TECHNICAL COMMENT

BIOPHYSICS

# Comment on "Extreme electric fields power catalysis in the active site of ketosteroid isomerase"

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**Fried *et al*. (Reports, 19 December 2014, p. 1510) demonstrated a strong correlation between reaction rate and the carbonyl stretching frequency of a product analog bound to ketosteroid isomerase oxyanion hole mutants and concluded that the active-site electric field provides 70% of catalysis. Alternative comparisons suggest a smaller contribution, relative to the corresponding solution reaction, and highlight the importance of atomic-level descriptions.**

We were excited to see the data of Fried *et al.* (*1*) demonstrating a strong correlation between reaction rate and the stretching frequency of the carbonyl group of a product analog bound to a series of ketosteroid isomerase (KSI) oxyanion hole mutants. These data were interpreted in terms of a model, calibrated using vibrational data and molecular dynamics simulations in a series of solvents, which led to the conclusion that the active-site electric field generated by the oxyanion hole and surrounding groups accounts for $10^5$-fold rate enhancement and 70% of the observed catalysis. Based on these findings, it was suggested that electrostatic forces are the dominant contributor to catalysis.

Below, we note that the conclusion of a dominant contribution to KSI catalysis relies on comparison to a hypothetical enzyme that provides zero electric field at the position of the carbonyl group and would not hold for a comparison to the corresponding reaction in aqueous solution. The accompanying analysis leading to an estimate of the rate advantage from positioning of KSI's general base is similarly affected. Finally, we note that electrostatic stabilization requires and is linked to positioning of the groups responsible for that stabilization.

Conservative mutations, such as the Tyr[16]Phe mutation in the construct employed by Fried *et al*., often transmute a polar group to a nonpolar group and generate an apolar or hydrophobic environment that is less favorable toward charge accumulation than the polar environment in aqueous solution, and are inhibitory as a result (*2*). Thus, a "conservative" mutation of the dominant

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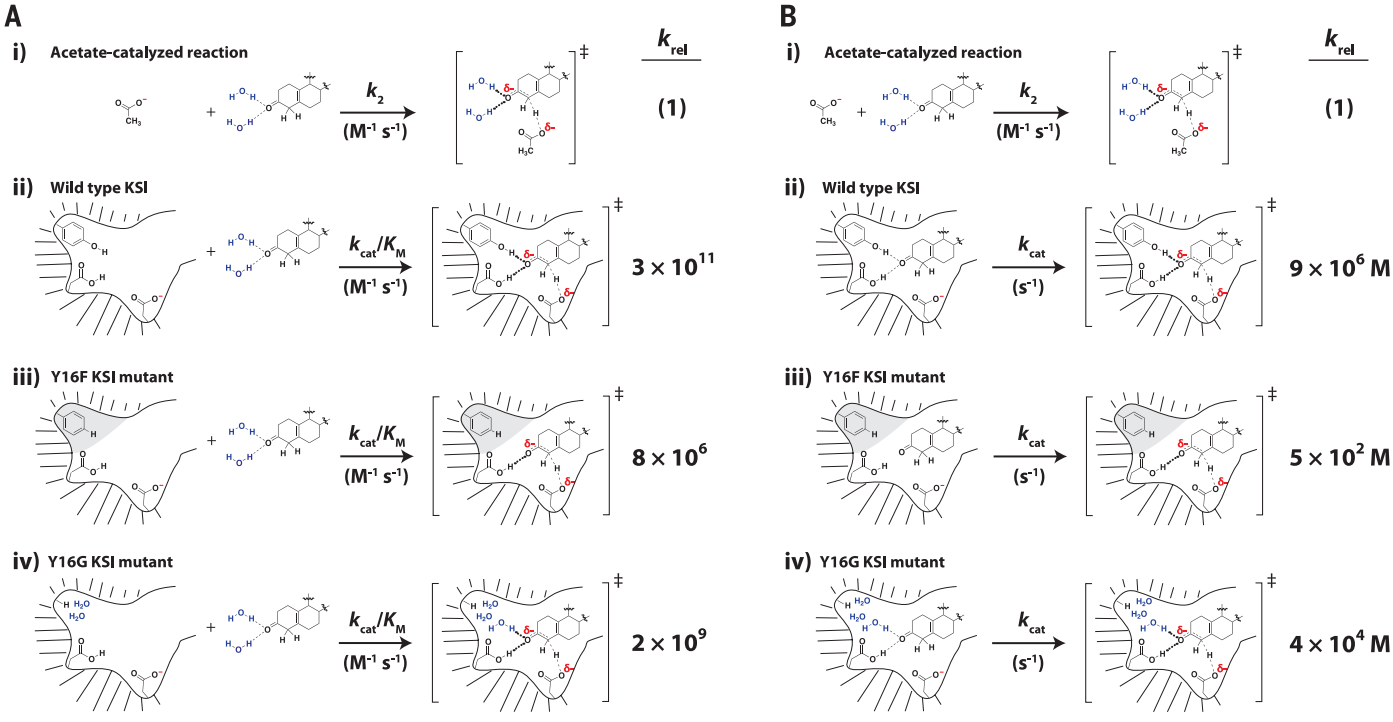


**Fig. 1. Rate enhancement provided by different KSI variants relative to the rate of the acetate-catalyzed reaction.** Rate enhancement provided by different KSI variants relative to the rate of the acetate-catalyzed reaction under (**A**) subsaturating and (**B**) saturating conditions. (i) Reaction between substrate and acetate in aqueous solution. (ii) Reaction between substrate and wild-type KSI. (iii) Reaction between substrate and KSI with a conservative Tyr[16]Phe mutation that replaces the oxyanion hole hydrogen-bond donor Tyr[16] with a hydrophobic environment. (iv) Reaction between substrate and KSI with a Tyr[16]Gly mutation, more drastic than the Tyr[16]Phe mutation above. The units of each rate constant are shown in parentheses under the corresponding reaction arrow. Values were computed from rate constants for variants of KSI from *Pseudomonas putida* tabulated in table S2 in Kraut *et al*. (*2*) and correspond to the substrate 5(10)-estren-3,17-dione, because a chemical step is rate-limiting for reaction of this substrate (*9*). Similar rate enhancements are provided for reactions with the substrate 5-androstene-3,17-dione referred to by Fried *et al*., although a nonchemical step is partially rate limiting for this substrate (*9*, *10*).

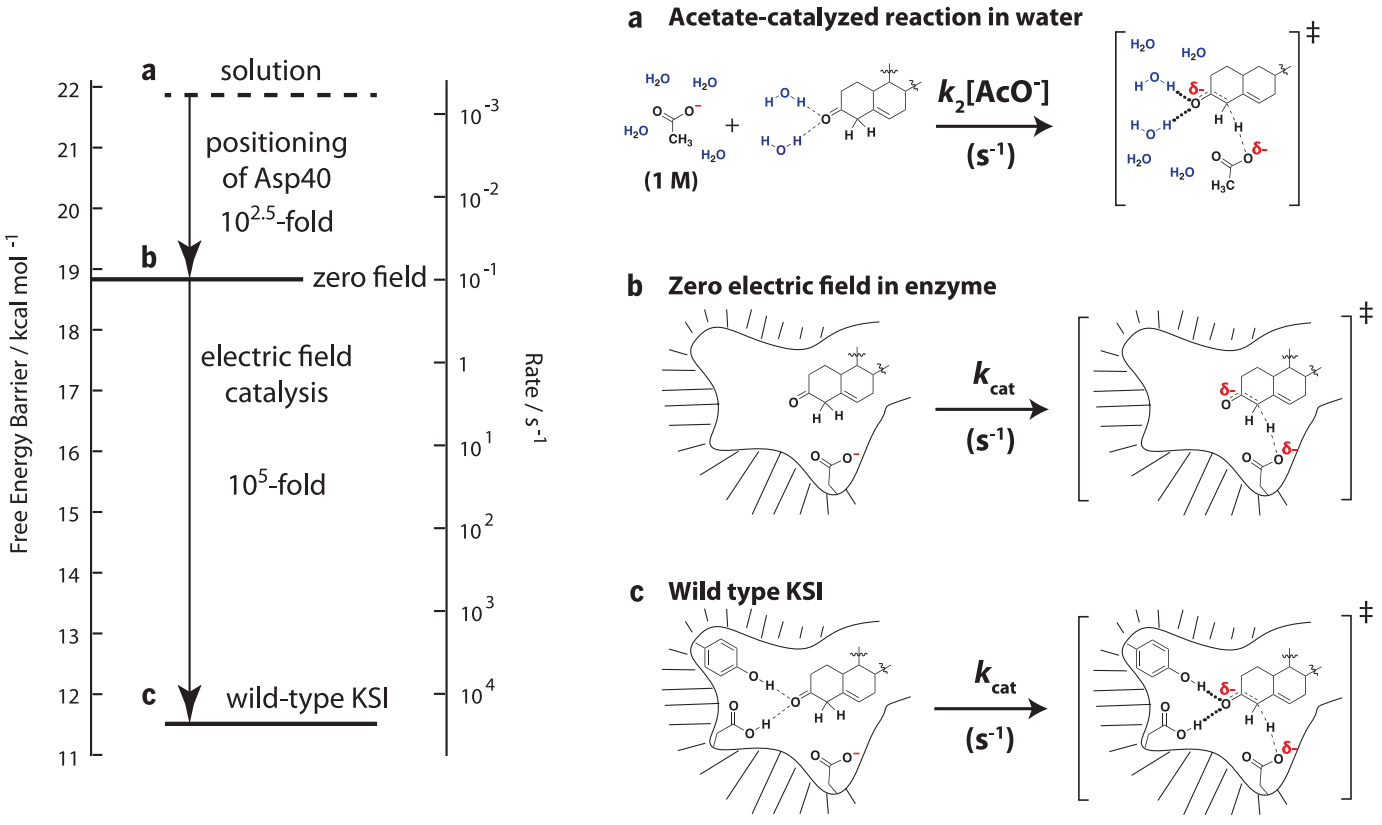


**Fig. 2. Reactions used by Fried *et al*. to estimate the fraction of the overall catalytic power from the electric field on the carbonyl group.** The left side of the figure is reproduced from Fried *et al*. with the reactions annotated by the lowercase letters and a schematic of each corresponding reaction shown on the right. The reactions are depicted schematically on the right and are as follows: (a) Nonenzymatic reaction between acetate and substrate in aqueous solution. Because this is a second-order process whose reaction rate depends on acetate concentration, Fried *et al*. used the observed rate constant at 1 M acetate (so that the calculated rate enhancement of $10^{2.5}$ is unitless but will vary with different concentrations of acetate used as the reference reaction). (b) Reaction with substrate bound to an enzyme that provides zero electric field at the carbonyl (and the same contribution from the general base as in wild-type KSI). (c) Reaction with substrate bound to wild-type KSI.

KSI oxyanion-stabilizing residue (*3*), such as Tyr[16]Phe, can exaggerate the catalytic contribution of active-site hydrogen-bonding groups relative to aqueous solution (Fig. 1A, reaction iii). Indeed, KSI variants with more extreme ablation of Tyr16 (e.g., Tyr[16]Ser, Ala, or Gly) are two orders of magnitude more active than the Tyr16Phe variant (Fig. 1A, reaction iv) (*2*), suggesting that replacing the hydrophobic Phe residue with what seems to be a relatively disordered water molecule is much less deleterious (*2*). In this mutant, only ~20% (log scale) of the catalytic power is lost and ~$10^9$-fold catalysis remains, suggesting that the oxyanion hole may not be the dominant contributor to KSI catalysis

Below, we lay out more explicitly the comparisons used by Fried *et al.* and demonstrate that there is likely more catalysis from the positioned general base than estimated by these authors, thereby helping to clarify the relative contributions of catalytic mechanisms and the underlying comparisons used to derive them.

Figure 3C from Fried *et al.* proposes a full accounting of catalysis, assigning a modest catalytic contribution from general base positioning and a dominant contribution from the enzyme's electric field. Figure 2 reproduces figure 3C in Fried *et al.*, annotated with the corresponding reactions that were used by these authors to estimate the catalytic contributions. Three reactions were considered:

a. Nonenzymatic reaction in aqueous solution between 1 M acetate and substrate.

b. Substrate bound to a hypothetical enzyme that provides zero electric field at the position of the carbonyl group.

c. Substrate bound to wild-type enzyme.

Fried *et al.* estimated the contribution from the positioned general base from comparison of reactions **a** and **b**, but these reactions have two differences, and thus, the comparison does not isolate the contribution from the positioned general base. Specifically, reaction **a** takes place with free general base (acetate ion) in aqueous solution, whereas reaction **b** takes place with positioned general base in an enzyme with a zero electric field at the oxyanion hole (Fig. 2). As noted above, a hydrophobic or zero-field oxyanion hole environment would be inhibitory relative to an aqueous environment, as also implied by the comparisons of figure 3, A and B, of Fried *et al.* Stated another way, charge localization is more difficult upon removal of the aqueous surroundings and placement in a zero field or hydrophobic environment. Thus, the effect from the positioned general base appears to be underestimated by the comparison of reactions **a** and **b**. Correspondingly, this result implies an overestimation of the catalytic effect from the oxyanion hole.

In addition, Fried *et al.* use the assumption that the oxyanion hole and general base contributions are independent to parse the catalytic contributions, although, as they note, there is no experimental evidence to support this assumption. The additivity of the catalytic features remains to be tested.

Fried *et al.* consider effects of positioning and electrostatics and state that "contrary to earlier views [(*4*, *5*)], electrostatic stabilization can be the more important of the two (Fig. 3C)." We emphasize that positioning is also important for electrostatic catalysis. As Fried *et al.* note elsewhere, "the active site achieves this large field by restricting H-bond conformations to those that are associated with the largest electric fields." If there were no positioning of the active site groups giving rise to the electric field, the fields would be lower, and if positioning of the oxyanion hole and surrounding residues were incorrect (e.g., backward with respect to substrate), the fields could even be inhibitory. The field is a consequence of

both the functional groups that are present and their positioning. This positioning arises from the folding of the protein, using favorable folding energy to orient and restrict the conformational mobility of these groups, and from binding of the substrate, in a pocket also formed due to folding of the protein (*6*–*8*). Turning to the substrate, if there were no pocket or if the substrate were sterically restricted from approaching the oxyanion hole, then there would be less or no catalysis; if the substrate were bound but positioned such that its carbonyl group faced away from the oxyanion hole, then the oxyanion hole and its surroundings would not contribute to catalysis. In summary, electrostatic catalysis, to be effective, requires positioning—proper positioning of the substrate via binding interactions into a pocket that is created via protein folding as well as proper positioning of enzymatic groups, again via protein folding and substrate binding, to make favorable electrostatic interactions in the reaction's transition state. Thus, catalytic contributions from electrostatics and positioning appear to be inextricably linked. Understanding this linkage, and catalysis, will likely require descriptions that extend beyond measures of apparent electric fields to atomic-level descriptions and models, including the multiple states present in the ensemble of an enzyme-substrate complex and the reaction probability from each state.

Another future challenge will be to understand the extent to which functional groups near and far from the active site contribute to the electric field perceived at the substrate carbonyl and how much they contribute to catalysis. Fried *et al.* (*1*) cite the observation of a nonzero field in the $Tyr^{16}Phe$ mutant to conclude that "a substantial electrostatic field contribution also arises from the environment fashioned by the enzyme scaffold." An alternative model that remains to be tested is that the nonzero field in this mutant arises largely from $Asp^{103}$, the other oxyanion hole hydrogen-bond donor. The observation of a negligible electrostatic field effect from mutation of the $Asp^{40}$ general base to Asn suggests a limited propagation distance (*1*).

Vibrational measurements, like those elegantly presented by Fried *et al.*, provide a powerful means to compare enzyme variants, and the observed vibrational properties can be compared to predictions from computation to assess those models. For example, Fried *et al.* note that molecular dynamics simulations with KSI did not reproduce the electric fields calculated from the experimentally observed vibrational frequencies [supplementary text 4 of (*1*)], providing a strong indication of features or factors lacking in the computation model, in its implementation, or in its underlying physical forces. Analogously, whether hydrogen-bond interactions can be quantitatively and accurately modeled as field effects or correlate with field effects and require more sophisticated atomic-level models remains to be determined. Regardless, vibrational data, as obtained by Fried *et al.* and others, provide an incisive window into the active site that will help test, develop, and refine increasingly accurate and predictive models for enzymatic catalysis.

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