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The Power of Integrating Kinetic Isotope Effects into the Formalism of the Michaelis-Menten Equation

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Abstract

The final arbiter of enzyme mechanism is the ability to establish and test a kinetic mechanism. Isotope effects play a major role in expanding the scope and insight derived from the Michaelis-Menten equation. The integration of isotope effects into the formalism of the Michaelis-Menten equation began in the 1970s and has continued to this day. This review discusses a family of eukaryotic copper proteins that includes dopamine β -monooxygenase, tyramine β -monooxygenase, and peptidylglycine α -amidating enzyme, responsible for the synthesis of the neuro-active compounds, norepinephrine, octopamine and C-terminally carboxamidated peptides, respectively. Highlighted are results that show how combining kinetic isotope effects with initial rate parameters permits an evaluation of: (i) the order of substrate binding to multi-substrate enzymes; (ii) the magnitude of individual rate constants in complex, multi-step reactions; (iii) the identification of chemical intermediates; and (iv) the role of non-classical (tunneling) behavior in C–H activation.

Keywords

Steady-state kinetics; kinetic isotope effects; mechanism of enzyme action; mechanism of two copper monooxygenases; enzymatic C–H activation

Introduction

Although it may seem as if the Michaelis-Menten equation (M-M) (1) has been with us forever, a mere one hundred years mark the beginning of a kinetic context for nature's catalysts. This brief article is primarily a tribute to the M-M kinetic analysis of enzyme reactions, and its considerable elaborations. The widespread role of remote protein interactions in catalysis, as encapsulated within the Monod-Changeux-Jacob description of allostery (2), is also discussed briefly.

Prior to the 1960s, the primary kinetic determinants for an enzyme reaction were considered to be the two limiting parameters, k_{cat} and K_{m} . At that early stage, K_{m} was often assumed to be a K_{d} for substrate binding and the formalism was restricted to enzymes that act on single substrates (1). Cleland's classic articles, published in the 1960s and 1970s, further extended kinetic analyses to [the majority of] enzymes that work on more than a single substrate (3, 4). This period also saw a major shift away from a focus on k_{cat} and K_{m} to the recognition

that the two primary kinetic parameters in enzyme reactions are k_{cat} [for reaction of the enzyme substrate complex] and k_{cat}/K_m [for reaction of free enzyme and substrate] (5). And, there is the corollary, that K_m *cannot be assumed* to be a dissociation constant (6).

Kinetic isotope effects [KIEs], which had played a role in the evolution of the field of physical organic chemistry, began to be applied “in force” to the field of enzymology during the 1970s (7, 8). While the measurement of the impact of isotopic substitution on initial rate parameters would seem, on the surface, to simply double the amount of experimental information, the impact of KIEs on our understanding of enzyme function has gone far beyond this expectation [a consequence, in part, of the distinctive properties of k_{cat} versus k_{cat}/K_m for complex, multi-step enzyme reactions]. A partial list of the information that can be gleaned from measurements of KIEs in combination with initial rate parameters is listed below:

- i. The determination of the order in which multiple substrates bind to an enzyme in the course of forming the productive catalytic complex;
- ii. The identification of rate-limiting steps, together with the extraction of rate constants for individual steps;
- iii. The ability to infer the structure of chemical intermediates and activated complexes; and
- iv. The detection of a role for barrier penetration, referred to as tunneling, in enzymatic C–H activation.

For the purposes of illustrating the enormous mechanistic insights that can come from combining isotope effects with initial rate kinetics, the properties of a single family of copper-containing, eukaryotic enzymes will be the focus of this review. This family is comprised of dopamine β -monooxygenase [D β M] (9), tyramine β -monooxygenase [T β M] (10, 11), and peptidylglycine α -hydroxylating monooxygenase [PHM] (12). As illustrated in eq [1], each enzyme generates an important cell-signaling molecule via an ascorbate and oxygen-dependent process:

where R is a substituted phenyl group, and

[1]

where R is an extended peptide. Ascorbate [Asc] serves as an exogenous two-proton, two-electron donor, being converted to dehydroascorbate [DHAsc], and oxygen undergoes a net four-electron reduction to yield hydroxylated product and water. The organic products of D β M and T β M are norepinephrine and tyramine, respectively, whereas PHM leads to the precursor of C-terminally carboxamidated neuropeptides.

Multiple X-ray structures have been obtained for PHM, and one such structure, solved in the presence of a substrate analog, is reproduced in Figure 1 (13, 14). From the extensive structural and kinetic studies on all three enzymes, it is now accepted that substrate and O₂ bind at the Cu_M center and that Cu_H, ca. 10 Å away, stores the second electron needed for completion of the reaction (15). A major goal has been to understand how the controlled oxygenation reactions of eq [1] can occur at a fully exposed solvent interface.

Note that the reactions shown in eq [1] all occur at a methylene carbon, introducing the possibility of measuring both a primary deuterium KIE for the bond cleaved and a secondary deuterium KIE for the hydrogen left behind on the reactive carbon:

[2]

Since these reactions involve the uptake of molecular oxygen, there is the additional possibility of examining the discrimination between oxygen-16 and oxygen-18 during the activation of O₂ and its subsequent insertion into the carbon bond of substrate. Both hydrogen and oxygen KIEs have been applied to DβM (16–18), PHM (19, 20) and, most recently, TβM (21, 22), providing significant insight into features [i] – [iv] outlined above.

[i] The determination of the order in which multiple substrates bind to an enzyme in the course of forming the productive complexes

Early investigations of this family of proteins were almost exclusively focused on DβM, in large part because of the recognized central importance of dopamine and norepinephrine in human physiology. Using commercially available dopamine that was labeled with trace amounts of tritium at the reactive C-2 position and an O₂ electrode to control the level of O₂ in the reaction vessel, a series of primary tritium KIE measurements were initiated in this laboratory (23). Two key features defined the experimental system. First, the use of trace labeling with tritium ensured that the measurements would be restricted to k_{cat}/K_m [cf. ref. (24)]. Second, since the parameter k_{cat}/K_m reflects all steps from substrate binding up through the first irreversible step leading to product which, in this case, is the isotopically sensitive C–H bond cleavage step, the measurements were expected to be independent of all slow steps subsequent to the C–H bond cleavage step, eq [3]:

[3]

Experimentally, the data showed a tritium KIE that is dependent on the concentration of O₂ undergoing a regular progression from ca. 10 in the limit of very low O₂ concentration

toward 4 in the limit of saturating O_2 concentration, Figure 2. There is nothing inherent in the simple equation for the tritium isotope effect, $T(k_{cat}/K_m)$, for the reaction of dopamine with D β M that can explain this observation, illustrated by eq [4] which shows that the observed KIE is a reflection of the intrinsic KIE on the C–H bond cleavage step, $Tk_{(C-H)}$, moderated by the commitment to catalysis represented by $k_{(C-H)}/k_{(off,S)}$:

$$(k_{cat}/K_m)T = \frac{k_{(C-H)}T + \frac{k_{(C-H)}}{k_{(off,S)}}}{1 + \frac{k_{(C-H)}}{k_{(off,S)}}} \quad [4]$$

However, the multi-substrate nature of the D β M reaction provides an explanation for the data. Up to the time of these studies, it had generally been assumed that substrate-binding would be a prerequisite for the binding and activation of O_2 , not only in the case of D β M but for all oxygenases. A binding of O_2 to D β M subsequent to formation of the enzyme-dopamine complex is, in fact, the reason for the decreasing value of the KIE as $[O_2]$ is increased. As $k_{(on,O_2)}$ becomes very large in relation to $k_{(off,O_2)}$, eq [5]:

[5]

the O_2 binding step becomes the first irreversible step, and the KIE is predicted to go to unity as $k_{cat}/K_m(S)$ becomes independent of the C–H bond cleavage step. Yet, Figure 2 shows that the KIE does not approach unity, plateauing at a value of 4. The full explanation for the behavior in Figure 2 is the existence of a productive “escape hatch” for loss of substrate prior to the dissociation of O_2 , thereby demonstrating a random binding mechanism for the addition of dopamine and O_2 to D β M [random bi-bi, using the nomenclature of Cleland], eq [6]:

[6]

We now know that some enzymes can actually activate O_2 in the *complete* absence of substrate [e.g., ref. (25)]. Subsequent studies of other non O_2 -dependent enzymes have made use of the dependence of the KIE for substrate 1 as a function of the concentration of substrate 2 to determine the order in which substrate binding leads to a productive ternary complex, [cf. ref. (26)].

[ii] The identification of rate-limiting steps, together with the extraction of rate constants for individual steps

The value of the experimental tritium KIE in the limit as O₂ concentration approaches zero [Figure 2] suggested the possibility of a very large KIE for the C–H bond cleavage step. In 1975, Dexter Northrop had introduced an elegant method for extracting the intrinsic KIE on a single chemical step free from the inherent kinetic complexity of enzyme reactions (27). Following his lead, the intrinsic deuterium KIE for DβM was shown to be 12 (16), with later studies indicating an almost identical intrinsic KIE in the case of PHM (19). Such an elevated primary KIE, in excess of the semi-classical limit of 7 (28), provided an early hint of the importance of quantum mechanical tunneling in C–H activating enzymes [see section [iv] below].

Although, the multi-step nature of enzyme reactions presents a serious impediment to extracting mechanistic information on single chemical steps, this limitation can be significantly alleviated once the value of the intrinsic KIE is known. As illustrated by eq [4] above, the comparison of an observed to an intrinsic KIE can tell us the value of the commitment to catalysis, i.e., the relative kinetic importance of C–H bond cleavage vs. loss of substrate from enzyme. Ideally, individual rate constants, not simply ratios of rate constants, would be available, and this becomes possible for the chemical reactions catalyzed by DβM, PHM, and TβM, in which the C–H abstraction is irreversible. As outlined in a series of kinetics papers (17, 29, 30), knowledge of the intrinsic deuterium KIE, $^Dk_{(C-H)}$, together with the deuterium KIE on k_{cat} , $^D(k_{cat})$, provides sufficient enough information to calculate the value for $k_{(C-H)}$ according to eq [7]:

$$k_{(C-H)} = \frac{k_{cat} \left(^D(k_{(C-H)}) - 1 \right)}{^D(k_{cat}) - 1} \quad [7]$$

The only limitation of the application of eq [7] is that $^D(k_{cat})$ must be statistically larger than unity. Using eq [7], the value of $k_{(C-H)}$ can be calculated as a function of substrate structure [providing insight into the impact of changing substrate on chemical reactivity], pH [providing insight into pK_a values that may control a single step, rather than the more complex steady-state kinetic pK_as that can be shifted either up or down by the presence of multiple rate-limiting steps], temperature [providing the enthalpic barrier for an isolated chemical step], and mutation [indicating the role of specific side chains in facilitating the C–H bond cleavage step]. Each of these features has seen application to DβM, PHM, and TβM, as well with mutants thereof [e.g., refs. (17, 22, 31, 32)]. Inspection of eq [4] and eq [7] shows that the amount of available information need not stop at understanding the chemical step: once $k_{(C-H)}$ is available, it then becomes possible to calculate, for example, the off rate constant for dissociation of substrate and so on [cf. ref. (22) for an illustration of how isotope effects were used to calculate the rate constant for long range electron transfer between the two copper sites of a mutant form of TβM]. Hopefully, at this juncture, the reader can begin to see just how expansive the M-M equation becomes, when linked to measurements of steady-state isotope effects.

[iii] The ability to infer the structure of chemical intermediates and activated complexes

One of the very surprising features of the enzyme family comprised of D β M, PHM and T β M is the repeated observation that product formation is completely coupled to the uptake of O₂ (33, 34). In direct contrast to many other oxygenases, which often undergo an uncoupling that leads to O₂ reduction in the absence of substrate hydroxylation (35, 36), all efforts to uncouple O₂ uptake from product formation in D β M and PHM have failed. For example, in the case of D β M, using a para-trifluoromethylphenethylamine substrate that undergoes very slow C–H activation, at a 10³-fold reduction relative to dopamine, the ratio for O₂ uptake to product formation remains at unity (33). Similarly, mutagenesis of PHM at one of the conserved ligands at the Cu_H site leads to a ca. 10³-fold reduction in the rate of C–H activation with no evidence for an increase in O₂ uptake in relation to product formation (34). These findings are particularly surprising in the context of the large degree of exposure of the enzyme active site to solvent water [cf. Figure 1]. The aggregate data suggest that the O₂-derived species responsible for C–H abstraction may be quite distinct from what is observed in other metallo-enzymes.

Over the years, this laboratory has explored the measurement of oxygen-18 KIEs for O₂-consuming enzymes (18, 37, 38), hoping to discern patterns that are mechanistically informative (39). These measurements are fairly generic, involving the isolation of unreacted O₂, its quantitative conversion to CO₂, and examination of the ratio of O-16 to O-18 in the resulting CO₂ by isotope ratio mass spectrometry (40). While the absolute magnitude of the resulting KIE can be difficult to interpret, trends are very informative. The O-18 KIEs measured for D β M and PHM using substrates of similar reactivity are summarized in Table 1. The most interesting and mechanistically informative aspect of these data is their dependence on substrate deuteration at the reactive carbon (18). Analogous to the tritium KIEs described in section [i] above, these O-18 measurements are carried out using gas at natural isotopic abundance, i.e. containing levels of O-18 that are low enough for the isotope to be considered a tracer. As a result, the KIEs are competitive measurements on $k_{\text{cat}}/K_{\text{m}}$ and, thereby, restricted to steps that involve O₂ binding up through the first irreversible step. In the majority of monooxygenases, it is generally expected that O₂ will undergo an irreversible reductive cleavage of O₂ to a highly reactive metal-oxo [cf. ref. (41)] or via a two-electron reduction of O₂ to a peroxide intermediate [cf. ref. (15)].

In the case of D β M and PHM, the combined observations of isotope effects on isotope effects [deuterium effects on O-18 KIEs], and the full coupling of O₂ uptake to product formation, under conditions designed to lead to uncoupling, has effectively ruled out an irreversible activation of O₂ prior to C–H activation (15). The final mechanism proposed, summarized in Scheme 1, involves the reversible formation of a superoxo-species as the substrate-hydroxylating agent. Such an O₂-derived species is well supported by DFT calculations for this class of enzyme (42). Recent observations on iron-supported hydroxylations at C–H bonds with relatively low bond dissociation energies [less than or equal to ca. 80 kcal/mol] have also led to the proposal of ferric-superoxo as the active hydroxylating species (43).

[iv] The detection of a role for barrier penetration, referred to as tunneling, in enzymatic C–H activation

One of the more prominent features of enzyme function to emerge from studies of KIEs is the role for non-classical behavior in enzymatic C–H bond activation. The implication of such behavior began many decades ago with findings of secondary KIEs that either exceeded their semi-classical limits (44) or by virtue of their size implicated a structure for the activated complex that was opposite from that inferred from Hammett structure function correlations (45). These deviant behaviors became a subject of increasing debate during the 1980s and led to an early model (46) that incorporated H-tunneling at the primary position of substrate that was coupled into the non-transferred secondary position [cf. eq [2]]. In the intervening years a role for room temperature tunneling has been implicated in virtually every enzymatic system studied, independent of the family class or nature of C–H activation, i.e., as a hydrogen atom, proton or hydride ion [cf. refs. (47–52) for illustrative references].

As noted in section [ii] above, the large size of the intrinsic KIE in the D β M reaction had suggested that tunneling was contributing to the reaction coordinate for hydrogen transfer. While the size of $D_{k_{\text{C-H}}}$ could be rationalized by the incorporation of a tunneling correction to a semi-classical treatment of rates and KIEs, a later study of PHM showed that a different type of tunneling was likely at play in enzymes. Following the lead with D β M, Francisco and co-workers proceeded to measure the value of the intrinsic KIE, $D_{k_{\text{C-H}}}$, for PHM as a function of temperature (32), and the results are shown in Figure 3. The data indicate an extremely small temperature dependence for $D_{k_{\text{C-H}}}$ that contrasts with either semi-classical predictions or the expected behavior for a tunneling correction to semi-classical transition state theory (53). These results cannot be attributed to kinetic complexity, since the KIEs shown are computed for a single C–H bond cleavage step (32).

The data for PHM were not the first to show a temperature-independent or weakly temperature-dependent KIE [cf. refs. (51, 54)], and since that time this property has emerged as a dominant feature in native enzyme-catalyzed C–H activation processes [cf. refs. (55, 56) for illustrative references]. Once again, a lively debate has ensued about the origins of such behavior, with a physical picture emerging in which the transferred hydrogen moves as a wave from the its donor to acceptor site in a temperature-independent manner. According to this model, the thermal activation barrier comes from the heavy atoms of the protein/solvent and substrate. These heavy atom motions tune the relative energies for reactant and product into a range that can support efficient wave function overlap [Figure 4, panels (a) and (b)]. This multi-dimensional treatment for tunneling also has the capability to explain weak or absent temperature dependencies for KIEs that can segue into highly temperature-dependent KIEs when the enzyme system is perturbed (51, 55–57). The temperature dependence of the KIE emerges when the initial distance between the H-donor and acceptor is altered beyond the range compatible with efficient tunneling, requiring a sampling of different donor-acceptor distances that depends on both temperature and the mass of the transferred atom [Figure 4, panel (c)]. The motions that facilitate the behavior in Figure 4 are expected to be fairly local to the reacting bonds and on time scales $\sim$ ns. A second class

of protein motions has also been implicated in the C–H activation catalyzed by optimized native enzymes, as a prerequisite to generate the very close H-donor/acceptor distances that are the hallmark of temperature-independent KIEs (55–57). These are expected to occur throughout the entire protein and involve a wide range of time scales (ms–fs) (cf. ref. 58). The more global motions will be independent of isotope labeling of the substrate but may be altered by the incorporation of heavy atoms into the protein (59).

The importance of protein motions that has emerged from studies of enzymatic C–H activation is likely to be applicable to all classes of enzyme reactions. The sampling of a wide range of protein conformational substates, to achieve the family of substates capable of supporting very rapid catalytic rates, should be a phenomenon that is independent of the nature of the transferred group (58–60). Such a model for enzyme catalysis can rationalize the often large impact of distal mutations on catalytic turnover numbers that show little or no accompanying changes in static X-ray structures. In the context of allostery, the concept of sampling among large numbers of conformational substates has also emerged as an important area of research [e.g., refs. (61–64)], expanding the traditional focus on the interconversion between protein states with highly different (static) structures (2) toward the impact of a modifier ligand on the responsive protein's overall conformational landscape.

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Abbreviations

KIE	kinetic isotope effect
DβM	dopamine β-monooxygenase
TβM	tyramine β-monooxygenase
PHM	peptidylglycine alpha-hydroxylating monooxygenase
M-M	Michaelis-Menten

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Figure 1.

X-ray structure of oxidized PHM. Coppers at the Cu_M and Cu_H sites are in the +2 oxidation state. The substrate analog, Ac-DiI-YG [acetyl-diiodo-Tyr-Gly] is in stick form. The conserved ligands at Cu_M [H242, H244, and Met314] and at Cu_H [H107, H108, and H172] are in green. The conserved tyrosines are yellow [Y318 and Y79] (13, 14). Reprinted with permission from the Journal of the American Chemical Society, Vol. 126, pp. 13168–13169. © 2004 American Chemical Society.

Figure 2.

The size of the tritium isotope as a function of O₂ levels in the DβM reaction. The y axis, $V/K_H/V/K_T$ is $T(k_{cat}/K_m)$ in eq [4] of the text (23). Reprinted with permission from the Journal of Biological Chemistry, Vol. 255, pp. 11648–11651. ©1980 The American Society for Biochemistry and Molecular Biology.

Figure 3.

Temperature dependence of the intrinsic deuterium isotope effect on $k_{(C-H)}$ for PHM (32). Reprinted with permission from the Journal of the American Chemical Society, Vol. 124, pp. 8194–8195. © 2002 American Chemical Society.

Figure 4.

Marcus-like model of H-tunneling (53). The position of the heavy atoms is shown in (a) and the position of the H-atom is in (b). The movement of the heavy atoms generates a tunneling-ready state that allows the H-wave function to be delocalized between the reactant and product wells [middle graphs in (a) and (b)]. When the donor-acceptor distance is non-optimal, a mass- and temperature-dependent sampling of the donor-acceptor distance enters into the reaction coordinate. This is illustrated in (c), and represents the origin of the temperature-dependent KIEs. Reprinted with permission from the Annual Reviews of Biochemistry, Vol. 82. © 2013 Annual Reviews.

Scheme 1.

The C–H activation step of D β M, T β M, and PHM is proposed to occur via a hydrogen atom transfer to an adjacent $\text{Cu}_M(\text{O}_2^{\cdot-})$. Though the latter has never been experimentally detected, all mechanistic probes converge on the illustrated, “consensus” mechanism (15).

Table 1 Observed k_{16}/k_{18} for O₂ activation.

Enzyme	Bond cleaved	
DβM ^a	1.0197 (0.0003)	1.0256 (0.0003)
PHM ^b	1.0167 (0.0032)	1.0216 (0.0014)

^a Substrate for DβM is dopamine: $k(\text{C-H}) = 1200 \text{ s}^{-1}$; BDE = 85 kcal/mol (18).^b Substrate for PHM is hippuric acid: $k(\text{C-H}) = 810 \text{ s}^{-1}$; BDE = 87 kcal/mol (20).