Supporting Information

Fried and Boxer 10.1073/pnas.1310964110

SI Discussion: The Validity of Approximating K_D^{LH} as K_D^{app}

In the main text, when entering differential acidity into the catalytic cycle, the approximation was made to replace K_D^{LH} (the dissociation constant of neutral ligand to ionized enzyme, Eq. **6**) with K_D^{app} (the ligand's apparent dissociation constant without regard to protonation states, Eq. **7**). This reformulation allowed one to clearly distinguish differential acidity as a separate contribution from differential binding. In the specific case of ketosteroid isomerase (KSI), the approximation is well-founded, as K_D^{LH} is indeed only 1.3-fold greater than K_D^{app}. In this section, we examine the range of validity of this approximation. Clearly, for some choice of thermodynamic parameters, K_D^{LH} will not be close to K_D^{app}. In the general case, the differential acidity effect is represented as the factor by which K_D^{L-} (the dissociation constant of ionized ligand to neutral enzyme) is lower than the apparent dissociation constant, K_D^{app}. The dependence of the log ratio of K_D^{L-} to K_D^{app} on the two acidity parameters is given in Fig. S1*A*. As pK_α^E (the internal acidity of the enzyme active site) increases, K_D^{L-} decreases, representing stronger effective binding and therefore greater stabilization. However, this effect plateaus (as shown by arrow 1) once pK_α^E is equal to the ligand's pK_a, or when the enzyme has equal affinity for protons as the bound ligand (1). The contribution from differential acidity also grows more pronounced as pK_a^E (the standard acidity of the enzyme active site to water) decreases (as shown by arrow 2). This effect has no bound, and as the active-site oxyanion hole (OAH) becomes more and more acidic in the apo state, K_D^{L-} continues to exponentially decrease, making the enzyme's effective stabilization of the intermediate greater and greater.

For a large portion of the acidity parameter space, K_D^{LH}/K_D^{app} is close to 1 (the yellow region of Fig. S1*B*), where the approximation is valid. As pK_α^E increases up to pK_a^L (following arrow 1), this remains the case. However, once pK_α^E exceeds pK_a^L, K_D^{LH} begins to exponentially increase, which implies a deleterious effect of the differential acidity on intermediate stabilization and marks where the approximation breaks down. Combined with the result from Fig. S1*A*, it is clear that for catalytic purposes the optimum value of pK_α^E is matched to the ligand's pK_a^L, as increasing it beyond that value no longer affects K_D^{L-}.

To summarize, differential acidity depends on the enzyme's internal acidity, pK_α^E, and the enzyme's solution acidity, pK_a^E. For catalysis, differential acidity is optimized when $pK_\alpha^E = pK_a^L$ and when pK_a^E is as low as possible (these two principles could be considered as design criteria for a hypothetical artificial enzyme). So long as $pK_\alpha^E < pK_a^L$, the approximation holds that $K_D^{LH} \simeq K_D^{app}$. In this case, the contribution from differential acidity, ΔG_3°, is proportional to the simple expression $pK_a^E - pK_\alpha^E$, in analogy to the expression for ΔG_2°, $pK_D^{E\cdot S} - pK_D^{E\cdot I}$.

A Note on tKSI

Interestingly, a homolog of KSI from a different organism (tKSI) does not have a remarkably low pK_a^E. However, unlike the variant of KSI^{D40N} considered wherein bound inhibitors exist as a mixture of neutral and anionic forms (1, 2), $tKSI^{D40N}$ appears to bind inhibitors (1, 3, 4) and the intermediate (3) preponderantly in the anionic form, indicating that its pK_α^E is larger than 9.7. Therefore, tKSI with an unexceptional pK_a^E may still exhibit a differential acidity effect if pK_α^E is correspondingly larger. Considering Fig. S1*A*, a beneficial effect from differential acidity (that is, $K_D^{L-}/K_D^{app} << 1$) is still expected with an unexceptional $pK_a^E \sim 9$, so long as pK_α^E is higher yet. However, unless pK_a^E and pK_α^E fortuitously take on values that render them measurable (as was the case for pKSI), the differential acidity effect will not be experimentally observable. Consideration of tKSI calls attention to the fact that differential acidity effects will not be measurable for all systems, which highlights another reason why computational methods are essential in thermodynamic analyses of enzyme catalytic cycles (5–7).

1. Childs W, Boxer SG (2010) Proton affinity of the oxyanion hole in the active site of ketosteroid isomerase. *Biochemistry* 49(12):2725–2731.
2. Fried SD, Boxer SG (2012) Evaluation of the energetics of the concerted acid-base mechanism in enzymatic catalysis: The case of ketosteroid isomerase. *J Phys Chem B* 116(1):690–697.
3. Petrounia IP, Pollack RM (1998) Substituent effects on the binding of phenols to the D38N mutant of 3-oxo-delta5-steroid isomerase. A probe for the nature of hydrogen bonding to the intermediate. *Biochemistry* 37(2):700–705.
4. Petrounia IP, Blotny G, Pollack RM (2000) Binding of 2-naphthols to D38E mutants of 3-oxo-delta 5-steroid isomerase: Variation of ligand ionization state with the nature of the electrophilic component. *Biochemistry* 39(1):110–116.
5. Warshel A (1981) Calculations of enzymatic reactions: Calculations of pKa, proton transfer reactions, and general acid catalysis reactions in enzymes. *Biochemistry* 20(11): 3167–3177.
6. Pierdominici-Sottile G, Roitberg AE (2011) Proton transfer facilitated by ligand binding. An energetic analysis of the catalytic mechanism of *Trypanosoma cruzi* trans-sialidase. *Biochemistry* 50(5):836–842.
7. Schutz CN, Warshel A (2004) The low barrier hydrogen bond (LBHB) proposal revisited: The case of the Asp ··· His pair in serine proteases. *Proteins* 55(3):711–723.

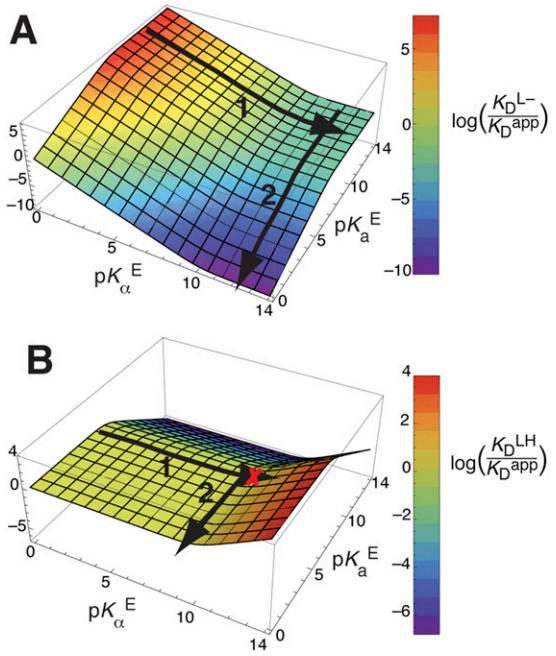

Fig. S1. Dependence of microscopic binding constants on differential acidity. Plots of Eq. **5** from the main text, showing the effect of the two acidity parameters (pK_a^E and pK_α^E) on the microscopic dissociation constants (*A*) K_D^{L-} and (*B*) K_D^{LH}. The vertical axis is on a log scale and is normalized to K_D^{app}; pH 7.2 and $pK_a^L = 10.0$. In *A*, arrow 1 shows that a higher effective affinity is achieved for increasing pK_α^E up to the pK_a of the ligand; arrow 2 shows that higher effective affinity accompanies a more acidic active site. In *B*, arrow 1 shows that K_D^{LH} is nearly equal to K_D^{app} and insensitive to pK_α^E until pK_α^E surpasses the pK_a of the ligand.

Table S1. Thermodynamic and kinetic parameters

Quantity	Description	Value	Determination	Ref(s).
$K_D^{D40/Andro}$	Binding of substrate to KSI	17 μM	UV-vis kinetics, K_M of a "slow" substrate	(1)
pK_a^{Andro}	pK_a of substrate	12.7	UV-vis kinetics, detailed balance	(2)
pK_a^{Enol}	pK_a of protonated intermediate	10.0	UV-vis kinetics, detailed balance	(3)
pK_a^{Asp40}	pK_a of general base in apo enzyme	3.75	pH-titration kinetics	(4)
$K_D^{N40/Enol}\left(K_D^{N40/Enol-app}\right)$*	Binding of intermediate to KSI^{D40N}	6 nM	UV-vis kinetics	(5)
K_{int}^*	The internal equilibrium constant for the process **E•S** ⇌ **E•I**	0.01–0.3	Thermodynamic analysis	(5)
pK_a^E	pK_a of OAH in KSI^{D40N}	6.3	^{13}C NMR, UV-vis titration	(6)
pK_α^E	Internal acidity of OAH in KSI^{D40N}	9.7	IR spectroscopy	(7)
$K_D^{N40/Equ}\left(K_D^{N40/Equ-app}\right)$	Binding of equilenin to KSI^{D40N}	1.0 nM	Fluorescence quenching	(8)
k_{cat}^*	Rate constant for **E•S** → **E•I**	1.7×10^5 s^{-1}	UV-vis kinetics; intermediate partitioning	(9)
k_{uncat}	Rate constant for AcO^- + **S** → AcOH + **I**	9.0×10^{-3} s^{-1}	UV-vis kinetics	(3)

All parameters were measured with a temperature between 20 and 25 °C and with a pH between 6.9 and 7.2, except for acid dissociation constants (pK_as), which are measured over a pH range.

*These parameters were determined for the homolog of KSI from *Comamonas testosteroni* (tKSI), a related but distinct form of KSI from the one on which the current analysis focuses (pKSI). The two are known to use the same mechanism, and possess very similar kinetic and thermodynamic specifications when subject to the same experimental conditions. Two of the parameters for which data are not available on pKSI (K_{int} and k_{cat}) do not affect the analysis directly because they are not used in the calculations, but are compared to calculated values. Note that the Michaelis–Menten k_{cat} for tKSI and pKSI are very similar (within a factor of 1.5; ref. 4). $K_D^{N40/Enol-app}$ is a key parameter for which data are not available on pKSI, but its value for tKSI is very close to the value of $K_D^{N40/Equ-app}$ for both tKSI and pKSI, justifying its use.

1. Schwans JP, Kraut DA, Herschlag D (2009) Determining the catalytic role of remote substrate binding interactions in ketosteroid isomerase. *Proc Natl Acad Sci USA* 106(34): 14271–14275.
2. Pollack RM, Zeng B, Mack JPG, Eldin S (1989) Determination of the microscopic rate constants for the base catalyzed conjugation of 5-androstene-3,17-dione. *J Am Chem Soc* 111(16): 6419–6423.
3. Zeng B, Pollack RM (1991) Microscopic rate constants for the acetate ion catalyzed isomerization of 5-androstene-3,17-dione to 4-androstene-3,17-dione: A model for steroid isomerase. *J Am Chem Soc* 113(10):3838–3842.
4. Yun YS, et al. (2003) Origin of the different pH activity profile in two homologous ketosteroid isomerases. *J Biol Chem* 278(30):28229–28236.
5. Hawkinson DC, Pollack RM, Ambulos NP, Jr. (1994) Evaluation of the internal equilibrium constant for 3-oxo-delta 5-steroid isomerase using the D38E and D38N mutants: The energetic basis for catalysis. *Biochemistry* 33(40):12172–12183.
6. Fafarman AT, et al. (2012) Quantitative, directional measurement of electric field heterogeneity in the active site of ketosteroid isomerase. *Proc Natl Acad Sci USA* 109(6):E299–E308.
7. Fried SD, Boxer SG (2012) Evaluation of the energetics of the concerted acid-base mechanism in enzymatic catalysis: The case of ketosteroid isomerase. *J Phys Chem B* 116(1):690–697.
8. Kraut DA, et al. (2006) Testing electrostatic complementarity in enzyme catalysis: Hydrogen bonding in the ketosteroid isomerase oxyanion hole. *PLoS Biol* 4(4):e99.
9. Hawkinson DC, Eames TC, Pollack RM (1991) Energetics of 3-oxo-delta 5-steroid isomerase: Source of the catalytic power of the enzyme. *Biochemistry* 30(45):10849–10858.