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Review Article

The use of solvent isotope effects in the pursuit of enzyme mechanisms†

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Abstract: The basic principles and illustrative applications to enzyme mechanisms of solvent isotope effects, with emphasis on the use of rate measurements in mixtures of protium and deuterium oxides ('proton inventories'), are reviewed over a period slightly shorter than the history of this Journal. The principles in their current formulation are traced to publications of the 1960s and the illustrations are taken from publications that have appeared in 2006–2007. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: isotope effect; deuterium oxide; heavy water; reaction mechanism; enzyme; catalysis

Introduction[1–13]

Beginning with the discovery and purification of deuterium oxide, curiosity about its biological properties began to motivate *in vivo* and *in vitro* experiments.[12] The purpose of this article for the 50th anniversary issue of the Journal is to review the development of solvent isotope effects as a technique of biochemical, and particularly enzymological, research and to give several examples of its employment from 2006 to 2007.

An emphasis of the article will be the use in studies of enzyme mechanisms of rate measurements in mixtures of protium and deuterium oxides, which our group began calling 'proton inventories,' after our coworker Cherie Behn introduced us in 1967 to the particularly striking formulation of the ideas of such experiments by Kresge[13] (see Figure 1).

Although the emphasis of the present article is on the application of solvent isotope effects to enzyme mechanisms, the use of this technique in other mechanistic studies remains active. Readers wishing information on such aspects should consult appropriate reviews.[1,2,4,5,9–11]

A few fundamentals of solvent isotope effects[1,6,7]

Terminology

Readers of this Journal are likely to be fully current with the terminology of isotope effects in general as well as with the main terms of the solvent isotope-effects field, but in the service of prudence, we will here define

$$\text{equilibrium isotope effect} = K_{HOH}/K_{DOD} \quad (1)$$

where the K's are equilibrium constants in protium oxide (HOH) and deuterium oxide (DOD);

$$\text{kinetic isotope effect} = k_{HOH}/k_{DOD} \quad (2)$$

where the k's are rate constants. Expressed in this way, isotope effects larger than unity (more favorable equilibrium or faster rate in HOH) are called *normal isotope effects* and those smaller than unity (more favorable equilibrium or faster rate in DOD) are called *inverse isotope effects*.

Sources of solvent isotope effects

In the case of solvent isotope effects, the observed values may in principle arise from (a) differences between HOH and DOD in general solvent properties such as dielectric constant or viscosity; (b) differences between free energies of reaction or activation arising from interactions of reacting species directly with water molecules in the reactant state, transition state, or

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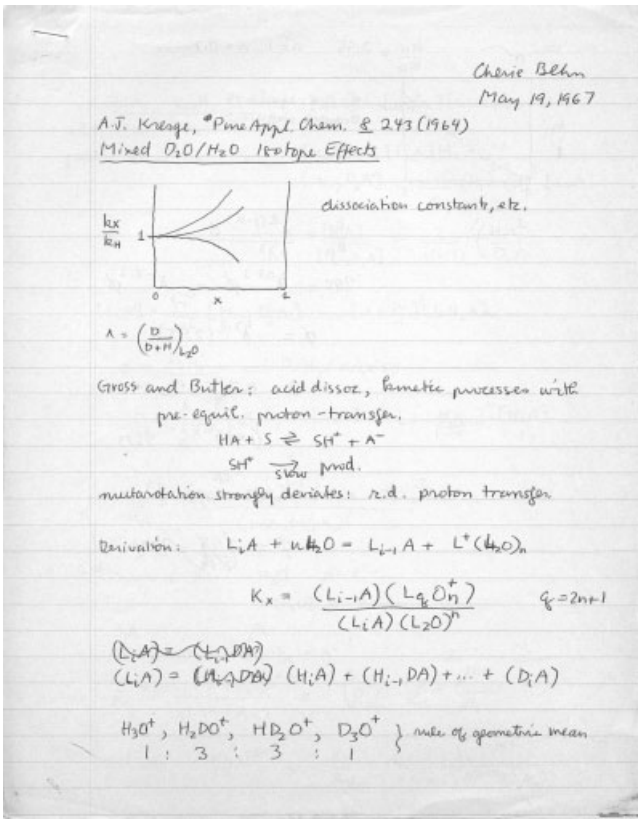

Figure 1 First page of the notes taken by R. L. Schowen at a group seminar presented by Ms Cherie Behn (M.S. in Chemistry, 1967, University of Kansas) on 19 May 1967. The subject was rate measurements in mixtures of protium and deuterium oxides as described by A. J. Kresge in Reference 13. The joint result of Kresge's clear writing and Behn's clear presentation was the publication of a series of papers on 'proton inventories' beginning[15–18] in 1973 and continuing in subsequent decades.

product state; (c) differences between free energies of reaction or activation arising from interactions involving exchangeable hydrogenic site of reacting species in the reactant state, transition state, or product state.

General solvent properties usually neglected

The general solvent properties of HOH and DOD are so similar that the differences have rarely been found to produce solvent isotope effects of reasonable magnitude. Unless evidence to the contrary is available, this source is usually assumed in enzymological studies to be negligible.

Direct participation of water molecules potentially important

Direct participation of water molecules in reactions may easily produce solvent isotope effects. For example, in hydrolytic reactions in which water attacks an electrophilic center, general-base catalysis by proton removal from the nucleophilic water molecule should result in a normal isotope effect. Studies in model reactions[5] have shown the magnitude of such effects to be around 2–4.

Participation of exchangeable protonic sites very important in enzymology

Protons attached to electronegative atoms such as O, N, or S generally exchange with solvent deuterons as rapidly as they become exposed to contact with labelled water molecules. For surface sites of proteins, this time is commonly within the mixing time of solutions of the two isotopic solvents or the time of dissolution of enzyme in either isotopic solvent (often enzymes are lyophilized from HOH and DOD solutions to optimize the degree of exchange but this is rarely actually necessary). Protonic sites within the structure of proteins are generally thought to exchange only when conformational fluctuations expose them and indeed the exchange reaction is used as a probe of the rates of such fluctuations. Relevant fluctuations may have characteristic times from less than a millisecond to days or weeks. It is generally unnecessary to achieve complete or even extensive exchange in kinetic or

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equilibrium experiments unless deep-lying sites are directly involved in, for example, catalytic reactions.

Examples of the involvement of exchangeable sites in enzymes often rest on rapidly exchanging sites. The serine residue in the active sites of serine hydrolases such as trypsin and acetylcholinesterase contains a hydroxyl group that rapidly exchanges with solvent hydrogenic sites; the hydroxyl group also attacks substrate peptide or ester sites nucleophilically with assistance from general bases of the enzyme structure. As in the model reactions referred to earlier, this interaction generates normal solvent isotope effects in the range of 2–4.

Some site-to-site variation in the degree of exchange into protein functional groups

While dissolution of an enzyme in pure DOD (or nearly pure: the commonly available material is about 99.5% pure) will result in essentially complete deuteration at all rapidly exchangeable sites, exchange with mixtures of HOH and DOD will not always lead to the degree of deuteration in all protein sites being equal to the degree of deuteration in the isotopic solvent mixture. The use of mixtures of isotopic solvents in the proton-inventory approach, described below, brings this point into prominence.

It is useful to think of the equilibrium exchange process in the following terms. For exchange with an isotopic solvent mixture of a protein functional group GH, the relevant relationships are

$$\mathrm{GH} + \mathrm{DOL} = \mathrm{GD} + \mathrm{HOL}, \quad K = X_{\mathrm{GD}}X_{\mathrm{HOL}}/X_{\mathrm{GH}}X_{\mathrm{DOL}} \qquad (3)$$

$$\text{isotopic fractionation factor} = \varphi_{\mathrm{GL}} = (X_{\mathrm{GD}}/X_{\mathrm{GH}})/(X_{\mathrm{DOL}}/X_{\mathrm{HOL}}) = K \qquad (4)$$

$$\varphi_{\mathrm{GL}} = (X_{\mathrm{GD}}/X_{\mathrm{GH}})/(X_{\mathrm{DOL}}/X_{\mathrm{HOL}}) = (X_{\mathrm{GD}}/X_{\mathrm{GH}})/(n/[1-n]) \qquad (5)$$

In Equations (3)–(5), the X's are mole fractions and the symbol L ('Label') refers to *either* H or D. The facts that we write GH and GD for a specific site in the protein and neglect the question of how many of all the remaining sites are occupied by H and D and that we write HOL and DOL, thus assuming that the equilibrium constant K is independent of whether L = H or L = D, represent a particularly strong form of the *Rule of the Geometric Mean.* This rule states that isotopic substitution at a single protic site in a molecule effects a free-energy change that is independent of the isotopic composition at all other protic sites in the molecule. The rule is to some degree correct as a result of the fact that isotopic free-energy changes are vibrational properties and are thus relatively local in sufficiently large molecules (a fact that is familiar from infrared spectroscopy). The fact that the rule is roughly valid in aqueous solutions of HOH and DOD results in a near-statistical distribution of D and H among the isotopic forms HOH, DOH and DOD: a mixture of equimolar HOH and DOD produces mole fractions of 0.25 HOH, 0.25 DOD and 0.5 HOD. Thus, the practice has arisen of defining an *atom fraction of deuterium* often denoted as n (sometimes x) as in Equation (5), which gives the most commonly encountered definition of the isotopic fractionation factor.

The magnitude of φ for a given functional group follows the general rule that 'the heavier isotope (here, D) accumulates in the tighter or more strongly bonded site while the lighter isotope (here, H) accumulates in the looser or more weakly bonded site.' As Equations (3)–(5) show, the standard of bonding for comparison is the average bulk-water site, where the D/H ratio will be $n/(1-n)$. If the hydrogen isotope in the GL site in Equation (3) is more tightly bound than in the bulk-water site, then at equilibrium, GD will predominate over GH compared with the bulk-water site and φ will be greater than unity. If, in contrast, the hydrogen isotope in the GL site in Equation (3) is less tightly bound than in the bulk-water site, then at equilibrium, GH will predominate over GD compared with the bulk-water site and φ will be smaller than unity.

Fractionation factors for common exchangeable sites

In much of organic chemistry and biochemistry, the relevant exchangeable sites are OL, NL, and SL groups. To a rough but useful approximation, the fractionation factors φ_{OL} and φ_{NL} are unity when the oxygen of OL is electrically neutral and regardless of the charge borne by the nitrogen of NL. That is to say, the strength of binding of these hydrogenic sites is very similar to the binding of the hydrogen in the average bulk-water site. When the oxygen of OL bears positive charge, as in L_3O^+, the binding at each of the three sites becomes looser and φ_{HO^+} has a value of 0.69. When the oxygen of LO bears a negative charge as in the solvated lyoxide ion $(\mathrm{LOL})_3\mathrm{LO}^-$ the three solvating waters develop strong hydrogen bonds with mobile, loosely bound sites while the internal site of the lyoxide itself develops a slightly stronger binding, leading to an overall φ_{LO^-} of 0.5. Finally, the longer, more loosely bound site in SL also produces $\varphi_{\mathrm{SL}} = 0.5$.

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Relationship of fractionation factors to equilibrium and kinetic isotope effects

Consider a chemical reaction that converts the species GL to the species JL. Then,

$$\mathrm{GL} = \mathrm{JL}, \quad K^{\mathrm{L}}_{\mathrm{GJ}} = X_{\mathrm{JL}}/X_{\mathrm{GL}} \tag{6}$$

$$\begin{aligned} K^{\mathrm{H}}_{\mathrm{GJ}}/K^{\mathrm{D}}_{\mathrm{GJ}} &= (X_{\mathrm{JH}}/X_{\mathrm{GH}})/(X_{\mathrm{JD}}/X_{\mathrm{GD}}) \\ &= (X_{\mathrm{GD}}/X_{\mathrm{GH}})/(X_{\mathrm{JD}}/X_{\mathrm{JH}}) \end{aligned} \tag{7}$$

$$\begin{aligned} K^{\mathrm{H}}_{\mathrm{GJ}}/K^{\mathrm{D}}_{\mathrm{GJ}} &= [(X_{\mathrm{GD}}/X_{\mathrm{GH}})/(n/1-n)]/[(X_{\mathrm{JD}}/X_{\mathrm{JH}})/(n/1-n)] \\ &= \varphi_{\mathrm{GL}}/\varphi_{\mathrm{JL}} \end{aligned} \tag{8}$$

Thus, the equilibrium isotope effect as usually defined is given by the ratio of reactant-state fractionation factor to the product-state fractionation factor: the initially surprising inversion of these factors is a result of defining the fractionation factor as *deuterium preference* rather than as *protium preference.*

In a perfectly similar fashion, the kinetic isotope effect is the ratio of reactant-state fractionation factor to transition-state fractionation factor:

$$\begin{aligned} k^{\mathrm{H}}_{\mathrm{GJ}}/k^{\mathrm{D}}_{\mathrm{GJ}} &= [(X_{\mathrm{GD}}/X_{\mathrm{GH}})/(n/1-n)]/[(X_{\mathrm{TD}}/X_{\mathrm{TH}})/(n/1-n)] \\ &= \varphi_{\mathrm{GL}}/\varphi_{\mathrm{TL}} \end{aligned} \tag{9}$$

where TL = TH or TD refer to transition-state species. This formulation assumes the adequacy of the transition-state theory in its so-called 'ultra-simple' version, and will possibly need modification if more modern versions of transition-state theory or other kinetic theories are to be applied.

Use of fractionation factors for experiments in mixtures of HOH and DOD: isotope effect at a single site

Consider an experiment in which the rate constant k is determined in mixtures of HOH and DOD for a reaction with a reactant-state RL (L = H or D, an exchangeable hydrogenic site), a transition-state TL and a product-state PL. The rate constant in a mixture of atom fraction of deuterium n should be given by

$$k_n = k_{\mathrm{RH}}(X_{\mathrm{RH}}) + k_{\mathrm{RD}}(X_{\mathrm{RD}}) \tag{10}$$

A useful set of relationships can be obtained by rearrangement of the definition of a fractionation factor in Equation (5):

$$X_{\mathrm{RH}} = (1-n)/(1-n+n\varphi_{\mathrm{RL}}) \tag{11}$$

$$X_{\mathrm{RD}} = (n\varphi_{\mathrm{RL}})/(1-n+n\varphi_{\mathrm{RL}}) \tag{12}$$

The terms in the denominators are customarily left uncollected to leave the role of the fractionation factor displayed explicitly. Rearranging Equation (10) and then introducing Equations (9), (11) and 12 yields

$$k_n = k_{\mathrm{RH}}\{(X_{\mathrm{RH}}) + (k_{\mathrm{RD}}/k_{\mathrm{RH}})(X_{\mathrm{RD}})\} \tag{13}$$

$$k_n = k_{\mathrm{RH}}\{(X_{\mathrm{RH}}) + (\varphi_{\mathrm{TL}}/\varphi_{\mathrm{RL}})(X_{\mathrm{RD}})\} \tag{14}$$

$$k_n = k_0\{(1-n+\varphi_{\mathrm{TL}})/(1-n+n\varphi_{\mathrm{RL}})\} \tag{15}$$

In Equation (15), we have noted that $k_{\mathrm{RH}} = k_0$, the rate constant in pure HOH. The function $k_n(n)$ is obviously complex in general, although this is the simple case in which the isotope effect $k_0/k_1 = (\varphi_{\mathrm{RL}}/\varphi_{\mathrm{TL}})$ arises from changes in binding at only a single hydrogenic site as the reactant state is converted to the transition state. Taking note of the fact, given above, that in a number of common circumstances $\varphi_{\mathrm{RL}} = 1$, we observe that Equation (15) then indicates that in such a circumstance $k_n(n)$ will be a linear function and $k_0/k_1 = (1/\varphi_{\mathrm{TL}})$.

Use of fractionation factors for experiments in mixtures of HOH and DOD: generalization to isotope effects at multiple sites

If the approach illustrated in the last section is applied to the case of multiple isotope effects at multiple sites (the Rule of the Geometric Mean being taken as true), then factors of the form $(1-n+n\varphi)$ appear in the numerator of $k_n(n)$ for each transition-state site with φ not equal to one and factors of the form $(1-n+n\varphi)$ appear in the numerator of $k_n(n)$ for each transition-state site with φ not equal to one:

$$k_n = k_0\{[\Pi^{\nu}(1-n+n\varphi_{\mathrm{Ti}})]/[\Pi^{\mu}(1-n+n\varphi_{\mathrm{Rj}})]\} \tag{16}$$

This is the general expression for isotope effects arising from the transformation of μ reactant-state sites Rj to ν transition-state sites Ti. It will obviously generate very complex forms of $k_n(n)$ but there are important special cases of considerable simplicity and special interest in enzymological applications.

Often, the approximation that reactant-state fractionation factors for enzyme structure beyond the reactive function groups of the active site has either unit fractionation factors or factors – whatever their magnitudes – that remain unchanged as the reactant state is transformed to the transition state. Then the denominator of Equation (16) reduces to unity. Under such circumstances, several instances are of special note (Figure 2):

$$\text{one-proton catalysis} : k_n = k_0(1-n+n/[k_{\mathrm{H}}/k_{\mathrm{D}}]) \tag{17}$$

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Here, a single transition-state site generates the observed isotope effect $k_H/k_D = k_0/k_1$ and a plot of k_n versus n will be linear.

two-proton catalysis :

$$k_n = k_0(1 - n + n/[k_H/k_D]_1)\ (1 - n + n/[k_H/k_D]_2) \quad (18)$$

Here, two transition-state sites each generate their own isotope effects, $[k_H/k_D]_1$ at one site and $[k_H/k_D]_2$ at the other site. Such results are expected, for example, if proton relay occurs across a chain of two H-bonds. The plot of k_n versus n will be parabolic ('bowl-shaped') and the overall isotope effect $k_0/k_1 = [k_H/k_D]_1\ [k_H/k_D]_2$, the product of the two individual isotope effects.

There is always the possibility that an overall isotope effect, particularly in enzyme systems, arises from a very large number of very small isotope effects. In this case, the value of each fractionation factor φ will be close to one so that the factors $(1 - n + n\varphi)$ in Equation (16), when written as $(1 - n[1 - \varphi])$, will become approximately $\exp(-n[1 - \varphi])$ since $1 - x$ is approximately $\exp(-x)$ when x is small. Thus, the products in Equation (16) become exponential sums and

$$k_n = k_0\ \exp\{\Sigma^v(-n[1 - \varphi_{Ti}]) - \Sigma^\mu(-n[1 - \varphi_{Rj}])\} \quad (19)$$

$$k_n = k_0\ \exp\ n\{\Sigma^v(-[1 - \varphi_{Ti}]) - \Sigma^\mu(-[1 - \varphi_{Rj}])\}$$
$$= k_0(Z)^n \quad (20)$$

where $Z = \sum^v(-[1 - \varphi_{Ti}]) - \sum^\mu(-[1 - \varphi_{Rj}])$, which is independent of n and thus a constant equal to k_1/k_0. This circumstance corresponds to

'multiproton catalysis : '$k_n = k_0(Z)^n$ (21)

Here, a plot of $k_n(n)$ versus n is exponential and the overall isotope effect is equal to $1/Z$.

Special considerations in the case of proteins[7]

The role of pH and pD in the magnitudes of enzymic rate constants: corresponding pL

Many enzymic reactions have pH-dependent rate constants and the question arises, how are the pH and pD to be selected for comparison in calculating the isotope effect or conducting proton-inventory studies? It is tempting but potentially disastrous to assume that the appropriate procedure is to take pH = pD.

Consider the case in which an enzyme exists in three protomeric states, EH_2, EH and E. A reasonably common situation is that EH, which has one ionizable residue in the protonated state (thus capable of acid catalysis), and a different ionizable residue in the unprotonated state (thus capable of base catalysis), is reactive while neither EH_2, which is incapable of base catalysis, nor E, which is incapable of acid catalysis, has any appreciable reactivity.

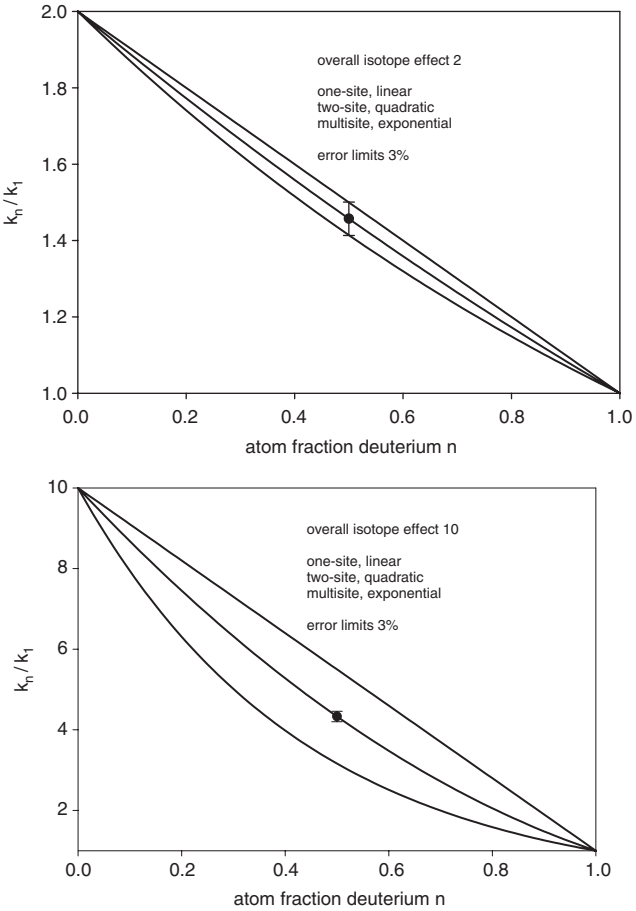

Figure 2 Expected proton-inventory curves [plots of $k_n(n)$ versus n, the atom fraction of deuterium in mixtures of HOH and DOD] for an overall isotope effect k_0/k_1 of 2 (upper graph) and an overall solvent isotope effect of 10 (lower graph). The three curves in each graph are for a single-site isotope effect (uppermost curves), a two-site isotope effect (middle curves) and a large number of sites (lowermost curves). Error limits of $\pm$ 3% are shown for a point on the two-site curves at $n = 0.5$, illustrating that this level of precision is barely adequate to distinguish the three curves for $k_0/k_1 = 2$ but is entirely adequate for $k_0/k_1 = 10$.

Let the rate constant for catalysis by EH in HOH be called $k_{max\,H}$, the ionization constant for formation of EH from EH_2 in HOH be called K_{a1H}, and the ionization constant for loss of EH to E be called K_{a2H}. Then, the corresponding quantities for the species in ED_2, ED, and E in DOD will be $k_{max\,D}$, K_{a1D}, and K_{a2D}. As a brief exercise will show, Equations (22) and (23) describe the dependence of the observed rate constants k_{HOH} and k_{DOD} on pH and pD, respectively:

$$k_{HOH} = k_{max\ H}K_{a1H}a_H/(a_H^2 + K_{a1H}a_H + K_{a1H}K_{a2H}) \quad (22)$$

$$k_{DOD} = k_{max\ D}K_{a1D}a_D/(a_D^2 + K_{a1D}a_D + K_{a1D}K_{a2D}) \quad (23)$$

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where a_H, the activity of the proton in HOH, and a_D, the activity of the deuteron in DOD, are of course related to pH and pD by $pL = -\log(a_L)$. Equations (22) and (23) generate the familiar bell-shaped curves with a maximum rate constant between pK_{a1L} and $pK_{a2}L$.

Laughton and Robertson[14] in 1969 produced a most valuable table (Table 7-3) in which values of pK_{aH} and pK_{aD} are recorded for many acids. For a large majority of the acids with pK_{aH} between 4 and 9, $pK_{aD} = pK_{aH} + 0.5$ to within a reasonable approximation. This is expected for OH and NH acids where the fractionation factor for the unionized acid is around unity and, if the conjugate base interacts weakly with solvating water molecules, giving little or no fractionation, then the entire equilibrium isotope effect on ionization will come from the lyonium ion. Since the fractionation factor for each of the three hydrogenic sites is 0.69 (see above), the isotope effect should be $1/(0.69)^3 = 3$, thus $pK_{aD} = pK_{aH} + 0.5$ just as above. This behavior was assumed in constructing the pL/rate profiles in Figure 3. It was also assumed that $k_{max\,H} = 2$ units and $k_{max\,D} = 1$ unit for an isotope effect on catalysis of 2.

Figure 3 shows that if comparisons of rate constants in HOH and DOD are made at pH = pD, then the apparent isotope effect will range from about 6 at low pL to inverse values at high pL, because the different ionization-state distribution of enzyme species in HOH and DOD at any common pL value has been confounded with the actual isotope effect on the catalytic process.

A workable procedure is to use *corresponding pL*, where the pH and pD are selected to obtain the same distribution of protomers in the two solvents. An effective operational approach is to control the pL with a buffer having a $pK_{buf\,H}$ near pK_{a1H} and pK_{a2H} (hardly an inconvenience). Then, the isotope effect on both enzyme ionizations *and* the buffer ionization is likely to be the same, say a value J (possibly 3 but any value will do). Then $a_H = K_{buf\,H}R$, where R is the ratio of conjugate base to conjugate acid in the buffer. In DOD, *if the same buffer ratio R is used*, $a_D = K_{buf\,D}R = K_{buf\,H}R/J$, $K_{a1D} = K_{a1H}/J$ and $K_{a2D} = K_{a2H}/J$. Substitution of all these relationships into Equations (22) and (23) gives

$$k_{HOH} = k_{max\,H}K_{a1H}K_{buf\,H}R/([K_{buf\,H}R]^2 + K_{a1H}[K_{buf\,H}R] + K_{a1H}K_{a2H}) \quad (22a)$$

$$k_{DOD} = k_{max\,D}K_{a1H}K_{buf\,H}R/([K_{buf\,H}R]^2 + K_{a1H}[K_{buf\,H}R] + K_{a1H}K_{a2H}) \quad (23a)$$

Thus because the isotope effects J, common to all the ionizations, cancel from the fraction of species, the fraction of EH in HOH and ED in DOD are equal to each other at all pL and $k_{HOH}/k_{DOD} = k_{max\,H}/k_{max\,D}$ when the ratio is taken at corresponding pL. For proton-inventory experiments, the same buffers may be used in HOH and DOD and all their mixtures to assure a corresponding pL throughout the range of mixtures.

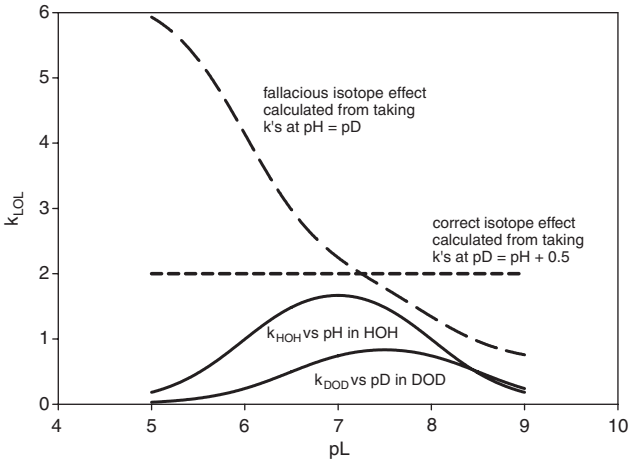

Figure 3 Rate constants in HOH and DOD as a function of pL (L = H, D) for a reaction in which an enzyme catalyst exists in three protomeric forms, EL_2 ($pK_{aHOH} = 6.0$, $pK_{aDOD} = 6.5$), EL ($pK_{aHOH} = 8.0$, $pK_{aDOD} = 8.5$), and E. EL_2 and E are catalytically inactive while EL is active with a rate constant equal to 2 units in HOH and 1 unit in DOD. Thus, the true isotope effect is $k_H/k_D = 2$. The two bell-shaped curves give the expected pL/rate dependences (notice that the actual values of the catalytic rate constants are not achieved even at the rate maxima). If the isotope effect is obtained erroneously by measuring the rate constants at pH = pD, then the values along the steep dashed curve, ranging from over 6 to inverse values, will result. If the rate constants at *corresponding pL* (pD = pH + 0.5) are used, then a constant, correct value of 2 is found independent of the pL.

Magnitudes of isotopic fractionation factors at protein sites[19–21]

Advances in NMR technology brought these methods to the point in the last decade or so that the determination of the equilibrium level of deuteration could be measured for all or nearly all backbone NH groups in moderately small proteins, i.e. the entire array of deuterium fractionation factors at each of the individual sites could be measured. The fractionation factors are often close to unity, as expected, but there are also some values both larger and smaller than unity by surprising amounts. These studies are proving to be of considerable interest for biomolecular structure but are only rarely relevant to mechanism when specific interactions that change between reactant state and transition state are identified.

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Kinetic complexity

This term has come to mean the contribution of more than a single step to limitation of the rate, a circumstance that arises frequently in enzymology. The equations shown above were derived for a single rate-limiting step and must therefore be suitably modified if that condition is not met (see Reference 7 for details).

Some examples[22–25] of applications of solvent isotope effects to mechanistic enzymology, 2006–2007

Simultaneous general-acid–general-base catalysis of nucleotidyl transfer in the action of nucleic-acid polymerases (Castro *et al.*[22])

Castro *et al.*[22] made use of the proton-inventory technique to examine the nature of general acid–base catalysis in the action of the four different kinds of nucleic-acid polymerases. These enzymes catalyze the sequential condensation of the next nucleic-acid unit to the growing polymer chain being built on the template of an existing nucleic-acid polymer. The template polymer can be either a DNA molecule or an RNA molecule. The product polymer can, in either case, be either a DNA or an RNA molecule, thus allowing for four different classes of such enzymes.

In perhaps the simplest case, a DNA-dependent DNA polymerase is involved in the replication of double-stranded DNA, for example to generate two daughter copies of the cellular genome before cell division, one copy for each of the resulting daughter cells. The chemical reaction catalyzed is the nucleophilic reaction of the 3′-OH group of the residue at the end of the growing chain (see the illustration in Figure 4) at the α-phosphorus center of a deoxyribonucleoside triphosphate that has been recognized and bound on the template molecule; the result is the displacement of pyrophosphate, with formation of the new P–O bond incorporating the new base into the growing polymer. Logically, the reaction could be accelerated by (a) general-acid catalysis by proton donation from an acidic enzyme residue to the departing pyrophosphate; (b) general-base catalysis by proton abstraction from the 3′-OH group by a basic enzyme residue; or (c) simultaneous general-acid–general-base catalysis.

The same considerations apply to RNA-dependent RNA polymerases that catalyze the replication of RNA molecules, the DNA-dependent RNA polymerases involved in transcription of the DNA code into messenger RNA, and the RNA-dependent DNA polymerases, such as the HIV 'reverse transcriptase' studied here, which transcribe the RNA genome of the virus into DNA that is then inserted into the genome of the host cell, making it a permanent factory for the replication of the virus.

The four proton-inventory plots in Figure 4, exhibiting data for representatives of the four classes of enzymes, each shows a persuasively quadratic data set for $k(n)$. The direct presentation shows that the rate constant is described by a quadratic equation, and a second plot shows that $[k(n)]^{1/2}$ is linear in n, suggesting that the data are at least roughly described by Equation (18) above with $(k_H/k_D)_1 = (k_H/k_D)_2$.

This finding is most easily consistent with the view that simultaneous general-acid–general-base catalysis, with the enzyme assisting a concerted displacement at phosphorus, is accelerating the enzyme-catalyzed reaction. This mechanism is shown by the four plots in Figure 4 to apply to all the four classes of enzymes studied.

The importance of environmental reorganization in the activation of prothrombin by Factor Xa of the blood coagulation cascade (Zhang and Kovach[23])

The coagulation of blood is effected by a huge complex cascade of enzymes, in which the later participants are activated by earlier participants by the hydrolytic removal of an inhibitory 'pro-moiety,' as in the conversion of prothrombin to α-thrombin. This conversion is catalyzed by the serine protease Factor Xa in a process that is further accelerated by the presence of a phospholipid surface and the accessory protein Factor Va. Zhang and Kovach[23] in 2006 examined solvent isotope effects in this system (Figure 5).

When the human proteins are employed in the full assembly, the reaction proceeds about 10–15% faster in DOD than in HOH (top of Figure 5). This is in contrast to the action of many serine proteases, which possess an array of 2–3 hydrogen bonds in the active site, linking the nucleophilic serine to a nearby histidine base and sometimes other groups as well. This machinery can function as a simple general catalyst with model substrates that do not interact extensively with the active site, generating one-site isotope effects around 2–4. Substrates more nearly approximating the structure of the physiological substrate can activate more of the machinery and commonly generate two-site isotope effects of the same rough magnitudes.

That this can also be the situation with thrombin activation was shown by Zhang and Kovach in a model experiment. Prothrombin activation requires hydrolysis of two peptide bonds, Arg^{322}–Ile^{323} and Arg^{273}–Thr^{274}. The graph in the bottom part of Figure 5 shows the proton inventory for the hydrolysis, with a catalysis by Factor Xa of a model peptide terminating in an Arg-*p*-nitroanilide linkage, thus roughly simulating the

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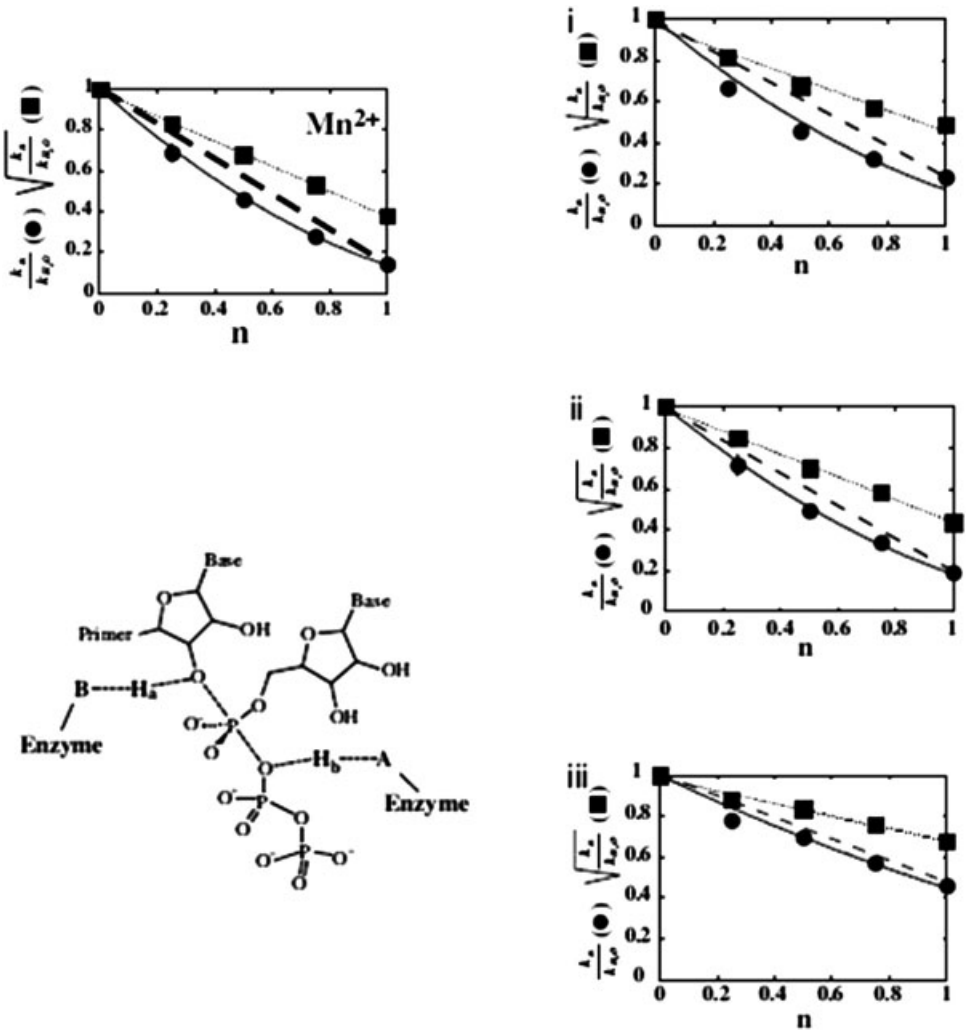

Figure 4 Proton inventories obtained by Castro *et al.*[22] for examples of nucleotide polymerases for circumstances under which the nucleotidyl-transfer step is essentially rate-limiting. Upper left, data for the poliovirus RNA-dependent RNA polymerase with Mn^{2+} as cofactor. The plot of $k_n(n)$ fits a quadratic function, as demonstrated by the linearity of a plot of $[k_n(n)]^{1/2}$. This signals that the isotope effect is arising from two transition-state sites, such as the acid–base catalytic bridges shown in the diagram at the lower left. The data at right show that the same conclusions can be drawn for the RB69 DNA-dependent DNA polymerase (i, top); the T7 DNA-dependent RNA polymerase (ii, middle); and the HIV reverse transcriptase (iii, bottom: an RNA-dependent DNA polymerase). Reproduced with permission from Reference 22.

steps of the physiological reaction that involves catalysis of the peptide linkage itself. The overall isotope effect is 2.3 and the plot suggests that it arises from two sites, each generating an isotope effect around 1.5. When the 'chemical steps' are thus exposed, Factor Xa behaves like an ordinary serine protease.

Thus, Zhang and Kovach conclude that, 'the rate-determining process in the FXa-catalyzed activation of prothrombin at the aqueous-lipid interface is dominated by the physical events, most likely a conformational change accompanied by rearrangement of water while poising the scissile bond for nucleophilic attack by Ser.'

Conformational dynamics in the action of ribonuclease-A (Kovrigin and Loria[24])

Kovrigin and Loria[24] document the increasing evidence that enzyme catalysis may involve the coupling of enzyme motions to substrate-reorganization events in the active site (see also Reference 25). They applied to this question an NMR method capable of measuring the rate of exchange of individual residues in a protein between two conformational states. For 14 residues in ribonuclease-A, an enzyme that catalyzes the hydrolytic fission of the phosphodiester bond in RNA, they found an identical rate constant for exchange

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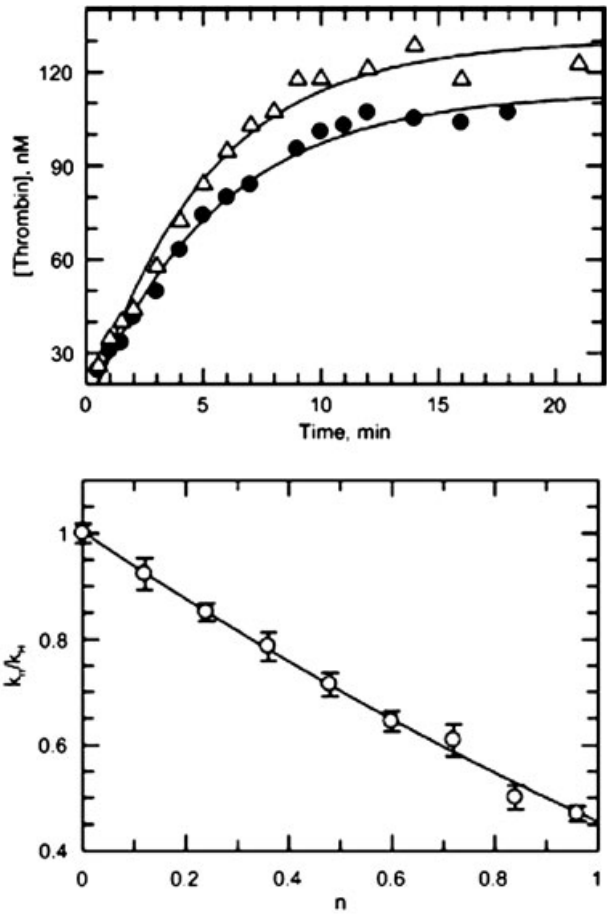

Figure 5 Results of Zhang and Kovach[23] for the hydrolysis of prothrombin to thrombin by the catalysis of the blood coagulation enzyme Factor Xa (top: time course of hydrolysis, 4-fold excess of the accessory Factor Va in the presence of large unilamellar vesicles, pH 7.5, 25°C, in protium oxide [filled symbols] and deuterium oxide [unfilled symbols]) and for the hydrolysis of the model substrate D-Ile-L-Pro-L-Arg-*p*-nitroanilide (bottom: k_{catn}/k_{cat0} versus n, atom fraction of deuterium in the solvent, pH 8.4 and equivalent, 25°C). The proenzyme activation at the top exhibits a small inverse isotope effect (10–15% faster in DOD) consistent with a conformational change as the rate-limiting step. The peptide hydrolysis at the bottom shows a normal isotope effect of about 2.5, which the proton inventory fit (solid line) indicates to arise from two sites, each generating an isotope effect around 1.5. This is consistent with observations for other serine proteases with quasi-physiological substrates, the 'chemical step' being rate limiting. Reproduced with permission from Reference 23.

between two states that have different arrangements of two structural loops and also some residues in the active site. The motions of these 14 residues, which are located not only in the active site but also throughout the enzyme, some more than 20 Å away from the active site, thus appear to be correlated during the conformational reorganization. Furthermore, the rate constant for the conformational exchange reaction

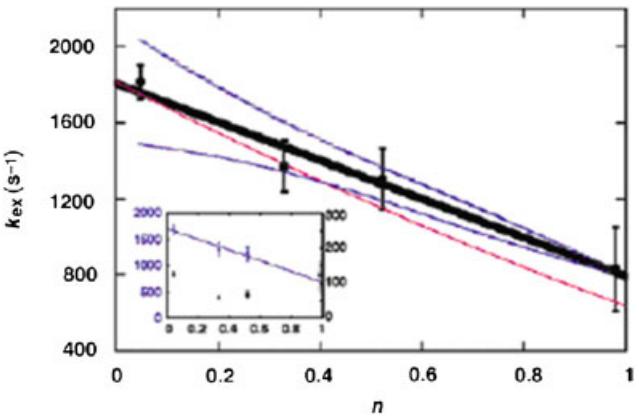

Figure 6 The rate constant in mixtures of HOH and DOD for the conformational exchange of 14 residues between two conformations of ribonuclease-A, as determined by Kovrigin and Loria.[24] The heavy black line is the fit to the one-proton model of Equation (17) with $k_H/k_D = 2.3$, with the blue lines showing the 95% confidence limits. The red line is the best fit to the two-proton model of Equation (18) with the two isotope effects being equal. The inset shows the values of the forward and reverse rate constants in the isotopic solvent mixtures, with a line similar to that for the exchange rate constants drawn through the points for the forward rate constants. Reproduced with permission from Reference 24. This figure is available in colour online at www.interscience.wiley.com/journaljlcr.

is equal in magnitude to the value of k_{cat} for which the rate-limiting step is believed to be the release of the product.

Figure 6 shows a proton-inventory plot for the exchange rate constant, for which the overall solvent isotope effect (H/D) is 2.3. The data are more nearly consistent with a single-site model than a two-site model, so that the transfer of a single proton or the formation of a single-site hydrogen bond with substantial weakening of the potential about the proton is indicated as the origin of the observed effect. The indicated interaction may serve as a trigger for the conformation change. The equilibrium constant for the exchange reaction shows no isotope effect, so the interaction is a transition-state interaction absent in both the stable conformations. The inset in Figure 6 shows the forward and reverse rate constants in the isotopic mixtures; a linear curve is drawn through the forward-reaction values, and although no line is shown for the reverse rate constants, the absence of an equilibrium effect requires a straight line of the same slope as for the forward constants.

The overall picture is thus that the development of a single site with a weakened potential about an exchangeable hydrogenic site in the protein triggers a large-scale, correlated reorganization of the enzyme structure that allows ('gates') product release and catalytic turnover of ribonuclease-A.

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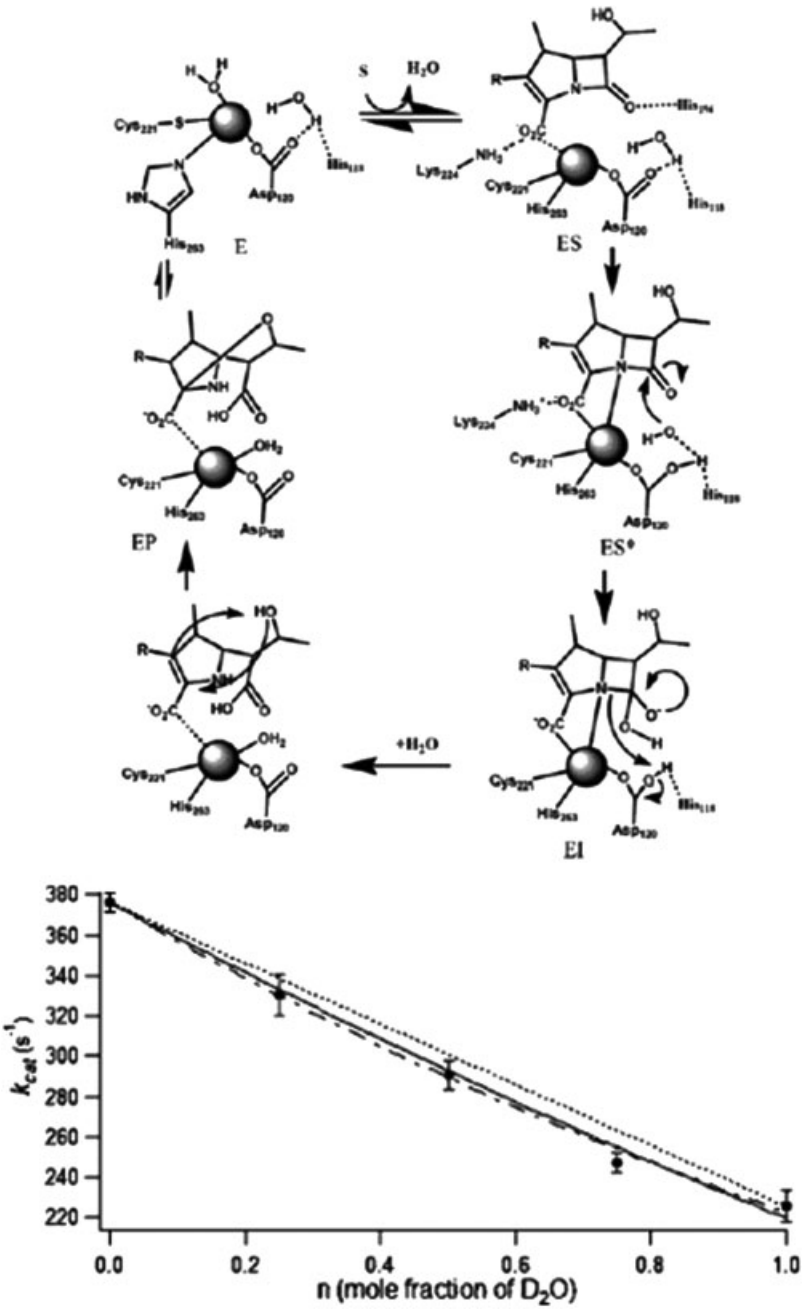

Figure 7 Mechanism of β-lactam hydrolysis proposed by Sharma *et al.* (2006)[25] catalyzed by ImiS, a Zn(II)-dependent class B2 β-lactamase originally obtained from *Aerobacter veronii* (top) and supporting evidence in the form of a plot of $k_{cat}(n)$ versus n. The three curves plotted in the proton-inventory graph are for one-proton catalysis (dotted line), two-proton catalysis (solid line) and multiproton catalysis (dashed line). The isotope effect is around 1.6 and the plots illustrate that, even with data of good precision, two-proton catalysis cannot be distinguished from multiproton catalysis for an isotope effect of this modest size. Reproduced with permission from Reference 25.

Mechanism of β-lactam hydrolysis catalyzed by ImiS, a Zn(II)-dependent class B2 β-lactamase from Aerobacter veronii (Sharma *et al.*, 2006)[25]

An important mechanism of microbial resistance to β-lactam antibiotics, such as penicillin, is the development of β-lactamases, enzymes that catalyze the hydrolytic destruction of the antibiotic molecules. Some β-lactamases possess a nucleophilic serine that attacks the lactam carbonyl, leading to an acyl-enzyme that is hydrolyzed in a second, deacylation stage. These β-lactamases are subject to inactivation by substances

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like clavulanic acid, which acylates the serine residue irreversibly. Drugs that combine a β-lactamase inactivator with a β-lactam antibiotic can thus be effective against resistant bacteria that rely on a serine β-lactamase for resistance. The drug Augmentin, for example, combines amoxycillin with clavulanic acid.

A different form of resistance has appeared, however, in the form of metallo-β-lactamases, which do not possess an active-site serine and are not susceptible to clavulanic-acid inactivation. One of these, a Zn(II)-dependent enzyme, was the subject of a solvent isotope-effect study in 2006 by Sharma *et al.*[25] The presumed mechanism of action of the enzyme, known as ImiS, is shown in the top part of Figure 7. As can be seen, protic interactions, including proton transfers, play a role in the mechanism and solvent isotope effects may be expected.

The lower part of Figure 7 shows a proton inventory for k_{cat} in imipenem hydrolysis. The overall isotope effect (H/D) is only 1.6, which as the authors showed, presents problems in use of the method. The data clearly fall below a straight line connecting the $n = 0$ point to the $n = 1$ point, suggesting that more than a single site accounts for the isotope effect. The lines for a two-site effect and an 'infinite-site' effect, however, lie very close together and within the error limits of most points. The data are thus consistent with the proposed mechanism but unfortunately cannot add much resolution to it.

Conclusion

Solvent isotope effects form an effective tool for mechanistic studies of enzyme-catalyzed reactions, and the extension to proton inventories can in some cases lend a further level of information.

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