# Supporting Information

Kamerlin et al. 10.1073/pnas.0914579107

## SI Text

**The Preorganization Concept.** In light of the crucial role of the preorganization concept, it is useful to briefly summarize it here. That is, as shown in (1), there exist multiple cases where most of the catalytic effect is clearly due to electrostatic interactions. Nevertheless, it is important to be able to establish that such effects are due to TS stabilization, as well as to determine why the protein is capable of providing such large effects. An awareness of the importance of electrostatic reorganization emerged (2) from exploring the change in the polarization of the solvent (or protein) dipoles during the reaction in the enzyme and in solution. It was found that during reactions in solution, the solvent must pay a major free energy penalty when reorienting its dipoles toward the TS charges while moving from the reactant state (RS) to the transition state (TS). On the other hand, the protein active site dipoles are already partially oriented toward the TS, and thus the protein has to pay much less reorganization energy. This concept is related to Marcus' reorganization energy (see discussion in ref. 1), but the crucial difference is that here, the protein must have fixed dipoles rather than a nonpolar environment in order to reach both small reorganization and a stable TS. The preorganization concept can be realized by looking at the shift of the Marcus parabolae or by considering the preorganization associated with binding the TS (1) and the IS. In the case of KSI, the relevant preoriented dipoles are provided by Tyr16 and Asp103, which are pointing toward the enolate negative charge (in the TS and IS), as well as other protein dipoles, plus a water molecule that stabilizes the charge (Asp 40) (3).

While the shift of the Marcus parabolae is relatively easy to understand, one has to formulate the preorganization effect in an equivalent but slightly more complex way, by trying to understand the TS binding. This is done in Fig. 8 of ref. 1 and reconsidered in part here. That is, the TS binding can be explored for both the TS and RS by means of the LRA expression (4):

$$\Delta G(Q^{\neq}) = 0.5(\langle U(Q=Q^{\neq}) - U(Q=0)\rangle_{Q=Q^{\neq}} + \langle U(Q=Q^{\neq}) - U(Q=0)\rangle_{Q=0} = 0.5(\langle \Delta U\rangle_{Q^{\neq}} + \langle \Delta U\rangle_0) \quad \textbf{[S1A]}$$

$$\lambda^{\neq} = 0.5(\langle \Delta U\rangle_0 - \langle \Delta U\rangle_{Q^{\neq}}) \quad \textbf{[S1B]}$$

Here, $U$ is the solute-solvent interaction potential, $Q$ designations the solute residual charges, $Q^{\neq}$ indicates the TS charges, and $\langle \Delta U\rangle_Q$ designates an average over configurations obtained from an MD run with the given solute charge distribution. The $\lambda^{\neq}$ in Eq. **S1B** denotes the reorganization energy for the solvation of the TS, which will be discussed below. Here, the first term of this equation (**S1A**) denotes the interaction energy at the TS, where $Q = Q^{\neq}$, and this term is similar in both enzymes and in solution. The second term of this equation expresses the effect of the environmental preorganization. That is, if the environment dipoles are randomly oriented toward the TS in the absence of a charge (as is the case in water), then this second term is zero, giving:

$$\Delta G(Q^{\neq})^{w}_{\text{sol}} = \frac{1}{2}\langle \Delta U\rangle_{Q^{\neq}} \quad \textbf{[S2]}$$

where the electrostatic free energy is half the average electrostatic potential (5). Note, however, that this does not hold in the preorganized environment of the enzyme, where the second term makes a significant contribution, giving an overall $\langle \Delta U\rangle_0$ that is more negative in the enzyme than in water. This situation arises as a result of the catalytic effect of the enzyme.

Another way to see this situation would be to consider the fact that in water, the solvent dipoles will be randomly oriented around the uncharged form of the TS, and thus the total activation free energy will include a contribution from the free energy cost associated with the reorganization of these solvent dipoles toward the charged TS. On the other hand, in the protein, the active site dipoles (which come from either polar groups, charged groups, and/or water molecules) are already partially oriented toward the TS charge (2), and thus the reaction costs less reorganization energy compared to its counterpart in water. This effect, which is shown schematically in Fig. S1, is related to Marcus' well-known reorganization energy (7). However, the two effects are not identical, as Marcus' reorganization energy is related to transfer from the reactant to the product states, whereas here, we are dealing with the charging of the TS. The conceptual and practical differences between these two issues have been discussed in detail in refs. 1 and 6.

In trying to understand the preorganization effect, one may wonder why we are focusing on the rotation of the ring rather than the polarization of the protein dipoles. As a start, it should be noted that the preorganization term of Eq. **S1** has the same nature when we have rotating dipoles with a fixed solute or a rotating solute and fixed dipoles. Here, one should realize that in the case of a reaction in water, we are interested in the rotation of the solvent relative to the solute, and this can be explored rigorously. In the case of a reaction in a protein, the trivial part is holding the solute fixed, and the fundamental issue is having fixed protein dipoles. So, in general, the issue is having fixed protein dipoles relative to the already (relatively) fixed solute. The confusion arises from the fact that whereas KSI evolved to fix its diploes relative to the bound steroid, the experiment (8) that led us to identify the rotational issue was done by using a ligand that is free to rotate in an already evolved preorganized protein active site. Remarkably, this attempt to disprove the preorganization idea inadvertently gave this proposal major support. However, this fact cannot be fully realized without using the concepts of Eq. **S1**.

**Additional Background.** As discussed in the main text, KSI provides an instructive example of the need for thorough theoretical studies when examining the catalytic effect of an enzyme. In this respect, it is useful to point out that some workers have attributed the catalytic effect of KSI to a low barrier hydrogen bond (LBHB) between the enolate and Tyr57 (e.g., ref. 9). However, a careful EVB study by Feierberg and Åqvist (3) (and that of ref. 10) demonstrated that the catalytic effect is not in fact due to an LBHB, but rather to the combined preorganization of the dipoles of Tyr57 and Asp103. Additionally, there exists experimental evidence that the enolate is stabilized by these hydrogen bonds, rather than by a delocalized bond to Tyr16 (11).

Because this work deals with both binding and catalysis, it is crucial to start from the thermodynamic cycles shown in Fig. S2, which make the same point as that made in ref. 10. That is, as can be seen from the figure, we are dealing with two entirely different thermodynamic cycles: one for the binding of a charge, and the other for charge transfer/proton transfer processes, and thus one has to be very careful when drawing assumptions from one cycle to the other.

**Thermodynamic Analysis of the Binding Energy of the TSAs and TSs.** A central part of this work involves a careful thermodynamic analysis of the binding of the different species involved. This starts with the thermodynamic analysis of Fig. S3, in which the different protonation states of the given TSA are considered. We also consider some of the key cases discussed in the text. For example, we have evaluated the charging free energy of equilenin and the phenolate (Fig. S4). Here, we reproduced the observed electrostatic contribution to the binding free energy (as determined by the observed $pK_a$ shift), and, using the previously evaluated nonelectrostatic contribution (10), we reproduce the experimentally determined binding free energies. It is also useful to point out that different experiments were done with different variants of the enzyme. For instance, the experiments of ref. 12 used KSI from *Pseudomonas putida*, whereas other workers have used KSI from *Pseudomonas testosteroni* (13). However, here we took the most reliable average result, regardless of the precise system used [because if the results are drastically different between different variants of KSI, then attempts (8, 12) to generalize the experimental results may not be so useful].

Of course, some may argue that there exist experimental facts about the nonpolar contributions that contradict our findings. However, this may amount to confusing interpretation of experiments with actual facts. First, we would like to establish that our estimate of the np term presents the most reliable analysis of the currently available experiments. That is, our estimate of the large (−8 kcal/mol) np term for phenolates is obtained not only by the theoretical calculation of ref. 10, or by subtracting the small calculated electrostatic contribution (of about −2.5 kcal/mol) from the −10.5 kcal/mol observed binding energy (see Table S1), but also by using the small observed electrostatic contribution deduced from the experiments of (8). If in some way the actual electrostatic contribution is large, then the arguments of ref. 8 are invalid (note that all of ref. 8 is about experimentally proving that the electrostatic effect is small in phenolates, and thus presumably also in the true TS, and we agree with this estimate for the phenolates). In that case, we would have little to talk about as our point about electrostatic effects is proven. On the other hand, if it is small, our estimate of the np term is reliable.

When we move to large multiring systems such as equilenin, we find (from the experimental $K_D$) that the binding of the uncharged form of D40N is $\sim -6$ kcal/mol (see Table S1). A similar estimate is obtained for $S_{full'}$, as can be seen from our analysis in ref. 10, and obviously, $S_{full'}$ and equilenin are the same size. Thus, we have strong reason to believe that it is a reasonable assumption that the np contribution of $S_{mini}$ is similar to that of the phenolate. We consider the analysis above to be far more relevant than using the observed $K_M$ in order to deduce hydrophobic contributions, and also believe that calculations of the type performed in ref. 10 (where the nonpolar ligand is mutated to nothing, see ref. 10 for the technical details of how this was achieved) are extremely informative and quite reliable, including the finding that, in this case, the np contribution does not increase with ring size.

We would also like to clarify that the perception that the larger ring system has a larger nonpolar term is the result of only considering the issue superficially, rather than the much deeper analysis of this term, which is common within the leading workers on this issue in the computational community [who attempt to quantify binding energies (10, 14, 15)]. In fact, whereas the hydrophobic term increases in water, this is not translated into a parallel increase in the binding energy, due to the penalty of removing water from the empty active site and other contributions. In the case of KSI, we should at least start by considering the observed $K_D$s discussed above. A point that might not be fully appreciated, if theoretical studies are dismissed, is the fact that the electrostatic calculations are at present far more stable and reliable than the computational evaluation of the np term (which nevertheless is superior to rough intuitive estimates). Using the electrostatic calculations and the observed binding is probably the best current way to estimate the np term (with known total binding free energy). However, our overall analysis is not dependent on the size of the np term at all, and all the discussion above is simply brought up in order to prevent a misleading discussion of our results. It is also crucial to point out that even if some of the items in the analysis above are not precise, nevertheless, these are not going to change the overall conclusions of our work, which consistently combines an analysis of both experimental and theoretical data. In fact, in some of the cases being considered here, having the ability to perform reliable calculations gave a clear advantage over just using experimental analyses that might not provide unique conclusions.

Another important issue is the interpretation of the measurement of the $pK_a$ of equilenin (16). That is, the finding that the $K_D$ of equilenin is pH independent can be in part be rationalized by considering the ionization of equilenin as involving proton transfer to Asp40. However, this feature does not change any of our conclusions. That is, what counts is the fact that at a pH of <3.5, equilenin becomes protonated. Thus, the experiment tells us about the *very* relevant protonation of equilenin in the presence of a protonated Asp40, which is what we actually need.

**Thermodynamic Analysis of the Catalytic Process.** The examination of the different TSs considered in this work requires a particularly careful thermodynamic analysis. That is, it is essential to consider both the binding and the catalytic steps, using $k_{cat}/K_D$ and $k_{cat}$. The overall analysis is summarized in Fig. S5, and the specific considerations are summarized in Table S1. The majority of the results in this table are directly based on very solid experimental findings, and, as far as the catalysis is concerned, we also reproduced the observed trend by the EVB calculations described in Fig. S6. Overall, both Table S1 and Fig. S5 illustrate the fact that the TS binding is much larger than that of the phenolate TSA.

**Additional Clarifications.** Proper appreciation of the preorganization idea is complicated by problematic assumptions about its nature. An example of such significant misunderstandings can be seen in the implication (12, 17) that our proposal is related to "the exclusion of water molecules by enzymes," as is suggested in the gas-phase model of Dewar (18) [which is mixed (12, 17) with our fundamentally different electrostatic idea]. Additionally, the confusion in missing the fact that the preorganization proposal is basically our original electrostatic proposal but simply formulated in more quantitative terms can be illustrated by the fact that, for instance, when listing workers who have supported the oxyanion electrostatic effect in ketosteroid isomerase, (12) overlooks the main proponents of the electrostatic stabilization idea.

It might also be useful to consider other problems with the alternatives to the preorganization idea. For example, Sigala and coworkers (19) suggested that proteins are able to induce large catalytic effects by pushing a C = O bond in the direction of the C-O bond observed in the TS. Both resonance Raman and computational studies are presented in support of this finding. However, the change in bond length in the serine proteases is not promoted by steric effects, but rather, it is the result of the stabilization of the ionic $C\text{-}O^-$ resonance form by the preorganized electrostatic environment of the oxyanion hole (20). Similarly, the authors of ref. 19 argued that rigid active site constraints promote catalysis by preventing favorable interactions in the ground state. However, this simply presents the incorrect ground state destabilization idea, rather than a transition state stabilization idea, and, furthermore, it is proposed that the catalysis is associated with preventing the hydrogen bonds from stabilizing the C = O state, rather than from the extra stabilization of the charged $C\text{-}O^-$ state. In fact, the hydrogen bond to the C = O

state is far less important than that to the charged C-O$^-$ state (and nothing prevents hydrogen bonding to the C = O). A part of the problem might be the focus on early simplistic gas-phase theoretical studies of different interactions [e.g., those of Kollman and coworkers (21)], rather than on actual quantitative free energy calculations (10) of exactly the same TSAs as those being considered by the authors in their work or on general quantitative studies (1).

In light of the findings in this work and in ref. 10, it is important to clarify that the discussion of the reorganization effect in (8) reflects a significant misunderstanding of this concept. That is, the preorganization idea reflects fully quantitative considerations, which are now well accepted in the electron transfer community (22) and are not related to the number of water molecules in the system or to the inability of some continuum models to evaluate this effect. At present, it appears that only microscopic computational approaches can quantify the preorganization contribution, and, of course, reproducing both the actual observed binding and catalysis must validate such models.

At this point it might also be useful to consider the argument that recent infrared field experiments using a thiocyanate vibrational probe incorporated into the KSI active site (17) support the arguments of ref. 12. In fact, the more relevant field experiments should involve placing a probe on the ligand rather than on the protein and examining the resulting effect with both large and small nonpolar ligands. This elusive uncharged state, which represents the end of the thermodynamic cycle, is the part overlooked by Herschlag and coworkers, though perhaps it is hard to understand that something that cannot be observed in a direct experiment nevertheless represents an absolute part of a charging cycle, whose thermodynamic result is very real.

On a related note, it should also be clarified that the rationalization of the catalytic power of KSI is unrelated to Jencks' concept of an "entropy-trap" (23), which is related to the entropy of aligning the reacting fragments (in this particular case is Asp40 and the steroid) or any other regular entropic proposal, which relates to the difference between the reactant and transition states in the enzyme in water. That is, the true preorganization lies in the uncharged state (which doesn't exist as a real nonmathematical state) and thus has no counterpart in any conventional entropic proposal. Furthermore, the electrostatic binding effect is not related to the degree of freedom of $S_{mini}$ in the enzyme site but only manifests itself in the binding of the product states. In this case, the rotational motion of the product state can also be converted to entropy in terms of its orientation toward the oxyanion hole, but the resulting expression is not related to the actual contribution from the preorganization term.


1. Warshel A, et al. (2006) Electrostatic basis for enzyme catalysis. *Chem Rev* 106:3210–3235.
2. Warshel A (1978) Energetics of enzyme catalysis. *Proc Natl Acad Sci USA* 75:5250–5254.
3. Feierberg I and Åqvist J (2002) The catalytic power of ketosteroid isomerase investigated by computer simulation. *Biochemistry* 41:15728–15735.
4. Lee FS, Chu ZT, Bolger MB, and Warshel A (1992) Calculations of antibody antigen interactions—Microscopic and semimicroscopic evaluation of the free-energies of binding of phosphorylcholine analogs to Mcpc603. *Prot Eng* 5:215–228.
5. Warshel A and Russel ST (1984) Calculations of electrostatic interactions in biological systems and in solutions. *Q Rev Biophys* 17:283–422.
6. Marcus RA (1956) On the theory of oxidation-reduction reactions involving electron transfer I. *J Chem Phys* 24:966–978.
7. Villà J and Warshel A (2001) Energetics and dynamics of enzymatic reactions. *J Chem Phys B* 105:7887–7907.
8. Kraut DA, et al. (2006) Testing electrostatic complementarity in enzyme catalysis: Hydrogen bonding in the ketosteroid isomerase oxyanion hole. *PLoS Biol* 4(4): 0501–0519.
9. Zhao QJ, Abeygunawardana C, Talalay P, and Mildvan AS (1996) NMR evidence for the participation of a low-barrier hydrogen bond in the mechanism of Delta(5)-3-ketosteroid isomerase. *Proc Natl Acad Sci USA* 93:8220–8224.
10. Warshel A, Sharma PK, Chu ZT, and Aqvist J (2007) Electrostatic contributions to binding of transition state analogues can be very different from the corresponding contributions to catalysis: Phenolates binding to the oxyanion hole of ketosteroid isomerase. *Biochemistry* 46:1466–1476.
11. Brooks B and Benisek WF (1994). Mechanism of the reaction catalyzed by Delta(5)-3-ketosteroid isomerase of comamonas (pseudomonas) testosteroni—Kinetic-properties of a modified enzyme in which tyrosine-14 is replaced by 3-fluorotyrosine. *Biochemistry* 33:2682–2687.
12. Schwans JP, Kraut DA, and Herschlag D (2009) Determining the catalytic role of remote substrate binding interactons in ketosteroid isomerase. *Proc Natl Acad Sci USA* 106:14271–14275.
13. Hawkinson DC, Pollack RM, and Ambulos NP, Jr. (1994) Evaluation of the internal equilibrium constant for 3-Oxo-D$^5$-steroid isomerase using the D38E and D38N mutants: The energetic basis for catalysis. *Biochemistry* 33:12172–12183.
14. Singh N and Warshel A (2010) A comprehensive examination of the contributions to binding entropy of protein-ligand complexes. *Proteins Struct Funct Bioinformat*, in press.
15. Hermans J and Wang L (1997) Inclusion of loss of translational and rotational freedom in theoretical estimates of free energies of binding. Application to a complex of benzene and mutant T4 lysozyme. *J Am Chem Soc* 119:2707–2714.
16. Zeng B, Bounds PL, Steiner RF, and Pollack R (1992) Nature of the intermediate in the 3-oxo-.DELTA.5-steroid reaction. *Biochemistry* 31:1521–1528.
17. Sigala PA, Fafarman AT, Bogard PE, Boxer SG, and Herschlag D (2007) Do ligand binding and solvent exclusion alter the electrostatic character within the oxyanion hole of an enzymatic active site? *J Am Chem Soc* 129:12104–12105.
18. Dewar MJS and Storch DM (1985) Alternative view of enzyme reactions. *Proc Natl Acad Sci USA* 82:2225–2229.
19. Sigala PA, et al. (2008) Testing geometrical discrimination within an enzyme active site: Constrained hydrogen bonding in the ketosteroid isomerase oxyanion hole. *J Am Chem Soc* 130:13696–13708.
20. Warshel A and Russell S (1986) Theoretical correlation of structure and energetics in the catalytic reaction of trypsin. *J Am Chem Soc* 108:6569–6579.
21. Andrea TA, et al. (1979) A model for thyroid hormone-receptor interactions. *J Med Chem* 22:221–232.
22. Warshel A and Parson WW (2001) Dynamics of biochemical and biophysical reactions: insight from computer simulations. *Q Rev Biophys* 34:563–670.
23. Jencks WP (1987) *Catalysis in Chemistry and Enzymology* (Dover, New York).
24. Hawkinson DC, Eames TCM, and Pollack RM (1991) Energetics of 3-oxo.-DELTA.5-steroid isomerase: Source of the catalytic power of the enzyme. *Biochemistry* 10849–10858.
25. Houck WJ and Pollack RM (2003) Activation enthalpies and entropies for the microscopic rate constants of acetate-catalyzed isomerization of 5-androstene-3,17-dione. *J Am Chem Soc* 125:10206–10212.
26. Houck WJ and Pollack RM (2004) Temperature effects on the catalytic activity of the D38E mutant of 3-oxo-Delta 5-steroid isomerase: Favorable enthalpies and entropies of activation relative to the nonenzymatic reaction catalyzed by acetate ion. *J Am Chem Soc* 126:16416–16425.

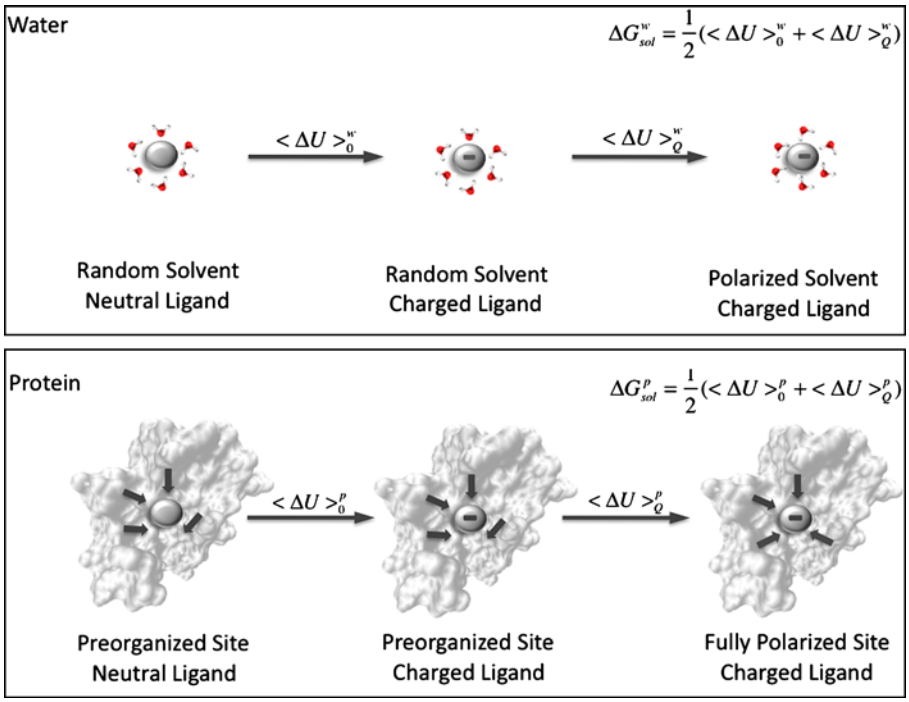


**Fig. S1.** Illustrating the preorganization effect, by first considering the charging of a substrate in an environment that is not polarized by the substrate (thus involving a large reorganization energy) and then considering the effect of the polarization of the solvent by the field of the substrate. This figure illustrates the difference between the preorganization contributions in (*A*) water, and (*B*) a protein. This figure is adapted from ref. 10.

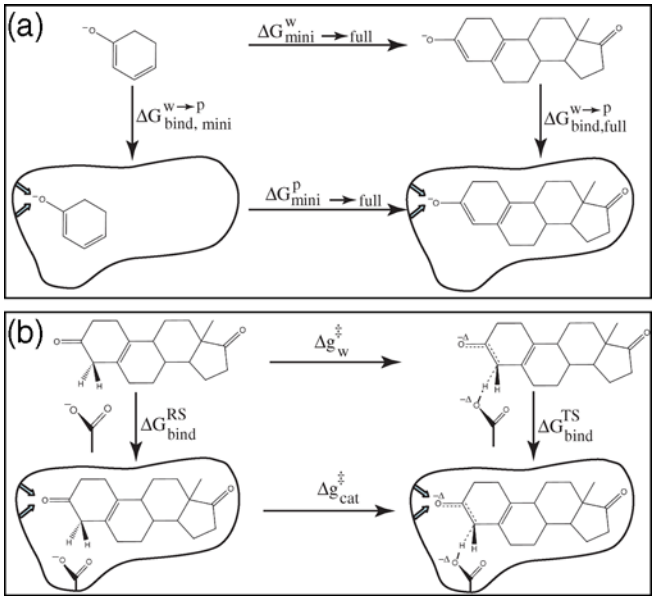


**Fig. S2.** Thermodynamic cycles corresponding to (*A*) the binding of a charged TSA (or TS), and (*B*) the transition from the RS to the TS (which constitutes a charge/proton transfer process). The figure uses a specific example, considering the cases of $S_{mini}$ and $S_{full}$.

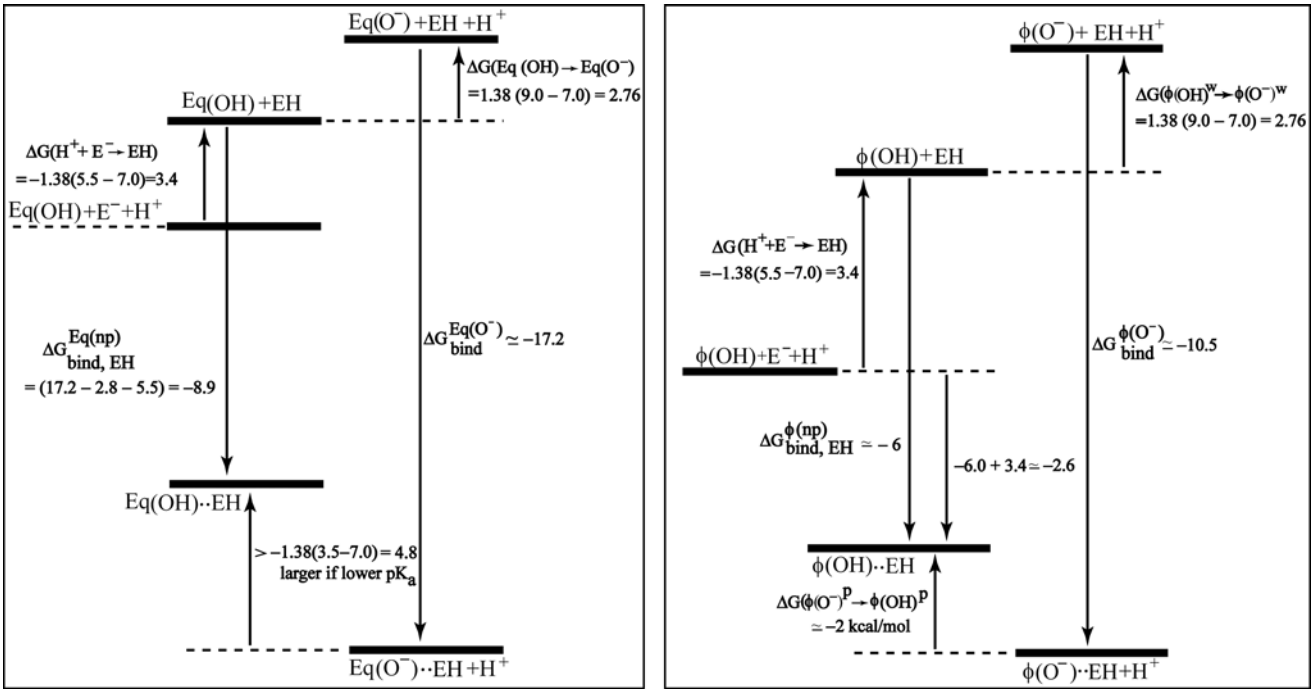


**Fig. S3.** (*A*) A thermodynamic description of the different protonation states of equilenin involved in the binding to KSI. Here, Eq(OH) and Eq($O^-$) designate the neutral and ionized forms of equilenin, respectively. (*B*) A thermodynamic cycle that includes the different protonation states involved in the binding of phenol to KSI. Here, $\phi$(OH) and $\phi$($O^-$) designate the phenol and phenolate, respectively.

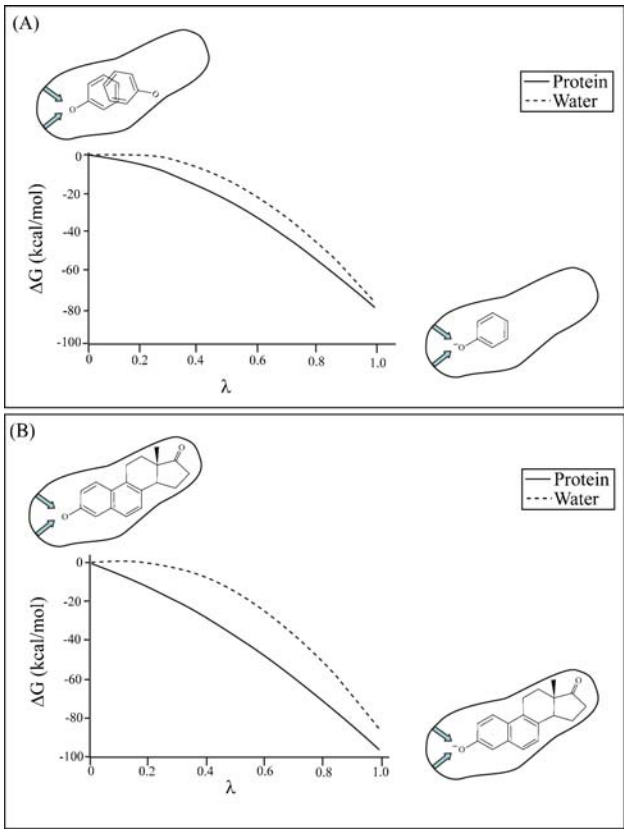


**Fig. S4.** A schematic illustration of the FEP charging of (*A*) the phenolate TSA and (*B*) equilenin. The electrostatic contribution to binding is given by the difference between the charging energy in water and in KSI.

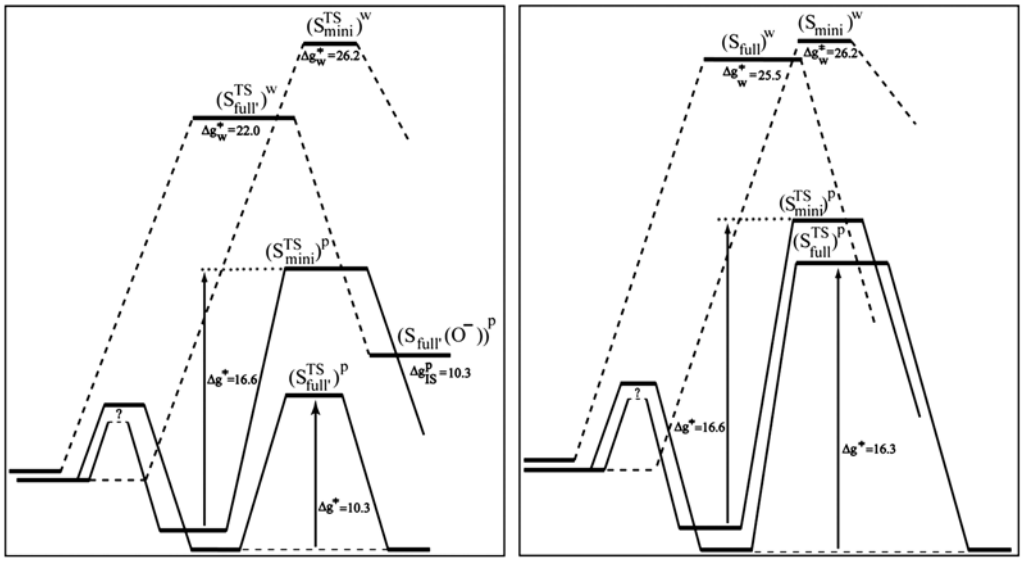


**Fig. S5.** An analysis of the barriers for the catalytic reaction of KSI for the relevant substrates.

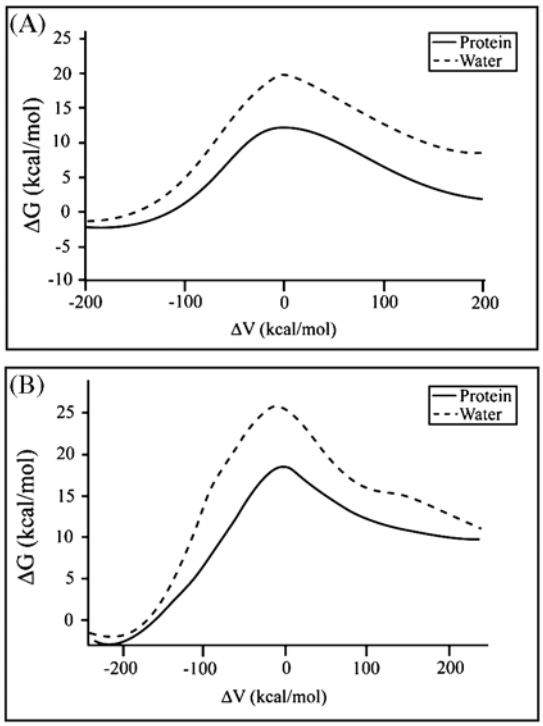


**Fig. S6.** Empirical valence bond (EVB) calculations of the energetics of the reactions of (*A*) $S_{full}$, and (*B*) $S_{mini}$. The difference between the two cases is discussed in the text.

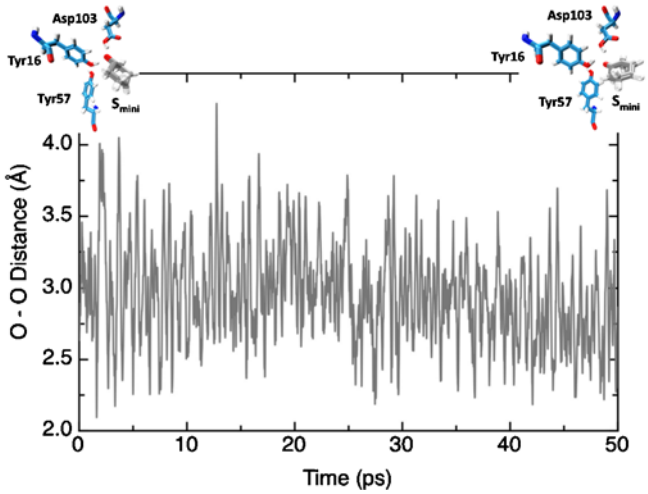


**Fig. S7.** The change in distance between the oxygen of Tyr16, and the oxygen of the TS of $S_{mini}$, during simulations where the protein sees zero charge on the TS. As seen from this figure, in contrast to the case of the IS (Fig. 6 of the main text), where the uncharged state is free to rotate in the active site, there is now very little change in the position of the TS relative to Tyr16.

**Table S1. Analyzing the binding energies of TSAs and TSs and their corresponding electrostatic contributions**

| System | $\Delta G_{bind}$ | $\Delta G^{elec}_{bind}$ | Source |
|---|---|---|---|
| $\phi^{(np)}$ (D40N) | −8 | 0 | [a] |
| $\phi(O^-)$ (D40N) | −10.5 | −2.5 | [a] |
| $S^{RS}_{mini}$ | −3.0 | −1 | [b] |
| $S^{TS}_{mini}$ | −12.7 | −10 | [b] |
| $Eq^{(np)}$ | −6.4 | 0 | [c] |
| $Eq(O^-)$ | −18.3 | > − 8 | [c] |
| $S^{RS}_{full'}$ | −5.5 | ∼0 | [d] |
| $S^{TS}_{full'}$ | −17.2 | −10 | [d] |
| $S^{RS}_{full}$ | −5.5 | 0 | [e] |
| $S^{TS}_{full}$ | −15.0 | −10 | [e] |
| $S^{(np)}_{full'}$ (D40N) | −5.8 | 0 | [f] |
| $S^{(O^-)}_{full'}$ (D40N) | −16.8 | −10 | [f] |

Energies in kcal/mol. Here, $\phi_{np}$, $\phi(O^-)$, Eq $S_{mini}$, $S_{full}$, and $S_{full'}$ designate phenol, the phenolate, equilenin, 3-Cyclohexen-1-one, 5(10)-estrene-3,17-dione and 5-androstene-3,17-dione, respectively.

[a]The binding energy of the ionized phenolate, $\phi(O^-)$, is taken from (8). The electrostatic (plus charge transfer) contribution is calculated in (10). The binding of the nonpolar form of the phenolate, $\phi(O^-)_{(np)}$ (or that of the phenol, $\phi_{(np)}$) is estimated by the calculations of Warshel et al. (10) and from the experimental considerations in the text. A lower limit is obtained from the thermodynamic cycle of Fig. S3.

[b]The binding of the reactant state (RS) of $S_{mini}$ is estimated to be ∼ − 3 kcal/mol, by considering the result from $K_m$ as a upper limit (> − 0.6 kcal/mol), and the requirement to not be drastically different than the binding of $S_{full}$ (see the text, and also note that the exact value would not change the analysis reported in this work). The TS binding is evaluated by $\Delta G^{TS}_{bind} = \Delta G^{RS}_{bind} + \Delta g^{\neq}_{cat} - \Delta g^{\neq}_{w} = -3.0 + 16.50 - 26.2 = -12.7$ kcal/mol. Here $\Delta g^{\neq}_{cat}$ and $\Delta g^{\neq}_{w}$ are taken from the rate constants reported in ref. 12. The electrostatic contribution is estimated from the present calculations, and the calculated contribution is ∼ − 10 kcal/mol.

[c]The binding of the $Eq^{(np)}$ is assumed to be equal to the observed $K_s$ (13) of $S_{full'}$ to the mutant D40N, where $K^{np}_s$ is the binding of the RS of the $S_{full'}$ to D40N. The binding of the ionized form is evaluated in this work from the available experiments. The electrostatic contribution is evaluated from the observed $pK_a$ shift (see the main text for discussion).

[d]The binding of $S_{full'}$ (5-androstene-3,17-dione) is taken from $K_D$ of (13). The binding of the TS is evaluated by $\Delta G^{TS} = \Delta G^{RS}_{bind} + \Delta g^{\neq}_{cat} - \Delta g^{\neq}_{w} = -5.5 + 10.3 - 22.0 = -17.2$ kcal/mol, where the activation barrier in the enzyme (10.3 kcal/mol) is taken from ref. 24, the activation barrier in water (22 kcal/mol) is taken from the analysis of refs. 25 and 10), whereas $\Delta G^{RS}_{bind}$ is taken from ref. 24). The electrostatic contribution is evaluated by the calculations of Warshel et al. (10).

[e]The binding of $S^{RS}_{full}$ is taken from the $K_D$ of $S^{RS}_{full'}$ and the TS binding is evaluated in the same way as for $S^{TS}_{full'}$ (i.e., $\Delta G^{TS} = -5.5 + 16.3 - 25.5 = -14.7$ kcal/mol), using the observed values of $\Delta g^{\neq}_{cat}$ and $\Delta g^{\neq}_{w}$, from the rate constants reported in ref. 12.

[f]The binding of $S^{(np)}_{full'}$ is estimated from the $K_D$ for the corresponding binding to D40N. The binding of $S^{(O^-)}_{full'}$ is taken (from figure 2 of ref. 26) to be the difference between the free energies of the bound intermediate and the intermediate in water (i.e., −11.0 − 5.8 = −16.8 kcal/mol).