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The enigmatic conservation of enzyme dynamics in evolution☆

Amnon Kohen

Department of Chemistry, University of Iowa, Iowa City, IA 52242, USA

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Summary Examination of the chemical step catalysed by dihydrofolate reductase (DHFR) suggested preservation of an ''ideal'' transition state as the enzyme evolves from bacteria to human. This observation is enigmatic: since evolutionary pressure is most effective on enzymes' second order rate constant (k_{cat}/K_M) and since the chemistry is not rate limiting on that kinetic parameter, why is the nature of the chemical step preserved? Studies addressing this question were presented in the 2015 Beilstein ESCEC Symposium and are summarized below.

Introduction

It has been observed that most enzymes that catalyse various H-transfer reactions present temperature-independent intrinsic isotope effects (KIEs). This observation seems to hold for well-evolved enzymes catalyzing reactions of their natural substrates under physiological conditions. Unnatural mutants, substrates, or non-physiological conditions often lead to temperature dependent KIEs. A physical interpretation suggests that temperature-independent intrinsic KIEs result from well-reorganized and narrowly distributed ensembles of transition states (TSs). Temperature-dependent KIEs, on the other hand, indicate a poorly reorganized TS with a broad distribution of states and is commonly associated with slower reaction rates.

The enzyme dihydrofolate reductase (DHFR) catalyzes a C—H→C hydride transfer and is found to have a well-reorganized TS across evolution from bacteria to human. A humanized bacterial enzyme only yields temperature-independent KIEs when the mutations are introduced in

Abbreviations: DHFR, dihydrofolate reductase; ecDHFR, *Escherichia coli* DHFR; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP+, oxidized nicotinamide adenine dinucleotide phosphate; DHF, 7,8-dihydrofolate; THF, 5,6,7,8-tetrahydrofolate; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(morpholino) ethanesulfonic acid; MTEN buffer, 50 mM MES, 25 mM Tris, 25 mM ethanolamine, and 100 mM sodium chloride; KIE, kinetic isotope effect; KIE_{int}, intrinsic KIE; KIE_{obs}, observed KIE; fs, femtosecond; ps, picosecond; QM, quantum mechanics; MM, molecular mechanics; MD, molecular dynamics; TS, transition state; GS, ground state.

E-mail address: amnon-kohen@uiowa.edu

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the same order as they appeared during evolution. However, mutations introduced in a different order lead to temperature-dependent KIEs. This observation is enigmatic because the chemical step is far from being rate limiting for most enzymes, including DHFR. If it is not rate limiting, why is there such a strong evolutionary pressure to maintain a well-reorganized TS?

In an attempt to resolve this enigma, current studies using accelerated evolution of primitive DHFR towards a mature enzyme are attempting to address the following questions: At what point during evolution does it stop being rate limiting (as it is in solution or the primitive enzyme)? When does the temperature dependence of the KIEs decrease, and when does it become temperature-independent? What are the relationships between the catalytic rate constants and the intrinsic KIEs?

The comprehension of evolution at the molecular level is quite challenging. In addition to common evolutionary issues like adaptation to new environmental conditions or means of selection at the phenotype level, the evolution of enzymes must address the question of which step in the catalytic cascade is rate limiting on rate constants that are relevant to evolution. The relation between measured rate constants and the rate of the chemical step is not trivial. Because several steps along the catalytic turnover are slower than the rate of the chemical step (bond activation, cleavage, or formation) in most enzymes, studies of measurable rate constants do not reflect effects on that step (Fig. 1).

One method that partly exposes effects on the chemical step is measuring substrate kinetic isotope effects (KIEs), where the bond to be cleaved is substituted with a heavier isotope. This needs to be done in a way that will not affect substrate binding or product release but will have a substantial effect on the chemical step. Below, I will discuss KIE measurements of C—H bond cleavage or, more specifically, enzyme-catalysed C—H→C hydride transfer reactions. For enzymes, KIEs can be measured on many different rate constants, e.g., the first order rate constant for steady state conditions, k_{cat}, the second order rate constant k_{cat}/K_M, or pre-steady state rates (via single turnover or burst experiments). However, measurements of a KIE on any measurable rate constant only yield an observed KIE (KIE_{obs}) rather than the intrinsic KIE (KIE_{int}, i.e., KIE on the chemical step per se). The general form of the relation between KIE_{obs} and KIE_{int} is presented in Eq. (1):

$$KIE_{obs} = \frac{KIE_{int} + C}{1 + C} \quad (1)$$

where C is the commitment to catalysis, and is the ratio between the isotopically sensitive rate forward or backward and the non-isotopically sensitive steps in the opposite direction (e.g., $C = k_{chemistry}/k_{dissociation\ of\ substrate}$ for a simple $E + S \rightleftarrows ES \rightarrow E + P$ process). Assessment of the KIE_{int} is critical for any attempt to use KIEs to elucidate properties of the chemical step or to compare measured KIEs to their computed values (most computations only examine the chemical step per se). Several methods for assessing KIE_{int} were discussed during the Beilstein ESCEC Symposium, and the one presented in the example below is based on the measurements of both KIE_{obs} for H/T KIEs (ratio of rate between protium and tritium) and D/T KIEs (ratio of rates between deuterium and tritium). Since T is the common isotope in

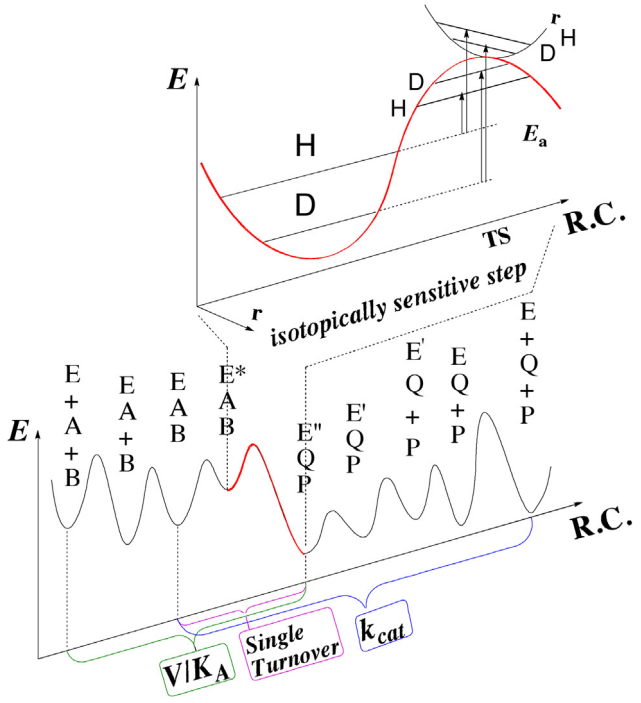

Figure 1 A schematic energy diagram of an enzyme (E)-catalysed reaction of reactants A and B to products Q and P. The chemical step is highlighted in red. The steps that are included in various rate constants (V/K or k_{cat}/K_M in green, k_{cat} in blue, and single turnover in magenta) are marked along the reaction coordinate (R.C.). A zoom into the chemical step is presented at the top with the zero point energy (ZPE) for light (H) or heavy (D) isotopes of hydrogen marked as well as the energies of activation (E_a) for the reaction with and without ''under the barrier'' QM tunneling. The take home message from this figure is that in the forward direction, barriers other than the chemical one are higher for all the measurable rate constants; thus, in this example the chemistry is not rate limiting for any of these measurable parameters.

both measurements, the commitment (C) for that isotope can be removed using the Northrop method and KIE_{int} can be assessed (Kohen, 2005; Liu et al., 2014; Sen et al., 2011).

Once KIE_{int} is determined, its temperature dependence can serve as a probe for the nature of the chemical step or, more specifically, how well-reorganized the reaction's TS is (Kohen, 2015). In short, the less temperature-dependent is the KIE_{int}, the better organized is the TS (also known as tunneling ready state, TRS, for reactions that involve QM tunneling) (Kohen, 2015). Fig. 2 graphically illustrates a model (addressed here as the Activated Tunneling Model) that encapsulates the effects of temperature on rates vs. its effect on KIEs.

The model proposed above can explain the temperature dependence of KIE_{int} whether the rates are temperature-dependent or not. The temperature dependence of the rates is mostly reflecting the pre- and re-organization of the whole system towards the tunneling-ready-state (TRS), i.e., columns A or A′ in Fig. 2. This process has little impact on the C—H bond to be cleaved and thus, involves no KIE. Since this process involves the motion of many atoms (the whole protein, solvent, reactants, etc.), little or no change in these