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Review

Combining solvent isotope effects with substrate isotope effects in mechanistic studies of alcohol and amine oxidation by enzymes☆,☆☆

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ABSTRACT

Oxidation of alcohols and amines is catalyzed by multiple families of flavin- and pyridine nucleotide-dependent enzymes. Measurement of solvent isotope effects provides a unique mechanistic probe of the timing of the cleavage of the OH and NH bonds, necessary information for a complete description of the catalytic mechanism. The inherent ambiguities in interpretation of solvent isotope effects can be significantly decreased if isotope effects arising from isotopically labeled substrates are measured in combination with solvent isotope effects. The application of combined solvent and substrate (mainly deuterium) isotope effects to multiple enzymes is described here to illustrate the range of mechanistic insights that such an approach can provide. This article is part of a Special Issue entitled: Enzyme Transition States from Theory and Experiment.

1. Introduction

Solvent isotope effects, in which one determines the effect(s) on a reaction of replacing water as the solvent with deuterium oxide, can have advantages over other kinds of isotope effects. There is no need to synthesize an isotopically labeled substrate, often the limiting factor in measurement of a kinetic isotope effect; instead one simply makes up solutions in D_2O instead of H_2O. As a result, solvent isotope effects can be simple to measure experimentally. In addition, in the case of exchangeable protons such as those on carbon, oxygen, or sulfur, use of D_2O as solvent is the only way to incorporate the heavier isotope. Conversely, interpretation of solvent isotope effects can be complex. Because all exchangeable protons are replaced in D_2O, multiple protons in the substrate can be replaced. In addition, many protons in the enzyme are exchanged in D_2O; this can have subtle effects on activity and structure. The properties of D_2O as solvent are not identical to those of H_2O; the former is ~24% more viscous than H_2O at 25 °C and 20% more viscous at 37 °C [1,2]. More critically, pK_a values are altered in D_2O and proper controls must be carried out to compensate for this. Despite these complications, when properly measured solvent isotope effects are an indispensable probe of reactions involving solvent-exchangeable protons, such as those in alcohols or amines.

The measurement of isotope effects arising from substrates that are isotopically substituted at non-exchangeable positions can be highly effective in addressing the inherent ambiguities in interpreting solvent isotope effects. The present review focuses on the combination of solvent isotope effects with other isotope effects to probe the mechanisms of amine and alcohol oxidations by enzymes. It is not meant to be a comprehensive review of the mechanisms of enzyme-catalyzed alcohol and amine oxidation or of the application of solvent isotope effects to study enzyme-catalyzed reactions.

2. Measurement of solvent isotope effects

There have been a number of comprehensive treatments of the theory and analysis of solvent isotope effects [3–5], and the reader is directed to those for a more comprehensive treatment. Typically, solvent kies arising from transfer of a single proton from nitrogen or oxygen are in the range 1.5–3, while transfer of a proton from sulfur yields an inverse solvent isotope effect that can be as low as 0.5 [4]. Inverse solvent isotope effects of a similar magnitude have also been associated with low-barrier hydrogen bonds and metal-bound hydroxide or alkoxide [6]. Normal and inverse solvent kies can also arise from the combined effects of a large number of small isotope effects; one possible origin for such effects is a change in the relative amounts of different conformational substates when a protein is transferred to D_2O.

For most enzymes key active-site residues must be properly protonated for tight binding and/or catalysis, so that the activity of most enzymes is sensitive to solution pH. The pK_a values of these residues will exhibit solvent isotope effects, in that the pK_a value will shift in D_2O,

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increasing by 0.3–0.7 [3]. Similar shifts will occur in the pK_a values for buffers. There is also a solvent isotope effect on the glass electrode commonly used to measure pH, so that 0.4 must be added to the pH meter reading to obtain the correct pD for a buffer in D_2O [7]. Because of the likelihood of a shift in the pH dependence of an enzyme in D_2O, measurement of a solvent isotope effects requires that the pH/D dependence of the kinetic parameter(s) of interest, usually k_{cat} and k_{cat}/K_m, be determined in both H_2O and D_2O. One can then determine the solvent isotope effect on the kinetic parameter in a pH/D-insensitive region of the profile.

A key question in interpretation of a solvent isotope effect is knowledge of the number of protons contributing to the observed effect. This is typically addressed by carrying out a proton inventory, in which one determines the solvent isotope effect in mixtures of D_2O and H_2O [5,8]. The data are then fit to a version of the Kresge-Gross-Butler equation (Eq. (1)), which describes changes in the state of the proton as it goes from the reactant (R) state to the transition (T) state. Here, k_0 is the kinetic parameter of interest in H_2O, k_n is the kinetic parameter of interest in a solution containing a mole fraction of D_2O of n, x is the number of protons in the reactant or transition state, ϕ is the respective fractionation factor, and $(Z)^n$ reflects a medium effect. The convention in treating solvent isotope effects has been to describe the properties of the proton in the R and T states in terms of fractionation factors, which are equilibria for isotope exchange reactions. For many reactions, the reactant has the same fractionation factor as solvent, so that the solvent isotope effect can be attributed only to a change in the state of the proton(s) in going to the transition state. The data can then be fit to the much simpler Eq. (2), where the isotope effect (kie) is simply the inverse of the transition state fractionation factor. A reaction in which a single exchangeable proton is in flight in the transition state ($x = 1$) will yield a linear relationship between the observed isotope effect and the fraction of D_2O. If more than one exchangeable proton is involved ($x > 1$), the inventory is bowl-shaped. It can be very difficult to measure the kinetics with sufficient precision to distinguish whether a curved inventory arises from 2 or more than 2 protons. A solvent isotope effect due to a combination of a large number of small effects, referred to as a medium effect, will yield the greatest curvature in the solvent inventory plot. While one could simply use Eq. (1) with a large and arbitrary value of a, such data should instead be fit to Eq. (3). Here Z is a fractionation factor, the inverse of the isotope effect. In some cases, curved proton inventories can occur if the solvent isotope effect being measured is suppressed from the intrinsic value due the presence of another solvent-insensitive step of comparable magnitude, so that there is a change in the commitments [9].

$$k_n = k_0 \frac{\prod_{i=1}^{x}\left(1-n+n\varphi^T\right)}{\prod_{i=1}^{x}\left(1-n+n\varphi^R\right)}(Z)^n \quad (1)$$

$$k_n = k_0(1-n+n/kie)^x \quad (2)$$

$$k_n = k_0(Z)^n \quad (3)$$

3. Utility of multiple isotope effects

A complete description of the structure of the transition state for a reaction requires knowledge of the changes in all of the bonds in the substrate when the transition state is formed. This is seldom done due to the amount of labor involved. However, at a minimum one would want to determine the effect of isotopic substitution of all atoms undergoing bond cleavage or formation. This is simplest if the reaction is concerted, with a single transition state. However, it is not uncommon for more than one step in an enzyme-catalyzed reaction to involve breaking of a bond. An important application of the use of multiple isotope effects is discrimination between concerted and stepwise reactions [10]. If a reaction is concerted, the isotope effect due to substitution of one reacting atom with a heavier isotope will be unaffected or increase if the isotope effect is measured using substrate in which another reacting atom is substituted with a heavier isotope. The former occurs if the isotope effect being measured is the intrinsic value, while the latter occurs if the measured isotope effect is decreased by kinetic complexity. Conversely, if a reaction is stepwise and the two steps exhibit isotope effects arising from substitution of different atoms, the isotope effect measured for one isotope will be smaller when the substrate is also substituted with the other heavier isotope.

4. Isotope effects on alcohol oxidation

4.1. Flavoenzymes

The application of isotope effects to study the mechanisms of flavoproteins that catalyze oxidation of alcohols or amines is often simplified due to their steady-state kinetic mechanisms. Typically, oxidation of the substrate and concomitant reduction of the flavin occur in the absence of any interaction with the second substrate; in addition, this reductive half-reaction is often irreversible. The subsequent oxidation of the reduced flavin then occurs by electron transfer to oxygen to form hydrogen peroxide in the oxidases or transfer to another redox cofactor in the dehydrogenases. A result of an irreversible reductive half-reaction is that the k_{cat}/K_m value of the alcohol or amine substrate is independent of the concentration of the oxidizing substrate. The separate reductive and oxidative half-reactions and the sensitivity of the flavin visible absorbance spectrum to redox state also makes these enzymes very amenable to single turnover methods, in that the reductive half-reaction can usually be analyzed in the absence of the oxidizing substrate, allowing the rate constant for flavin reduction by the substrate to be measured directly.

A number of flavoproteins that catalyze the oxidation of alcohols to aldehydes belong to the GMC oxidoreductase family. These enzymes have a common fold that was first identified from the sequences of glucose dehydrogenase, glucose oxidase, methanol oxidase, and choline dehydrogenase [11] and subsequently confirmed by three-dimensional structures [12]. Methanol oxidase (also known as alcohol oxidase) appears to be the first GMC oxidoreductase for which solvent isotope effects were used as a mechanistic probe. Sherry and Abeles reported that the enzyme from *Hansenula polymorpha* was inactivated by cyclopropanol, and that the inactivated enzyme contained an adduct of the flavin with a ring-opened form of the inhibitor [13]. Two mechanisms were considered for the inactivation (Fig. 1). In both the reaction is initiated by abstraction of the hydroxyl proton by an active-site base. In path i opening of the cyclopropyl ring in the alkoxide generates a transient carbanion that attacks the flavin. In path ii an electron is transferred from the alkoxide to the flavin to generate the cyclopropoxy and flavin radicals. Rapid opening of the ring in the former to yield a 3-propanol radical would be followed by recombination of the two radicals to form the adduct. The radical mechanism was favored because no adduct was detected when the native FAD was replaced with 5-deazaFAD as the cofactor. This synthetic flavin is generally considered incompetent in radical reactions [14].

The mechanism of methanol oxidase was re-examined by Menon et al. [15] using isotope effects on the oxidation of 2-substituted ethanols and p-substituted benzyl alcohols. The k_{cat}/K_m values for substituted benzyl alcohols showed a good correlation with the σ-value of the substrate yielding a ρ value of 1.9, consistent with an electron-rich transition state as in alkoxide formation. With benzyl alcohol as substrate, the $^{D}(k_{cat}/K_m)$ value was 1.2 and the $^{D2O}(k_{cat}/K_m)$ value 2.0, consistent with little change in the CH bond during cleavage of the OH bond. The solvent inventory with benzyl alcohol was linear, consistent with a