Isotope Effects in Enzymology

27

So far we saw how chemically identical behavior of isotopes is exploited in elucidating enzyme mechanisms. Despite their remarkable chemical similarity, isotopic substitution does affect reaction rates that directly involve them. Isotopic substitution can influence the equilibrium position, affect reaction rate, or both. The former (affecting the equilibrium constant) is termed *equilibrium isotope effect* while the latter (affecting the rate constant) *kinetic isotope effect* (KIE). Commonly encountered types of isotope effects in enzyme study are given with examples below.

Equilibrium Isotope Effect Consider the following exchange reaction where D^+ is exchanged with two hydrogen atoms (as H^+ ions) of water:

$$2D^+ + H_2O \rightleftarrows D_2O + 2H^+$$

The equilibrium constant for this reaction is 8.2. The D–O bond is stiffer (shorter by 0.04 Å and stronger) than the H–O bond because of the heavy deuterium atom. Thermodynamic stability of D_2O is relatively higher (hence has a lower zero-point energy) than H_2O. Therefore, at equilibrium there will be much more of D_2O than H_2O. For the same reasons, D_2O as a solvent can affect the ionization of an acid group. The dissociation of acetic acid in D_2O is relatively less favored than in H_2O.

$$CH_3COOD \rightleftarrows CH_3COO^- + D^+$$

The acid dissociation constant (itself an equilibrium constant!) is given by the ratio of the two rate constants, k_1/k_{-1}. We can consider k_{-1} (reverse) as a simple bimolecular collision rate constant; as this process is purely diffusion controlled, it is unaffected by the isotopic substitution. However k_1 (forward) involves breaking of the CH_3COO–D bond, is affected by isotopic substitution and is higher for

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$CH_3COO–H$. As a direct consequence of this, pKa of acetic acid is raised in D_2O (and therefore, pD = pH + 0.4). These two simple examples describe how an equilibrium isotope effect comes about. It should be mentioned that equilibrium isotope effect is presented as a non-unity ratio of the equilibrium constants ($K_{\text{light isotope}}/K_{\text{heavy isotope}}$).

Solvent Isotope Effect Isotopic substitution of solvent protons by the heavy isotope (such as in D_2O) can affect the ionization of an acid group and more importantly the rate of the enzyme reaction itself. Such solvent isotope effects are particularly important when solvent protons participate directly or are exchanged via the ionizable groups on the enzyme/substrate during catalysis. Solvent isotope effects are often useful in distinguishing between nucleophilic versus general base catalysis (Chap. 6 Origins of enzyme catalytic power, in Part I and Chap. 31 Nucleophilic Catalysis and Covalent Reaction Intermediates, in Part IV).

Kinetic Isotope Effect The KIEs reflect changes in the vibrational frequencies of reactants as they pass through the rate-determining transition states to form products. Because they directly report on the kinetic reaction path, the KIE is perhaps the most powerful tool available for a mechanistic enzymologist. A KIE is usually written as a ratio of rate constants for the light and heavy isotopic reactants. For example, the isotope effect for a C-H bond versus C–D bond cleavage may be written as $k_{\text{C-H}}/k_{\text{C-D}}$ (or simply k_H/k_D). When the isotopic substitution is at the reaction center (and directly participates in bond-breaking/bond-making events), then the observed KIE is termed as primary isotope effect (1°KIE). Consider the following schematic example:

$$X - C_\alpha - C_\beta - C_\gamma -$$

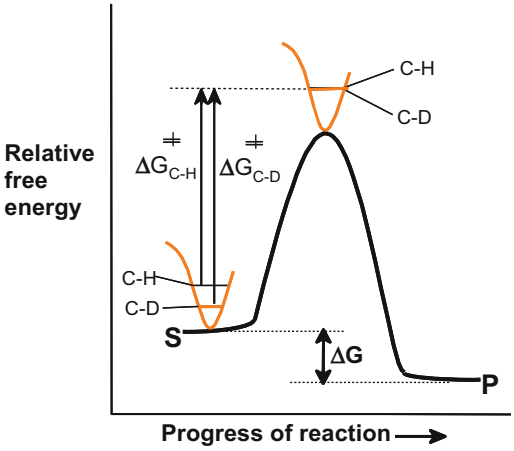
The effect due to isotopic substitution at X – in the X-C_α bond-breaking event – is a *primary* KIE. Secondary kinetic isotope effect (2°KIE) arises when the isotopic substitution is further removed from the scene of action. Accordingly they are denoted as *α-secondary* or *β-secondary* KIEs, etc. – where α and β denote the position of the isotopic substitution relative to the atom undergoing bond cleavage. KIEs typically decrease in magnitude as the point of isotopic substitution lies further from the reaction center. Most enzyme KIE studies are confined to primary and α-secondary effects – expectedly these are the ones giving most useful information on the enzyme mechanisms.

Normal Versus Inverse Kinetic Isotope Effect An isotopic substitution by a heavier atom (like H by D) makes that bond stiffer and stronger. If this bond is to be broken during reaction, then that isotope will have less restrained bonding environment in the transition state when compared to the reactant state. In such

cases, the reactant bearing the heavy isotope reacts less rapidly and a normal isotope effect is observed – the KIE value is above unity. An inverse isotope effect (and KIE value below unity) results in case the isotope in question experiences a more restricted bonding environment in the transition state. It is obvious that such information provide valuable insights into the nature of the transition state itself.

27.1 Magnitude of the Observed Isotope Effect

As full theory of the origin of KIEs is quite complex and involves many factors, we will consider a simple approximation to evaluate it from first principles. The elementary theory for the case of C–H bond versus C–D bond cleavage step is considered below. In this treatment, the C–H and C–D bonds have very little difference in terms of their electronic, translational, and rotational properties. Their vibrational motion is considered harmonic and that one of these stretching modes becomes the bond-breaking event in the transition state. The vibrational frequencies (as seen in the infrared region) representing their respective zero-point energies are distinct for the two bonds; this is the major factor contributing to KIEs because the energy level of the transition state for both the reactions is approximately same. These conditions are schematically represented in the Fig. 27.1.



Bond	Vibrational frequency
C-H	around 2,900 cm^{-1}
C-D	around 2,100 cm^{-1}

Fig. 27.1 Reaction coordinate diagram for the C–H bond cleavage in an exergonic reaction. The heavier isotopomer (C–D) lies at lower energy as expected for a shorter bond length. However the transition state for both C–H and C–D bond cleavages are of similar energy. Therefore ΔZPE significantly contributes to the difference between $\Delta G^{\neq}$ for C–H and C–D bonds. The accompanying table gives the vibrational frequencies (observed in infrared region) for the C–H and C–D bonds

The zero-point energy (ZPE) for a C–X bond (considered as a harmonic oscillator) is given by

$$\mathrm{ZPE} = \frac{1}{2}h\nu = \frac{1}{2}hc\overline{\nu}$$

where h is the Plank's constant (6.64×10^{-34} J.s), c is the velocity of light (3×10^{10} cm.s^{-1}), and $\overline{\nu}$ is the vibrational frequency in cm^{-1}. Based on these definitions, we could write the ZPE for the C–H and C–D bonds as follows:

$$\mathrm{ZPE}_{\mathrm{C-H}} = \frac{1}{2}hc\overline{\nu}_{\mathrm{C-H}} \quad \text{and} \quad \mathrm{ZPE}_{\mathrm{C-D}} = \frac{1}{2}hc\overline{\nu}_{\mathrm{C-D}}$$

and hence the energy difference between the two bonds as

$$\Delta\mathrm{ZPE} = \frac{1}{2}hc\overline{\nu}_{\mathrm{C-H}} - \frac{1}{2}hc\overline{\nu}_{\mathrm{C-D}}$$

From Hook's law, the vibrational frequency is given by:

$$\overline{\nu} = \frac{1}{2\pi c}\sqrt{\frac{\kappa}{\mu}}$$

where κ is the force constant of the bond and μ is reduced mass $\left(= \frac{m_1 m_2}{m_1 + m_2}\right)$. The difference between the C–H and C–D bonds thus boils down to differences in their reduced masses. Therefore,

$$\overline{\nu}_{\mathrm{C-H}} = \frac{1}{2\pi c}\sqrt{\frac{\kappa}{\mu_{\mathrm{C-H}}}} \quad \text{and} \quad \overline{\nu}_{\mathrm{C-D}} = \frac{1}{2\pi c}\sqrt{\frac{\kappa}{\mu_{\mathrm{C-D}}}}$$

We thus obtain

$$\frac{\overline{\nu}_{\mathrm{C-H}}}{\overline{\nu}_{\mathrm{C-D}}} = \sqrt{\frac{\mu_{\mathrm{C-D}}}{\mu_{\mathrm{C-H}}}} \quad \text{and} \quad \overline{\nu}_{\mathrm{C-D}} = \overline{\nu}_{\mathrm{C-H}}\sqrt{\frac{\mu_{\mathrm{C-H}}}{\mu_{\mathrm{C-D}}}}$$

and the ZPE difference between the two bonds is obtained by substituting these values in the equation above.

$$\begin{aligned} \Delta ZPE &= \frac{1}{2}hc\overline{\nu}_{\mathrm{C-H}} - \frac{1}{2}hc\overline{\nu}_{\mathrm{C-H}}\sqrt{\frac{\mu_{\mathrm{C-H}}}{\mu_{\mathrm{C-D}}}} \\ &= \frac{1}{2}hc\overline{\nu}_{\mathrm{C-H}}\left(1 - \sqrt{\frac{\mu_{\mathrm{C-H}}}{\mu_{\mathrm{C-D}}}}\right) \end{aligned}$$

We can evaluate ΔZPE from this equation (in fact, for any two isotopic pairs) by substituting respective parameters. Typical $\overline{\nu}_{C-H}$ is obtained from IR frequency data (2900 cm^{-1}, from table in the Fig. 27.1 above) and the respective reduced masses are calculated [where $\mu_{C-H} = (12 \times 1)/(12 + 1) = 0.923$ and ($\mu_{C-D} = (12 \times 2)/(12 + 2) = 1.714$]. Therefore,

$$\Delta ZPE = \frac{1}{2} \times 6.64 \times 10^{-34} \times 3 \times 10^{10} \times 2900 \left(1 - \sqrt{\frac{0.923}{1.714}}\right) = 7955 \times 10^{-24} J$$

This corresponds to 4789 $J.mol^{-1}$ (when multiplied by the Avogadro number, 6.02×10^{23} mol^{-1}). Since the reaction rate constant is given by $k = (kT/h)\, e^{-\frac{\Delta G^{\neq}}{RT}}$, we can find the ratio:

$$\frac{k_{C-H}}{k_{C-D}} = \frac{e^{-\frac{\Delta G^{\neq}_{C-H}}{RT}}}{e^{-\frac{\Delta G^{\neq}_{C-D}}{RT}}} = e^{\frac{\Delta G^{\neq}_{C-D} - \Delta G^{\neq}_{C-H}}{RT}} = e^{\frac{\Delta ZPE}{RT}}$$

At 25 °C, this value is around 7.0 $\left(= e^{\frac{4789}{8.314 x 298}}\right)$. When a deuterium isotope effect is fully manifest, one obtains a k_{C-H}/k_{C-D} value of around 7.0. Most often this value ranges from 2 to 15 and if no isotope effect is observed then it will be unity. As a rule of thumb, an observed k_{C-H}/k_{C-D} value between (a) 4–7 is indicative of a symmetric *TS* for that C-H bond cleavage event and (b) 1–4 means an asymmetric *TS* (for cleavage of this bond) or it is a secondary effect (2°KIE with no bond cleavage).

The magnitude of any KIE thus depends on the following factors that influence the bond-breaking/forming events at the transition state:

(a) The actual mass difference (in terms of reduced mass – μ) due to the isotopic substitution. We have noted that the reduced mass for C-D bond is almost double than that for C–H bond. For other isotopic substitutions, this difference is much smaller and consequently the KIE is smaller. For example, the reduced mass for ^{12}C-^{12}C bond is 6.00 [$\mu_{C-C} = (12 \times 12)/(12 + 12)$] while for ^{12}C–^{13}C bond it is just 6.24 [$\mu_{C-C} = (12 \times 13)/(12 + 13)$]. Therefore a maximum KIE of 1.06 may be observed for ^{12}C–^{13}C bond cleavage. The expected KIEs for other examples are $-k_{12C}/k_{14C} = 1.09–1.15$; $k_{14N}/k_{15N} = 1.04$; and $k_{O16}/k_{O18} = 1.08$.

(b) The force constant – that is how tightly the atom is held. This has a bearing on the bond order (and in turn bond length) associated with that bond.

(c) Whether any other step, other than due to the isotopic substitution, is more rate-limiting. If another step is significant in determining the reaction rate, then the anticipated isotope effect may be either small or not observed at all. Indeed such information can be actually used in elucidating enzyme reaction mechanisms.

27.2 Experimental Approaches to Measure Isotope Effects

It is obvious, from the theory on how the isotope effects are manifest, that very sensitive and accurate methods are required to measure them. Three different experimental approaches may be taken to measure KIEs.

27.2.1 Direct Comparison

A common strategy is to synthesize many different substrate molecules, each with a specific position isotopically labeled. Then the various kinetic constants (V_{max} and V_{max}/K_M) can be measured for the normal as well as the suitably isotope-labeled substrate. These rate constants can be directly compared to note the isotope effects on different kinetic parameters. In the case of lactate dehydrogenase, for example, the hydrogen on the C2 (asymmetric) carbon of lactate (Fig. 27.2a) can be substituted by deuterium.

Using the two kinds of substrates, the deuterium isotope effect on V_{max} and V_{max}/K_M can be directly measured (Fig. 27.2b, below). This approach of direct comparison is excellent as it measures all the required kinetic parameters in one go. However the method suffers in that it requires very high degree of label substitution. Impurities in the labeled substrate affect the measurement of V_{max} effect whereas accurate measurement of substrate concentration is crucial as it influences the KIE on V_{max}/K_M. Apart from the factors listed before, in practice, the extent of isotopic labeling possible at the given position (of substrate structure) also determines the

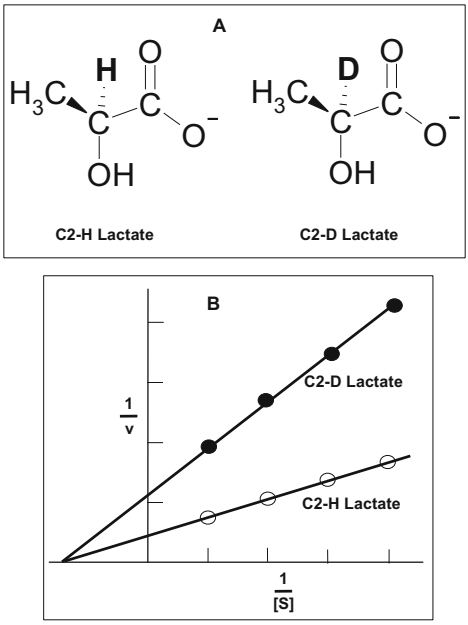


Fig. 27.2 Deuterium isotope effects in lactate dehydrogenase reaction. (**A**) Lactate structure with two different isotopic substitutions on its C2 carbon and (**B**) Schematic double reciprocal plots when these two isotopomers are used separately as lactate dehydrogenase substrates

actually measured effect. 90% label enrichment at the C2–H of lactate (by C2–D) gives only 90% of the maximal KIE. While high degree of isotopic enrichment at one position may be possible with deuterium, this is almost always not feasible for other isotopes.

27.2.2 Equilibrium Perturbation

In this approach, labeled substrate (S^*) and unlabeled product are mixed at a calculated equilibrium ratio in the presence of the enzyme. One observes a temporary displacement of this equilibrium with time as $S^* \rightarrow P$ conversion is slow compared to that from $S \rightarrow P$. The system thus takes time to reach equilibration of the label on both sides; and from this time, transient KIE can be obtained. This method is useful only for reversible reactions. Further, high label substitution in the substrate is required and temperature maintenance is crucial as it affects the equilibrium.

27.2.3 Internal Competition Method

This method exploits the fact that S and S^* (labeled S) compete with each other to form *ES* complex and subsequently for turnover. There are different ways one can set this competition, but we will exemplify this with glucose-6-phosphate dehydrogenase reaction.

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \rightarrow \text{6-Phosphogluconate} + \text{NADH}$$

The natural abundance of ^{13}C at C1 of glucose-6-phosphate is 1.1%. If there is discrimination by the enzyme (say ^{13}C glucose-6-phosphate is slowly converted), then with time the substrate remaining is enriched with ^{13}C at C1 of glucose-6-phosphate; its abundance rises above 1.1%. Simultaneously, the abundance of ^{12}C in the product (6-phosphogluconate) increases. The C1 of 6-phosphogluconate can be quantitatively converted into carbon dioxide by oxidation (enzymatic or chemical method). The corresponding enrichment of ^{12}C in carbon dioxide (actually the ratio of $^{12}C/^{13}C$ in the CO_2 gas) can be directly measured in an isotope ratio mass spectrometer. This protocol can be used to measure the KIE due to ^{13}C at the C1 position, for glucose-6-phosphate dehydrogenase reaction.

Use of an additional *remote label* in the reactant, apart from the atom expected to experience KIE, has made this method much more versatile.

27.3 Applications of KIEs in Enzymology:

The value of KIE studies in enzymology can be better appreciated through specific examples. Rather than giving a mere list of these applications, we shall simply present them as selected case studies. Therefore, in no way such a treatment can be exhaustive but can only be representative. For more details, the reader may wish to look up recent literature on this enzyme frontier (Cleland 2003).

27.3.1 Elucidating Kinetic Mechanism

Enzymes achieve catalytic power by setting up multiple steps, often of comparable energetic barriers, along the reaction coordinate. The isotope-sensitive chemical step may be buried between other rate-limiting enzymatic steps like a rate-limiting product release, a rate-limiting enzyme conformational change, etc. A large magnitude of the observed primary isotope effect on V_{max} is indicative of the fact that the isotopic substitution is part of a major rate-limiting step. A full deuterium isotope effect of 7.0 on V_{max} suggests that bond cleavage to that hydrogen determines the overall rate of the reaction; no isotope effect indicates that some other step is rate-limiting. Often a single step is not rate-limiting and hence deuterium isotope effects on V_{max} are in the range 1.5–2.0; the KIEs are between 2.0 and 4.0 for hydrolytic reactions in water. Physical binding steps are insensitive to isotope effects; hence no isotope effect on the K_M is expected whenever K_M equals K_S. As K_M is a complex of rate constants – effect of isotopic substitution on any one of its contributing rate constants will manifest as a KIE on K_M. As expected, only when the K_M is altered by heavy isotopic substitution the KIEs on V_{max} and V_{max}/K_M are different. From such studies, it should be possible to tease out some of the individual rate constants along the enzyme reaction scheme.

There are a few examples of how KIEs are influenced by the presence of allosteric regulators. The mammalian glutamate dehydrogenase is allosterically activated by ADP and inhibited by GTP. The presence of ADP increases the deuterium isotope effects on V_{max} and V_{max}/K_M from 1.05 to 1.3. AMP nucleosidase is activated by Mg-ATP and more bond order remains to the leaving group (a C1′-N9 bond order of 0.16 versus 0.21), in the presence of this activator.

27.3.2 Deciding Chemical Mechanism

KIEs provide valuable information into the chemical mechanism of the enzyme reaction under consideration. As enzyme chemical mechanisms are covered in detail later (Part IV), only a reference to applications of isotope effects is made here. A

major use of isotope effects is to decide whether the given reaction follows a concerted or stepwise mechanism. We will take two examples to demonstrate this concept.

Malic Enzyme The reaction catalyzed by malic enzyme is as shown:

$$\text{Malate} + \text{NADP}^{+} \rightleftarrows \text{Pyruvate} + \text{CO}_2 + \text{NADPH}$$

Mechanistically, this oxidative decarboxylation of malate involves two bond-breaking events – the cleavage of the C2–H bond with concomitant hydride transfer to $NADP^+$ and the cleavage of C3–C4 bond leading to CO_2 release (see Fig. 27.3 below).

Respective isotopic substitutions, namely, C2–H by C2–D and C3–C4 by C3–^{13}C4, give expected primary isotope effects indicating that both these bond cleavages contribute toward determining the overall rate of this reaction. We may wish to know whether the two bond cleavage events occur simultaneously (concerted) or follow one after the other (sequential). This can be verified by a *double kinetic isotope effect* study. Here we monitor the change in observed ^{13}C KIE due to a deuterium substitution (at C2). With malic enzyme, a ^{13}C KIE (for C3–^{13}C4) of 1.0302 was found. However, when the substrate C2–H was replaced by C2–D, this ^{13}C KIE decreased to 1.0250. This is indicative of the fact that hydride transfer and decarboxylation occur in different steps of the kinetic mechanism. If they were to occur in the same step (i.e., if concerted), then replacement of C2–H by C2–D should have made that step more rate-limiting and increased the size of the observed ^{13}C KIE. As the mechanism is stepwise, the C2–H to C2–D substitution made some other step rate-limiting and hence decreased the size of the observed ^{13}C KIE. In the reaction sequence therefore, the ^{13}C-sensitive step comes later than the deuterium-sensitive step – the hydride transfer occurs first and is followed by decarboxylation (Fig. 27.4).

This means, on the enzyme, malate is first oxidized to oxaloacetate and then decarboxylated! It goes with the same intuition that such double isotope effects are not symmetric – in the reverse direction (reductive carboxylation of pyruvate), ^{13}C-sensitive step arrives before the hydride transfer.

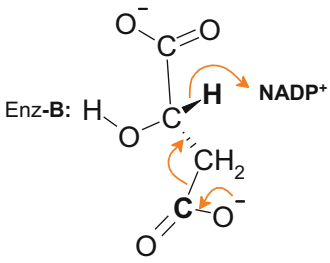


Fig. 27.3 The malic enzyme reaction mechanism where hydride transfer and decarboxylation occur in concert. The atoms of malate where isotopic substitutions are used are shown in bold

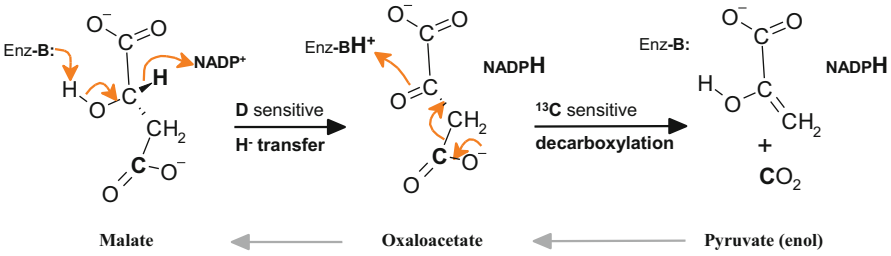


Fig. 27.4 Deuterium- and ^{13}C-sensitive steps in the reaction mechanism of malic enzyme. The dotted arrows indicate the sequence of events in the direction of reverse reaction

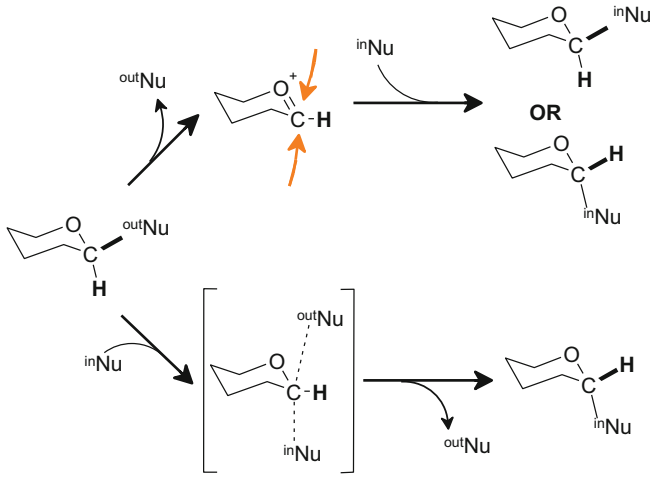


Fig. 27.5 Mechanism of glycosyl transfer: The two possible paths for this reaction are a stepwise mechanism (via an oxycarbonium; upper route) or a S_N2 mechanism (second-order nucleophilic substitution; lower route). The atoms where α-secondary KIE is measured are in bold

Glycosyltransferases Secondary isotope effects are very useful in determining the enzyme chemical mechanism in many cases. Two possibilities exist in the glycosyltransferase chemistry (Fig. 27.5 above). One can visualize a S_N1 reaction (a stepwise mechanism involving first the formation of an oxycarbonium intermediate; the upper path) or a S_N2 reaction (second-order nucleophilic substitution; the lower path). A detailed treatment on nucleophiles and nucleophilicity may be found in a later section (Chap. 31 Nucleophilic Catalysis and Covalent Reaction Intermediates, in Part IV).

In the S_N1 mechanism, because the outgoing nucleophile (^{out}Nu:) leaves first, the C1 carbon of the sugar changes from a tetrahedral (sp^3) arrangement to a flattened sp^2 configuration. This is subsequently attacked by the incoming nucleophile (^{in}Nu:).

During reaction, the hybridization state of glycosidic C1 goes from $sp^3 \rightarrow sp^2 \rightarrow sp^3$. The S_N2 mechanism (as shown) always results in an inversion of configuration at C1 (the Walden inversion). Also the C1 atom simultaneously experiences the effects of incoming and outgoing nucleophiles. Clearly by comparison, the bond order changes around the C1 atom are different for S_N1 and S_N2 mechanisms. This is exploited, through α-secondary isotope effects, to distinguish the two reactions. The α-secondary KIE can be measured by an isotopic substitution in the substrate C1–H (to C1–D). Typically, such an effect for S_N1 reaction (oxycarbonium ion mechanism) is larger (between 1.07 and1.13) than that observed for S_N2 reaction (1.00 ± 0.06). The observed α-secondary isotope effect (a $k_H/k_D = 1.11$) for lysozyme-catalyzed hydrolysis is best accommodated by an oxycarbonium ion mechanism. In this sense, isotope effects serve as a guide in choosing the most likely mechanism.

27.3.3 Understanding Enzyme Transition State

By definition, the transition state (*TS*) is the highest energy point on the lowest energy path between reactants and products. We have seen earlier (Chap. 6 Origins of Enzyme Catalytic Power, in Part I) that one of the major reasons enzymes catalyze reactions is by binding/stabilizing the *TS* in preference to either the substrate or the product. Rate accelerations are attributed to tightness of *TS* binding by an enzyme.

Enzymatic transition states are dynamic entities with lifetimes defying direct physical/experimental observation. Early work on analysis of *TS* and reaction mechanism relied on the introduction of various chemical substituents near/around the reaction center and monitor their effects on the reaction rates. This information was interpreted through linear free energy relationships – like Hammett equation and Bronsted relation – to arrive at mechanistic details about the reaction and its *TS*. Such a chemical approach through systematic use of structural homologues is not suitable for the study of enzyme *TS*. The substrate selectivity/specificity of an enzyme severely limits the number of structural variants that can be employed.

Isotopic substitutions lead to more subtle changes and are eminently suited to probe the active site chemistry. In this background, kinetic isotope effects have the potential to provide direct information on the enzymatic *TS*. However this path has been less traversed by researchers and for very few enzyme reactions. A major problem is that the intrinsic KIE for the chemical step, whose *TS* we wish to understand, may be difficult to access. Enzymes achieve catalytic power by setting up multiple steps, often of comparable energetic barriers, along the reaction coordinate. The isotope-sensitive chemical step may be buried between other rate-limiting enzymatic steps which could be rate-limiting product release, rate-limiting enzyme conformational change, etc. It would therefore be necessary to understand and uncover or account for them in order to obtain relevant intrinsic KIE from the observed KIE. For instance, a high commitment-to-catalysis (Chap. 25) results in underestimation of intrinsic KIE. Over the last three decades, these difficulties are

being surmounted, and it is now possible to approach the *TS* of almost any enzyme with some preparation.

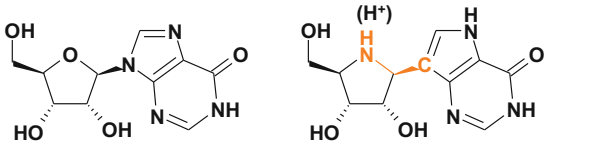
Once the intrinsic KIEs are available for an enzyme reaction, then the *TS* structure can be deduced in the usual physical organic chemistry sense. The experimental steps involved in *TS* analysis through KIEs are as follows.

(a) Synthesize substrates with appropriate isotopic labels at every position around the reaction center.
(b) Accurately measure the KIEs using these substrates. Correct them to get intrinsic values.
(c) A truncated *TS* is computed by fixing bond lengths and bond angles to match the observed KIEs.
(d) From this partial structure, generate the complete *TS* structure by optimization through semiempirical methods (best fit to data by trial and error) and computational enzymology.

In effect, isotopic substitution at different positions in the substrate structure reports (via KIEs) on what actually happens there during reaction. If an atom remains in the same binding environment, both in the substrate and in the *TS*, then there will be no KIE observed. Atoms that become vibrationally less constrained in the *TS* give normal KIE ($k_{light}/k_{heavy} > 1$) with the heavier isotopic substrate reacting more slowly. Conversely, atoms more constrained at the *TS* cause inverse KIE ($k_{light}/k_{heavy} < 1$) with the heavy isotope-labeled substrate reacting more rapidly. A qualitative picture of the *TS* can be constructed based on all such observed KIEs. Taken together, KIEs and computational chemistry provide a conceptually complete picture of the *TS*.

Arriving at the nature of enzyme *TS* complex is challenging, as typically positions of more than 10,000 atoms would have to be determined. A two-pronged approach is needed to decipher the features of enzyme transition states: (a) measuring KIEs (as mentioned above) and (b) computational quantum chemistry. The KIEs give information about the geometry (the shape of the electron cloud surrounding the atoms – van der Waals surface of the *TS*) and the electrostatic charge distribution. The two together lead us to the atomic structure of the *TS*. Computational chemistry is then used to sift through many possible *TS*s to find the one that matches the experimentally observed KIEs. The best fit structure contains the information about both geometry and electrostatic charge – the complete description of the *TS*.

Apart from a clear understanding of the reaction chemistry involved, *TS* analysis has a practical value. Knowledge of the *TS* for an enzymatic reaction provides information to design stable analogs as *TS* inhibitors (Schramm 1998, 2011). A comparison of molecular electrostatic potential surface of the substrate with the *TS* is possible. It may then be feasible to design molecules bearing electrostatic potential surfaces similar to the *TS*. They can be synthesized and tested for their potential for enzyme inhibition. More recently, through the observed KIEs and computational chemistry, *TS*-like structures for several N-ribosyltransferases were defined. For



Inosine (K_M = 15 μM)

9-Deazainosine iminoribitol (K_I = 20 pM)

Fig. 27.6 **Structures of the substrate (inosine) and the synthesized *TS* inhibitor of purine nucleoside phosphorylase**

example, a potent inhibitor of purine nucleoside phosphorylase (9-deazainosine iminoribitol with a K_I of 20 pM) was achieved by this approach (Fig. 27.6).

Drug design using *TS* analysis is in its infancy but has much to promise. This approach differs from the two traditional methods, namely, structure-based drug design and screening of chemical and/or natural product libraries. These two are general in that they can be used against most pharmaceutical targets of interest – including ion channels, receptors, and enzymes. However, the *TS* analysis approach to drug discovery is limited to enzyme targets. But then enzyme catalysis is at the heart of life processes.

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