

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