

Isotope Effects in Peptide Group Hydrogen Exchange

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ABSTRACT Kinetic and equilibrium isotope effects in peptide group hydrogen exchange reactions were evaluated. Unlike many other reactions, kinetic isotope effects in amide hydrogen exchange are small because exchange pathways are not limited by bond-breaking steps. Rate constants for the acid-catalyzed exchange of peptide group NH, ND, and NT in H₂O are essentially identical, but a solvent isotope effect doubles the rate in D₂O. Rate constants for base-catalyzed exchange in H₂O decrease slowly in the order NH>ND>NT. The alkaline rate constant in D₂O is very close to that in H₂O when account is taken of the glass electrode pH artifact and the difference in solvent ionization constant. Small equilibrium isotope effects lead to an excess equilibrium accumulation of the heavier isotopes by the peptide group. Results obtained are expressed in terms of rate constants for the random coil polypeptide, poly-DL-alanine, to provide reference rates for protein hydrogen exchange studies as described in Bai et al. [preceding paper in this issue]. © 1993 Wiley-Liss, Inc.

Key words: hydrogen exchange, equilibrium isotope effects, kinetic isotope effects, protein structure, poly-DL-alanine

INTRODUCTION

Hydrogen exchange (HX) rates of the many peptide NH hydrogens distributed throughout every protein molecule are determined by, and therefore can provide information about, local structure, structure change, dynamics, and energetics. The richness of protein HX information has begun to be realized in a variety of protein studies that use different hydrogen isotope combinations. Tritium exchange in H₂O, monitored by liquid scintillation counting, has been used to study allosteric structure change.^{2–6} In experiments designed to exploit high resolution ¹H nuclear magnetic resonance (NMR) analysis, the exchange of hydrogen and deuterium isotopes has been used. Peptide group NH exchange in D₂O has been used to resolve structure and measure stability in equilibrium protein folding intermediates.^{7–10} Peptide group ND exchange in H₂O has been used to observe structure formation during protein folding.^{11,12}

The use of hydrogen isotopes in proteins and solvent may impose a variety of effects on measured HX behavior. *Equilibrium* isotope effects reflect the equilibrium partitioning of hydrogen isotopes between peptide NH and solvent, and therefore may affect the number of protein sites measured when isotopic species compete in a labeling experiment. *Kinetic* isotope effects modify exchange rates, and therefore can affect measurements of protein stability. To place the various HX approaches now being used in protein structural studies on a quantitative and consistent scale, we integrate here new and previously available information on the isotope dependence of the peptide group HX reaction.

MATERIALS AND METHODS

Poly-DL-alanine (PDLA; dp ~ 28) was obtained from Sigma Chemical Co. (St. Louis, MO), D₂O (99.9%) from Isotech (Miamisburg, OH), tritiated water at 1 Ci/ml from New England Nuclear (Boston, MA), and salts and pH buffers from Fisher (Fair Lawn, NJ). To remove possible small molecule contaminants, PDLA solutions were initially passed through a centrifugal gel filtration column.¹⁰ Experimental solutions used contained 0.02 M NaCl, were buffered at the desired pH or pD with 50 mM phosphate, and were equilibrated at 20°C before mixing to initiate HX reactions, except as otherwise noted. Sample pH was set before and checked after each HX series. All pH measurements were made with a glass electrode (Radiometer, Copenhagen, Denmark) and are reported in D₂O either as pD_{read}, without correction for the glass electrode solvent isotope artifact, or as pD_{corr}, to indicate the corrected D⁺-ion concentration, according to Eq. (1).¹³

$$\text{pD}_{\text{corr}} = \text{pD}_{\text{read}} + 0.40. \quad (1)$$

NH/H₂O

H-H exchange was measured using a ¹H NMR magnetization transfer method. The water signal

Abbreviations: HX, hydrogen exchange; PDLA, poly-DL-alanine; H, ¹H; D, ²H; T, ³H.

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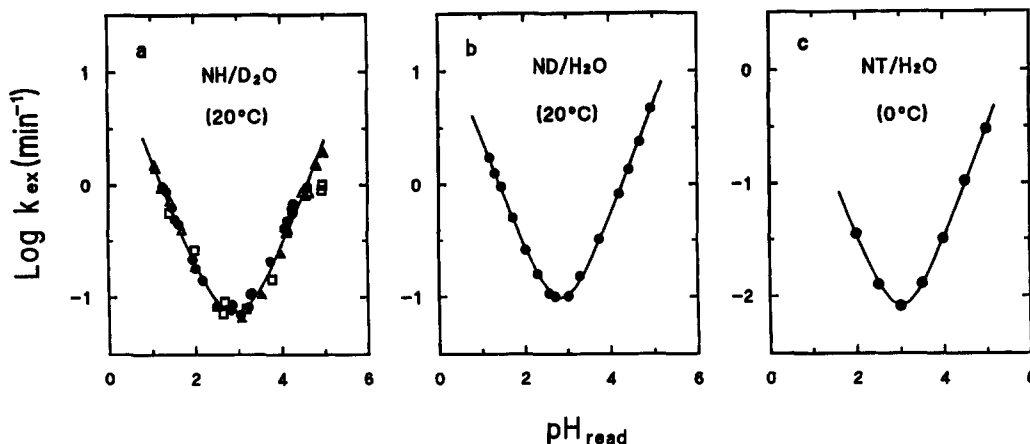


Fig. 1. HX behavior of PDLA with various isotope combinations. **a**: NH-PDLA exchange in D_2O at $20^\circ C$ measured by NMR and UV absorption methods. (\bullet) Data from Jeng and Englander¹⁰; (\blacktriangle) data from Englander et al.¹⁷; (\square) data from Loftus et al.²⁷ **b**: ND-PDLA exchange in H_2O measured at $20^\circ C$ by absorbance at 220 nm. **c**: NT-PDLA exchange in H_2O measured at $0^\circ C$ by gel filtration methods¹⁵.

was presaturated for increasing lengths of time ($t_{sat} \sim 50$ msec to 5 sec) during which the initial NH resonance intensity (I_0) decreased as in Eq. (2)¹⁴ due to exchange with the saturated H_2O protons, ultimately reaching a steady state value at times long relative to the spin-lattice relaxation time (T_1) and/or exchange lifetime ($1/k_{ex}$).

$$I(t_{sat}) = I(t_0)[(k_{ex}/k_s)\exp(-k_s t) + (1/k_s T_1)] \quad (2a)$$

$$k_s = k_{ex} + 1/T_1. \quad (2b)$$

To obtain the exchange rate constant, k_{ex} , the exponentially decaying NH peak heights were fit to Eq. (2a) using three fitting parameters (k_s , I_0 , $I_\infty = I_0/k_s T_1$). The experiment was repeated over a range of pH values where k_{ex} competes effectively with T_1 relaxation in order to obtain k_{ex} as a function of pH and thus define the rate constants for specific acid and base catalysis of the exchange reaction. T_1 measured independently in saturation-recovery and inversion-recovery experiments agreed with the calculated values to within 5% (measured at low pH to make exchange much slower than T_1 recovery). PDLA concentrations were typically 60 mM in peptide NH concentration.

NT/ H_2O

Tritium-HX data were obtained by a gel filtration method at $0^\circ C$.¹⁵ Here we refit the original data and extrapolate the rate constants to $20^\circ C$ as described by Bai et al.¹ using activation energies of 14 kcal/mol for the acid-catalyzed reaction and 17 kcal/mol for the base-catalyzed reaction. These values lead to correction factors between $0^\circ C$ and $20^\circ C$ of 5.8 and 8.5, respectively. Also, the small temperature-dependent shift in buffer pK_a s was taken into account.

ND/ H_2O

The exchange of peptide ND in H_2O was measured by ultraviolet (UV) absorbance in a Cary 118c spectrophotometer.^{16,17} PDLA was initially incubated in D_2O (~ 80 mg/ml) to obtain full deuteration. ND to NH exchange was initiated by mixing 0.02 ml of deuterium-exchanged PDLA into 1 ml H_2O , the exchange reaction was monitored by the absorbance change at 220 nm, and kinetic data were processed using ASYST software.

NH/ D_2O

The exchange of peptide NH in D_2O was monitored by one-dimensional (1D) 1H NMR and by a UV spectrophotometric technique^{16,17} as just described. For NMR measurements, HX was initiated by mixing 0.05 ml of PDLA (~ 200 mg/ml) into 0.5 ml D_2O . Sequential 1D spectra were recorded until the NH peak was fully exchanged, and the decrease in NH peak area, normalized internally against the non-exchanging methyl group of PDLA at 1.55 ppm, was fit to a single exponential decay. Measurements stated to be in D_2O correspond to 90–98% D_2O .

RESULTS

The racemic polymer PDLA provides a good model for uncomplicated peptide HX behavior. PDLA exists as an unstructured random chain in solution¹⁵ and does not contain bulky hydrophobic side chains, local charged groups, potentially catalytic side chain moieties, or other exchangeable hydrogens. We consider here the isotope effects exhibited by the exchanging hydrogens of this polypeptide model for various hydrogen isotope combinations.

Figure 1 shows HX data for PDLA, exchanging as NH into D_2O and ND or NT into H_2O . These data

TABLE I. HX Rate Constants for PDLA*

	$\log k_A$ ($M^{-1} \text{ min}^{-1}$)	$\log k_B$ ($M^{-1} \text{ min}^{-1}$)	$\log k_W$ (min^{-1})
NH/H ₂ O	1.39	9.95	— [†]
ND/H ₂ O	1.40	9.87	-1.6
NT/H ₂ O	1.27	9.59	-1.6
NH/D ₂ O	1.62	10.05	-1.5

*Rate constants for acid (k_A), base (k_B), and water (k_W) catalysis are at 20°C. Base catalytic constants were computed from measured data using the pH meter reading, corrected in the case of D₂O ($pD_{\text{corr}} = pD_{\text{read}} + 0.4$), and the molar dissociation constant for H₂O or D₂O (14.17 and 15.05, respectively). For temperature dependence see Bai et al.¹ These reference values can be used as specified by Bai et al.¹ to compute rates to be expected for unstructured peptide groups in polypeptide HX reactions.

[†]Not available. See Figure 2.

were fit by Eq. (3) to obtain catalytic rate constants for the acid (k_A)-, base (k_B)-, and water (k_W)-catalyzed reactions.

$$k_{\text{obs}} = k_A[H^+] + k_B[OH^-] + k_W. \quad (3)$$

For measurements in D₂O, [H⁺] and [OH⁻] in Eq. (3) are replaced by [D⁺] and [OD⁻]. Table I lists the rate constants obtained when [D⁺] was calculated as in Eq. (1) to correct for the glass electrode deuterium isotope effect¹³ and [OH⁻] and [OD⁻] were computed using pK_W values at 20°C for H₂O and D₂O of 14.17 and 15.05, respectively.

To determine solvent isotope effects (NH/H₂O vs. NH/D₂O), rate constants for peptide NH exchanging in H₂O were obtained by saturation-transfer and inversion-recovery methods. In these experiments, exchange rates compete with spin-lattice relaxation [Eq. (2); $1/T_1 \sim 1 \text{ sec}^{-1}$], so that measurements were made at pH values far from the pH minimum where k_{ex} is relatively large (Fig. 2). Figure 2a compares results for base catalysis of ¹H-PDLA exchange in H₂O with results in D₂O. In the acid-catalyzed region, PDLA reaches satisfactorily fast rates only below pH 0. Therefore, the solvent kinetic isotope effect for the acid reaction was evaluated using two amino acid dipeptides studied before,¹ which have a more favorable rate-pH dependence. Figure 2b and Figure 2c show NH/H₂O exchange data measured for the R peptide NHs of N-acetyl-alanine-N'-methylamide and N-acetylmethionine-N'-methylamide, and compares these results with data for the same peptide groups exchanging as NH in D₂O.

On a scale that uses pH_{read} and pD_{read} as in Figure 2, the base-catalyzed reaction appears faster in H₂O than in D₂O by 2.5-fold (Fig. 2a; $\Delta \log k_{\text{ex}} = 0.39$). For the acid-catalyzed reaction (Fig. 2b,c) the results for both dipeptides show that the HX rate appears faster by 1.5-fold for the NH exchanging in H₂O ($\Delta \log k_{\text{ex}} = 0.18$). Table I translates these measured increments into calculated rate constants for PDLA after correction for the pH electrode artifact

Eq. (1) and the different ionization constants of H₂O and D₂O.

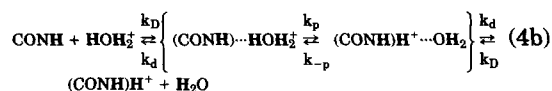
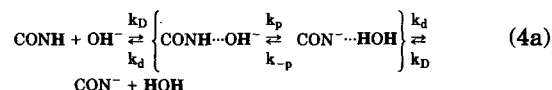
Figure 3a displays the relative rates found in all these experiments when raw data are plotted, i.e., when an uncorrected pH meter scale is used. Figure 3b takes into account the glass electrode correction [Eq. (1)] necessary to place [H⁺] and [D⁺] on the same concentration scales so that the catalytic power of H⁺ and D⁺ can be directly compared. Similarly, Figure 3c places [OH⁻] and [OD⁻] on a comparable basis by using pH and pD_{corr} together with the ionization constants of H₂O and D₂O to exhibit measured rates on a scale of catalytic base concentration.

Corrected rate constants obtained for the PDLA peptide group in the form of NH, ND, and NT exchanging in H₂O and D₂O are listed in Table I.

DISCUSSION

HX Reaction

Amide HX is catalyzed by hydrogen and hydroxide ions¹⁸ and minimally by water.¹⁷ The rate-determining proton transfer sequence for amide HX can be written as in Eq. (4a) for catalysis by specific base and Eq. (4b) for specific acid.¹⁹



The brackets in Eq. (4) set off events within the encounter complex, k_p and k_{-p} refer to proton transfer reactions within the complex, k_D represents the second-order diffusion-limited rate for complex formation, and k_d is the first-order dissociation rate constant.

The base-catalyzed sequence [Eq. (4a)] involves direct abstraction of the peptide H. Acid-catalyzed HX [Eq. (4b)] is rate-limited by protonation of the peptide group, which may occur at the carbonyl oxygen^{20,21} or the imidic nitrogen.^{22,23} The encounter complex, shown in brackets, represents association by H-bonding of the peptide group with aqueous species that can engage in facile proton transfers, including specific base [Eq. (4a)] and specific acid [Eq. (4b)]. Associated waters may also be involved. General acids and bases appear not to participate in aqueous peptide HX reactions owing to the extreme pK_a s for peptide group protonation and deprotonation.¹⁵ In the present context, the isotope presented in boldface as **H** in Eq. (4) can represent any hydrogen isotope combination in the peptide group, the catalysts, and solvent. When different hydrogen isotopes are used, equilibrium and kinetic differences may appear.

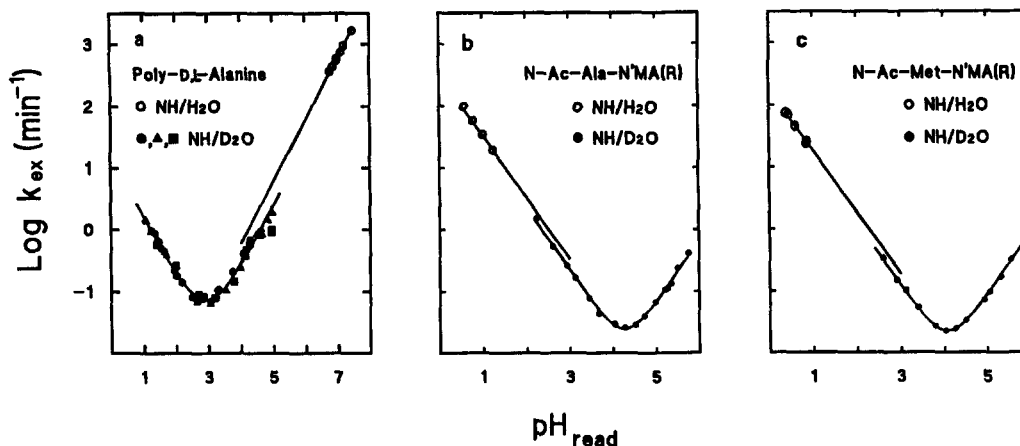


Fig. 2. Comparison of NH exchange in H_2O and D_2O . NMR measurements used saturation-transfer and saturation-recovery methods. a: Base catalysis results measured for PDLA. b,c: Acid catalysis results measured using dipeptide models. These results lead to rate constants for PDLA $NH-H_2O$ exchange (Table I, Fig. 3).

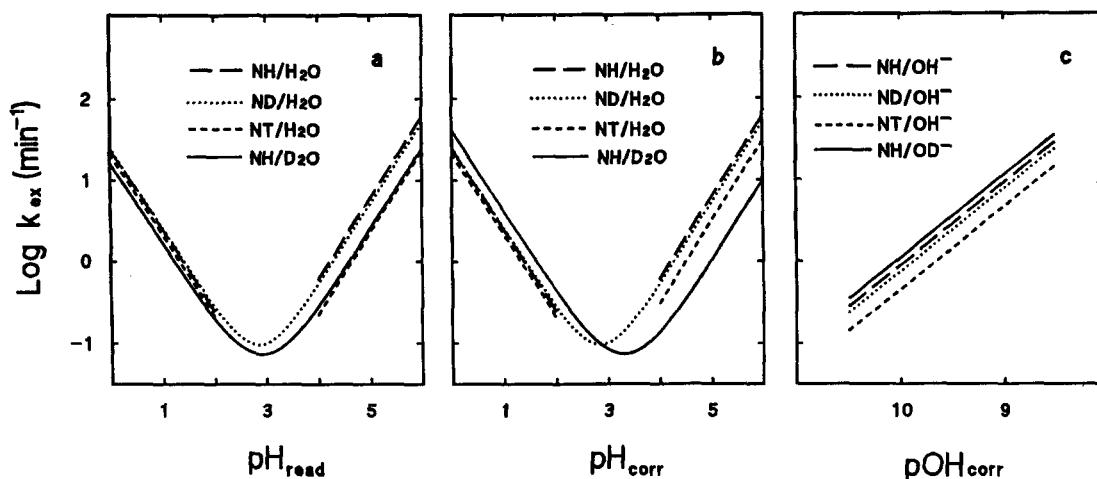


Fig. 3. Kinetic isotope effects in peptide group HX. a: Comparison of the results obtained here as they appear on an uncorrected pH meter reading scale. b: Results plotted on a pH or pD scale corrected for the glass electrode D_2O artifact. c: Base-catalyzed exchange rates plotted against true specific base concentration (pOH or pOD).

HX Rate Determination

A sequence similar to Eq. (4) can be written for many organic chemical reactions. The pathway can in principle be rate-limited by formation of the encounter complex, by the proton transfer step, or by separation of the complex.

The encounter complex considered here represents an essentially electrostatic H-bonded association between polar groups, thus can be expected to form on collision at the second-order diffusion-limited rate, $\sim 10^{10} M^{-1} sec^{-1}$, written as k_D in Eq. (4). The subsequent proton transfer step in both the acid and base pathways requires the making and breaking of covalent bonds. When bond-making and -breaking reactions involving hydrogen atoms are

rate-limiting, one expects large kinetic isotope effects since the 2- and 3-fold differences in mass between H, D, and T can strongly affect zero point energies and vibrational levels in the ground and transition states and thus alter the activation energy for the proton transfer step. We find that kinetic isotope effects are minimal (Table I). This indicates that the proton transfer steps in Eq. (4) are not rate-limiting. It has been thought that the transferred proton redistributes rapidly within the donor-acceptor encounter complex, probably by proton tunneling.^{19,24}

When redistribution is faster than dissociation, an equilibrium distribution is reached by the proton in the double well potential between the HX catalyst and the peptide group that is determined by their

relative affinities for the proton. If we express proton affinity in terms of pK_a , the equilibrium distribution of the proton between donor and acceptor groups in the encounter complex can be written as $k_p/k_{-p} = 10^{\Delta pK_a}$, where ΔpK_a is acceptor pK_a - donor pK_a . The fraction in the forward direction is then $k_p/(k_p + k_{-p}) = 10^{\Delta pK_a}/(1 + 10^{\Delta pK_a})$. Ultimately the complex separates, either to the right of Eq. (4) in a fruitful collision or to the left in a wasted one. The separation rate constant in the two alternative directions is likely to be nearly identical ($\sim 10^{10} \text{ sec}^{-1}$)¹⁹ since the separating species are so similar.

When separation of the complex is limiting, the observed HX rate can be written as in Eq. (5).

$$k_{ex} = k_D[\text{cat}]10^{\Delta pK_a}/(1 + 10^{\Delta pK_a}). \quad (5)$$

Eq. (5) states that the HX rate is determined by the rate of formation of the encounter complex ($k_D[\text{cat}]$) multiplied by the probability for the forward distribution within the complex. For example, since base-catalyzed exchange in PDLA is slower than the diffusion-limited rate by about two orders of magnitude, one can infer that the equilibrium proton distribution within the peptide- OH^- complex [Eq. (4a)] has $k_p/k_{-p} \sim 10^{-2}$ because the peptide group is a stronger base than water by about two units in pK_a .

The relationship suggested by Eq. (5) has been experimentally demonstrated for the OH^- -catalyzed exchange of a series of substituted amides.²³ These workers found a slope approaching unity (0.87 ± 0.14) for the dependence of $\log(k_{ex})$ on the inferred pK_a of a number of substituted amides, and demonstrated the approach of k_{ex} to a limiting rate (k_D) of approximately $10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ for specific base catalysis of the most strongly acidic amides.

The dependence of k_{ex} on pK_a in Eq. (5) provides an explanation for the inductive effect of polar amino acid side chains on amide HX rate.^{1,25} Polar side chains withdraw electron density from the peptide group and make it a stronger acid. This disfavors peptide protonation, and thus reduces the rate of acid-catalyzed proton deposition, but it promotes deprotonation, and thus increases the rate of base-catalyzed proton removal. The net effect appears as a leftward shift in the V-shaped curve of \log rate vs. pH.¹ Eq. (5) also indicates that the side chain blocking effect described in the preceding paper¹ must operate either by reducing the rate for forming the encounter complex or by affecting the equilibrium distribution within it. The former seems more likely.

Kinetic Isotope Effects

The proton transfer mechanisms just described explain the surprisingly small kinetic isotope effects found in amide HX. Formation of the hydrogen-

bonded encounter complex is diffusion-limited, and therefore depends only on the concentration of the solvent species, H^+ or D^+ and OH^- or OD^- , and their diffusion coefficients which are very similar for similar species. Within the encounter complex, k_p and k_{-p} are faster than separation, so the forward rate depends not on k_p itself but only on the equilibrium distribution ($k_p/k_{-p} = 10^{\Delta pK_a}$). For example, consider base catalysis of NH vs. ND by OH^- . D tends to be bound more tightly than H to the peptide donor (pK_a for ND > pK_a for NH), but D is also bound more tightly than H to the OH^- acceptor in the complex. Thus ΔpK_a will be relatively insensitive to isotopic mass. The kinetic isotope effect, which depends on the difference between these differences ($\delta\Delta pK_a = \Delta pK_a(\text{D}) - \Delta pK_a(\text{H})$), will be even smaller. Therefore, one can expect only small kinetic differences between the exchange rates of NH, ND, and NT. The solvent kinetic isotope effect, however, e.g., the difference between catalysis by OH^- and OD^- , depends on the relative basicities of these species and may be more substantial.

The present results show nearly identical rate constants for acid catalysis of NH, ND, and NT exchange in H_2O ($19\text{--}25 \text{ M}^{-1} \text{ min}^{-1}$; Table I, Fig. 3a). The rate-limiting sequence, protonation of the peptide group by H_3O^+ [Eq. (4b)], is wholly insensitive to the identity of the peptide-bound isotope, which is not involved in the protonation step. A solvent isotope effect is seen (Table I, Fig. 3b); the exchange of NH is faster by 2-fold in D_2O than in H_2O , evidently because D_3O^+ is a stronger acid than H_3O^+ . Small differences are seen in the base-catalyzed exchange of NH, ND, and NT by OH^- ; rates decrease slightly in the order NH > ND > NT.

On the scale of pH_{corr} (Fig. 3b), the base-catalyzed exchange of NH in D_2O appears slower than in H_2O . This is misleading. When rates are compared on a scale that properly represents OH^- and OD^- concentrations (Fig. 3c), it is clear that OD^- is at least as effective as OH^- . (One might have expected OD^- to be even more effective than OH^- due to its greater basicity.)

Equilibrium Isotope Effects

In protein HX experiments, an equilibrium isotope effect occurs when the hydrogen isotopes used partition unequally between protein and solvent so that the isotopic ratio in the protein, e.g., protein-T/protein-H, is unequal to solvent-T/solvent-H at exchange equilibrium. As for kinetic isotope effects in HX, an equilibrium isotope effect depends on a second-order difference, the difference between the unequal affinity of protein and solvent for one isotope (e.g., T) and their unequal affinity for a second isotope (e.g., H).

Equilibrium isotope effects are seen in tritium exchange experiments which use the T isotope in tracer amounts. In competition with H_2O , the un-

structured peptide group selectively accumulates T to the extent of 20%, i.e., $(T/H)_{\text{protein}}/(T/H)_{\text{solvent}} = 1.2$ at exchange equilibrium; in D_2O the equilibrium isotope effect for protein (T/D) is 1.13.¹⁵ This effect, though apparently small, becomes important in tritium exchange experiments intended to measure the number of peptide groups involved in some HX behavior.²⁶ Since H-D exchange experiments are normally done in solvents that are close to 100% of either H_2O or D_2O , equilibrium isotope effects are not easily detected. The unequal distribution of D and H, though not yet measured, is bound to be smaller than for T and H.

A different kind of isotope effect can occur in structured proteins. Hvidt and Nielsen²² pointed out that the presence of different isotopes in hydrogen-bonded positions may strengthen or weaken a hydrogen bond depending on the favored length of the bond. An individual site may then selectively accumulate one isotope relative to another, imposing an apparent equilibrium isotope effect that will operate in addition to the chemical partitioning just noted. Recent NMR experiments with staphylococcal nuclease in 50:50 mixtures of H_2O/D_2O solvent now show small effects of this kind (J. Markley, personal communication). Conversely, the presence of different isotopes may then affect hydrogen bond strength. Since this effect appears to be small for any individual site, it is likely to affect local stability and HX rates minimally, but may exert a significant summed effect on global stability.

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REFERENCES

- Bai, Y., Milne, J.S., Mayne, L., Englander, S.W. Primary structure effects on peptide group hydrogen exchange. *Proteins* 17:75–86, 1993.
- Rosa, J.J., Richards, F.M. An experimental procedure for increasing the structural resolution of chemical hydrogen exchange measurements on proteins: Application to ribonuclease S peptide. *J. Mol. Biol.* 133:399–416, 1979.
- Allewell, N.M. Hydrogen exchange studies of proteins: Recent advances in medium and high resolution methods. *J. Biochem. Biophys. Methods* 7:345–357, 1983.
- Kaminsky, S.M., Richards, F.M. Differences in hydrogen exchange behavior between the oxidized and reduced forms of *Escherichia coli* thioredoxin. *Protein Sci.* 1:10–21, 1992.
- Englander, J.J., Rogero, J.R., Englander, S.W. Protein hydrogen exchange studied by the fragment separation method. *Anal. Biochem.* 147:234–244, 1985.
- Englander, S.W., Englander, J.J. Structure and energy change in hemoglobin by hydrogen exchange labeling. *Methods Enzymol.* (in press), 1994.
- Baum, J., Dobson, C.M., Evans, P.A., Hanley, C. Characterization of a partly folded protein by NMR methods: Studies on the molten globule state of guinea pig α -lactalbumin. *Biochemistry* 28:7–13, 1989.
- Hughson, F.M., Wright, P.E., Baldwin, R.L. Structural characterization of a partly folded apomyoglobin intermediate. *Science* 249:1544–1548, 1990.
- Jeng, M.F., Englander, S.W., Elove, G.A., Wand, A.J., Roder, H. Structural description of acid-denatured cytochrome c by hydrogen exchange and 2D NMR. *Biochemistry* 29:10433–10437, 1990.
- Jeng, M.F., Englander, S.W. Stable submolecular folding units in a non-compact form of cytochrome c. *J. Mol. Biol.* 221:1045–1061, 1991.
- Englander, S.W., Mayne, L. Protein folding studied by hydrogen exchange labeling and 2D NMR. *Annu. Rev. Biophys. Biomol. Struct.* 21:243–265, 1992.
- Baldwin, R.L. Pulsed NH exchange studies of folding intermediates. *Curr. Opin. Struct. Biol.* 3:84–91, 1993.
- Glasoe, P.F., Long, F.A. Use of glass electrodes to measure acidities in deuterium oxide. *J. Phys. Chem.* 64:188–193, 1960.
- Forsen, S., Hoffman, R.A. Study of moderately rapid chemical exchange reactions by means of nuclear magnetic double resonance. *J. Chem. Phys.* 39:2892–2901, 1963.
- Englander, S.W., Poulsen, A. Hydrogen-tritium exchange of the random chain polypeptide. *Biopolymers* 7:329–339, 1969.
- Takahashi, T., Nakanishi, M., Tsuboi, M. Hydrogen-deuterium exchange study of amino acids and proteins by 200–230 nm spectroscopy. *Bull. Chem. Soc. Jpn.* 51:1988–1990, 1978.
- Englander, J.J., Calhoun, D.B., Englander, S.W. Measurement and calibration of peptide group hydrogen-deuterium exchange by ultraviolet spectrophotometry. *Anal. Biochem.* 92:517–524, 1979.
- Berger, A., Loewenstein, A., Meiboom, S. Nuclear magnetic resonance study of the protolysis and ionization of N-methylacetamide. *J. Am. Chem. Soc.* 81:62–67, 1959.
- Eigen, M. Proton transfer, acid-base catalysis, and enzymatic hydrolysis. *Angew. Chem. Int. Ed. Engl.* 3:1–19, 1964.
- Perrin, C.L., Arrhenius, G.M.L. Mechanisms of acid-catalyzed proton exchange in N-methyl amides. *J. Am. Chem. Soc.* 104:6693–6696, 1982.
- Martin, R.B., Hutton, W.C. Predominant N-bound hydrogen exchange via O-protonated amide. *J. Am. Chem. Soc.* 95:4752–4754, 1973.
- Hvidt, A., Nielsen, S.O. Hydrogen exchange in proteins. *Adv. Protein Chem.* 21:287–386, 1966.
- Molday, R.S., Kallen, R.G. Substituent effects on amide hydrogen exchange rates in aqueous solution. *J. Am. Chem. Soc.* 94:6739–6745, 1972.
- Hibbert, F. Mechanisms of proton transfer between oxygen and nitrogen acids and bases in aqueous solution. *Adv. Phys. Org. Chem.* 22:113–212, 1986.
- Molday, R.S., Englander, S.W., Kallen, R.G. Primary structure effects on peptide group hydrogen exchange. *Biochemistry* 11:150–158, 1972.
- Englander, S.W., Downer, N.W., Teitelbaum, H. Hydrogen exchange. *Annu. Rev. Biochem.* 41:903–924, 1972.
- Loftus, D., Gbenle, G.O., Kim, P.S., Baldwin, R.L. Effects of denaturants on amide proton exchange rates: A test for structure in protein fragments and folding intermediates. *Biochemistry* 33:1428–1436, 1986.