# Exploring challenges in rational enzyme design by simulating the catalysis in artificial kemp eliminase

**Maria P. Frushicheva, Jie Cao, Zhen T. Chu, and Arieh Warshel[1]**

Department of Chemistry, 418 SGM Building, University of Southern California, 3620 McClintock Avenue, Los Angeles, CA 90089-1062

Contributed by Arieh Warshel, July 19, 2010 (sent for review June 25, 2010)

**One of the fundamental challenges in biotechnology and in biochemistry is the ability to design effective enzymes. Doing so would be a convincing manifestation of a full understanding of the origin of enzyme catalysis. Despite an impressive progress, most of the advances on this front have been made by placing the reacting fragments in the proper places, rather than by optimizing the environment preorganization, which is the key factor in enzyme catalysis. Rational improvement of the preorganization would require approaches capable of evaluating reliably the actual catalytic effect. This work takes apreviously designed kemp eliminases as a benchmark for a computer aided enzyme design, using the empirical valence bond as the main screening tool. The observed absolute catalytic effect and the effect of directed evolution are reproduced and analyzed (assuming that the substrate is in the designed site). It is found that, in the case of kemp eliminases, the transition state charge distribution makes it hard to exploit the active site polarity, even with the ability to quantify the effect of different mutations. Unexpectedly, it is found that the directed evolution mutants lead to the reduction of solvation of the reactant state by water molecules rather that to the more common mode of transition state stabilization used by naturally evolved enzymes. Finally it is pointed out that our difficulties in improving Kemp eliminase are not due to overlooking exotic effect, but to the challenge in designing a preorganized environment that would exploit the small change it charge distribution during the formation of the transition state.**

computer aided enzyme design | empirical valence bond | directed evolution

Rational enzyme design is expected to have a great potential in industrial application and eventually in medicine (1). Furthermore, the ability to design efficient enzymes might be considered as the best manifestation of a true understanding of enzyme catalysis. However, at present there has been a limited success in most attempts of rational enzyme design, and the resulting constructs have been much less effective than the corresponding natural enzymes (1). Furthermore, despite the progress in directed evolution (e.g., ref. 2), we do not have unique rationales for the resulting rate enhancements.

Most attempts to identify the problems with the current rational design approaches (for review, see ref. 1) have not been based on actual simulations of the given effect. In fact, it has been argued (3, 4), that the problems are due to the incomplete modeling of the transition state (TS) and to the limited awareness to the key role of the reorganization energy. Even a recent attempt to use a molecular orbital-combined quantum mechanical /molecular mechanics (MO-QM/MM) approach (5) has not provided a reasonable estimate of the observed catalytic effect or the trend of the mutational effects in an artificially design enzyme. Thus, reproducing the effect of directed evolution and eventually obtaining better performance in enzyme design, are crucial for understanding what is exactly missing in current approaches.

Semiquantiative computational studies of the effect of mutations on enzyme catalysis date back to the empirical valence bond (EVB) simulations of the anticatalytic effect of mutations of trypsin (6). Subsequent calculations of known and predicted mutational effect include EVB studies (e.g., refs. 4 and 7–9) and more recent (MO-QM/MM) studies (e.g., refs. 10–13). The above studies, and in particular the quantitative one, have established the importance of the changes in reorganization energy upon mutations (4, 8). Recent study (3) has explored the ability of the EVB approach to be used in quantitative screening of design proposals. However, such approaches have not been used in actual predictive design studies, and we are not aware of any study that used the ability to determine activation barriers quantitatively as a successful guide in subsequent enzyme design experiments.

We would like to clarify that it is not extremely difficult to use the EVB to reproduce the overall catalytic effect or to suggest mutations with large anticatalytic effect that will reduce the overall catalysis. This was demonstrated in several enzymes (e.g., refs. 3 and 7). However, it is much harder to suggest mutations that will drastically increase catalysis. Thus we tried to explore this challenge and chose as a test system the Kemp elimination reaction (14). The design of enzymes that catalyze this reaction has been the center of recent excitements (15, 16), but as will be argued below the design has not obtained effective transition state stabilization. Our study focused on both validation of the power of the EVB and more importantly on exploring the effect of the directed evolution and on the requirement for improving the catalysis of the previously designed enzymes.

## 1 Results

**1.1 Systems.** The system chosen as a benchmark for the present study is the Kemp elimination reaction (14) of 5-nitrobenzioxazole with a carboxylic acid as a base (Fig. 1). To explore the catalysis in this system we quantified first the energetics of the reaction in water (4). Here one may try to use the rate constant of the uncatalyzed reaction in water [$1.2 \cdot 10^{-6}$ $M^{-1}$ $s^{-1}$ at pH = 7.25 (16)], but this reaction involves a large contribution from the hydroxide ion $OH^-$ available at this pH (14). Furthermore, as discussed repeatedly (e.g., ref. 4) it is much more useful and relevant to consider the "chemically filtered" reference reaction (4), which is defined as the solution reaction that follows exactly the same mechanism as the one in the enzyme. The corresponding rate constant was determined by taking the estimate (17) of the rate of the reaction of 5-nitrobenzioxazole in acetate buffer ($5 \cdot 10^{-5}$ $M^{-1}$ $s^{-1}$) and extrapolating it to 55M to obtain the rate constant ($k_{cage}$) of 0.0025 $s^{-1}$, for the case when the donor and acceptor [the substrate (see SI Text for more rigorous consideration of $k_{cage}$) and a carboxylic acid] are placed at the same solvent cage. Similar conclusions were obtained by other estimates (*SI Text*), including ab initio calculations that determine the solution surface (Fig. S1) and charge distribution (Fig. S2). The relevant activation barrier is given in *SI Text* and Table 1. It must be emphasized that we are not talking on $k_{cat}/K_M$ relative to the

Author contributions: A.W. designed research; M.P.F., J.C., Z.T.C., and A.W. performed research; M.P.F., J.C., and A.W. analyzed data; and M.P.F., J.C., and A.W. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: warshel@usc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1010381107/-/DCSupplemental.

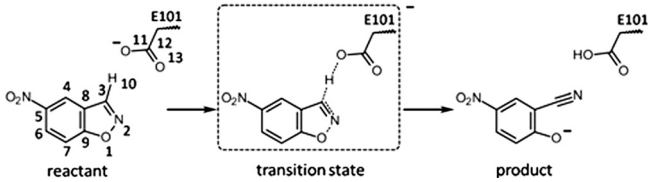


**Fig. 1.** A schematic description of the kemp elimination reaction.

uncatalyzed reaction, where the effect of the enzyme is larger, because this rate enhancement includes the well understood effect of binding to the active site (4) and the real challenge is to optimize $k_{cat}$.

Interestingly, the reference reaction in a solvent cage is not much slower than the reaction catalyzed by the originally designed enzyme ($k_{cat} = 0.02–0.3\ s^{-1}$) (see ref. 16). This indicates that a major part of the catalytic effect is due to just placing the donor and acceptor in a contact distance (see also ref. 15).

**1.2 Validation on Existing Constructs.** To validate our EVB approach we started by trying to reproduce the observed $k_{cat}$. This was done by calibrating an EVB surface for the reaction in solution (by forcing it to reproduce the observed activation barrier and calculated exothermicity) and using the calibrated EVB parameters (Table S1) in studies of the reaction in the protein. The initial structures of the "native" and mutant proteins were taken from the corresponding X-ray structures (2). The reacting substrates were placed in each active site in a similar orientation to that in the design KE07 model and relaxed. The resulting orientations (e.g., Fig. 2) are similar to those in the original design. Twenty structures were saved during each relaxation process, and then used to generate the EVB surfaces and obtain the activation free energies. The calculated activation barriers and the corresponding observed values are given in Table 1 and Fig. 3, while some reaction profiles are shown in Fig. S3. As seen from the table, we obtained a good agreement between the calculated and observed catalytic effects. We also explored the catalytic effect in the structure of the initial design and 34E4 catalytic antibodies (see *SI Text*).

One of the interesting results of our analysis is the finding that the initial design (KE07) has not lead to significant rate enhancement above that effect expected from placing the acid and the base at the same cage (see Table 1).

**1.3 Initial Screening.** To obtain an improved rational design it is crucial to be able to explore the contribution of each residue to the TS energy. Because the electrostatic effect is by far the most important factor in enzyme catalysis (4), we started with an attempt to estimate the electrostatic contributions expected from each residue. This was done by evaluating the effect of changing

**Table 1. The $\Delta g^{\ddagger}_{cat}$ for the systems explored in this work***

| System: | $\Delta g^{\ddagger}_{obs}$ kcal/mol | $\Delta g^{\ddagger}_{calc}$ kcal/mol |
|---|---|---|
| Water (cage) | 21.2 | 21.1 |
| KE07 (wt, PDB: 2RKX) | 20.1 | 19.5 (20.2) |
| KE07 (wt, PDB: 2RKX)+H2O | 20.1 | 20.4 |
| R6 3/7 F (PDB: 3IIP) | 17.8 | 16.1 |
| R7 2/5 B (from R6 3/7 F) | 17.6 | 18.0 |
| KE07 variant (PDB: 1 THF) | — | 24.0† |
| A9S (from wt) | — | 19.3 (17.2) |
| A9N (from wt) | — | 19.5 (17.5) |
| 34E4 (PDB: 1Y0L) | 17.9 | 17.3 |
| Y32K (34E4 mutant, from 34E4) | 19.5 | 19.9 |

*In brackets, the results with the qualitative charge set (set B) whereas all other results correspond to the quantitative set (set A).

†Note this calculation corresponds to the template structure rather than the structure of the actual KE07 (see *SI Text*).

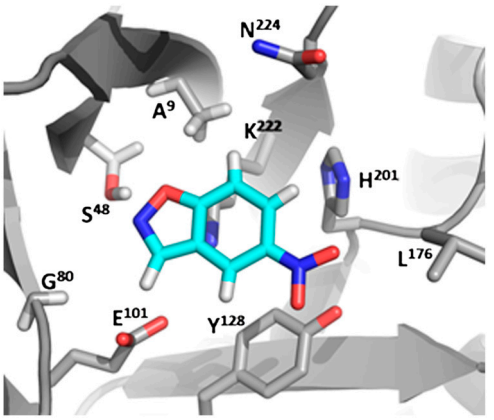


**Fig. 2.** The structure of the native enzyme (PDB: 2RKX) with the docked substrate.

the substrate charges [upon moving from the reactant state (RS) to the TS] on the contribution of each residue. More specifically we assigned (artificially) to each protein residues a charge of +1 and then calculated the change in the corresponding "group contribution" when the residual charges (or polarity) of the reacting substrate changes. The change, $\Delta G_i$, is then used to estimate the optimal change in the charges (or polarity) of the protein residues and thus the optimal mutations.

Our approach is based on the realization that the electrostatic contributions of the protein residues to the activation barrier can be very roughly approximated by (3, 18)

$$\Delta\Delta g^{\neq}_{elec} \cong 332 \sum_{ij} \sum_{k} (q^{k}_{j} \Delta Q_i)/r_{ij}\epsilon_{ij} \qquad [1]$$

where $q^{k}_{j}$ are the residual charges of the protein atom, $j$ runs over the protein residues , $k$ runs over the atoms of the $j^{th}$ residue, and $i$ over the substrate atoms, whereas the parameter $\epsilon_{ij}$ is the dielectric constant for the specific interaction. The $\Delta Qs$ are the changes in the substrate charges upon going from the RS to the TS (these charges are given in Fig. S2).

As described in *SI Text*, we can use Eq. **1** for a estimating the most effective charge changes in each site and this was done in examining the trend with two charge sets (set B that used the ab initio RS and PS as the corresponding EVB charges for the RS

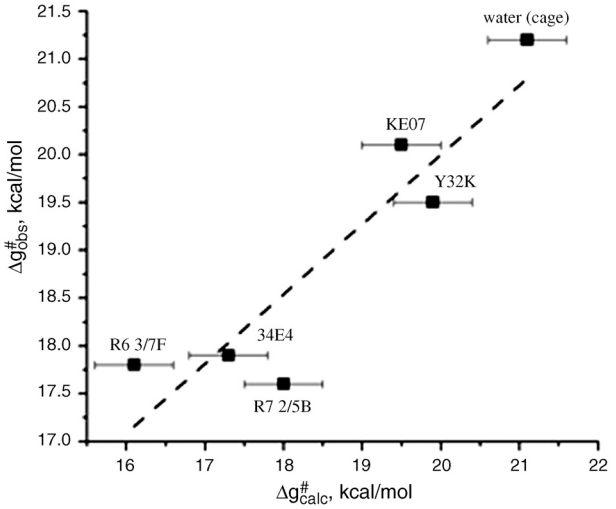


**Fig. 3.** Correlation between the calculated and observed activation barriers.

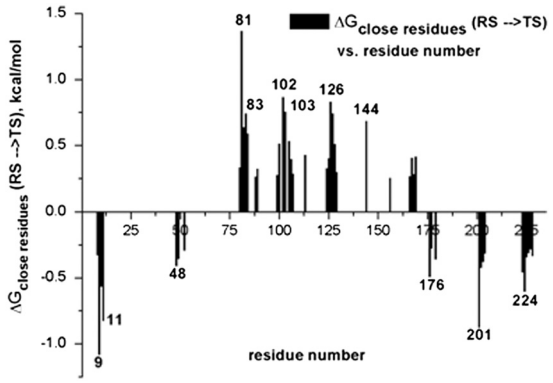


**Fig. 4.** The group contributions (in kcal/mol) that reflect the interactions between the protein residues with the charge change upon moving from the RS to the TS (the $\Delta Q$s in Eq. **1**). The calculations are done with $\varepsilon_{ij} = 4$ for all residues. The largest negative contributions provide a rough guide for the optimal sites for effective mutations that would enhance the catalytic effect.

and PS, and a much more rigorous set (set A), that forced the EVB TS charges to reproduce the ab initio TS charges. The resulting group contributions for set A is given in Fig. 4. As pointed out in the *SI Text*, it appears that the TS charges in the Kemp elimination reaction do not follow a simple rule of Set B [being the average of the RS and product state (PS) charges] and apparently the charge change on O1 is unexpectedly small (see Fig. S2).

It is instructive to examine the potential due to the $\Delta Q_i$ of each of the atoms of the reacting system, and the corresponding group contributions. These features, which are depicted in Fig. 5 suggest that the TS may be stabilized by placing a positive charge or a hydrogen bond near the regions were the negative charge is developing during the proton transfer process. There is also an option to stabilize the positive charge developing on Glu101. This can be also done by destabilizing the reactant charge. Unfortunately, the group contributions are quite small in the case of the Kemp elimination reaction and do not provide a clear hint for a mutation with a large effect (see below).

**1.4 Analysis of Suggested Mutations.** Trying to exploit the hints from the calculated group contributions we examined first the intuitive hints of the qualitative TS charge set (Set B). This was done by performing EVB calculations with set B, for mutations of residue 9 to polar residues that may stabilize the charge change in O1 at the TS. The initial calculated increase in TS stabilization for the mutations is given in brackets in Table 1. As seen from the table the A9S mutant (with the qualitative set B) is predicted to increase the catalytic effect.

Considering the very large predicted catalytic effect at residue 9 as a likely overestimate we looked for pitfalls, starting with the possibility of having a water molecule in the space between Ala 9 and O1. However, our calculations (with set B) did not change significantly the activation barrier of the native enzyme. We also explored the possibility (and concern) that the ligand orientation is incorrect (see *SI Text*) and found reasonable support for the current model. Therefore, it seems that set B charges predicted a large effect of polar groups on the supposedly developing TS charge of O1. However, this still seems to be an overestimate because the calculated effect was larger than the effect found in related studies in catalytic antibodies. Thus we considered the fact that the TS charges cannot be represented by the intuitive set and we moved to the more realistic (and nonintuitive) set A. Now we obtained the results listed in Table 1 (without brackets), where almost all the predicted effect of polar stabilization of the O1 developing charge disappeared.

Considering the difficulties in obtaining a major effect by improving the active site polarity, we looked for possible hints from the directed evolution experiments (here we focused on R6 3/7 F). Our starting point was the fact that we succeed,

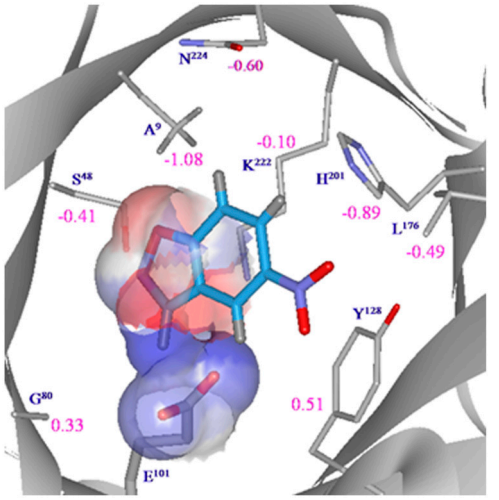


**Fig. 5.** The electrostatic potential form the change of the substrate charges upon moving from the RS to the TS (red and blue for negative and positive potential respectively) and group contributions (in kcal/mol) of key residues, where the contributions are defined for the case where each residue is positively charged.

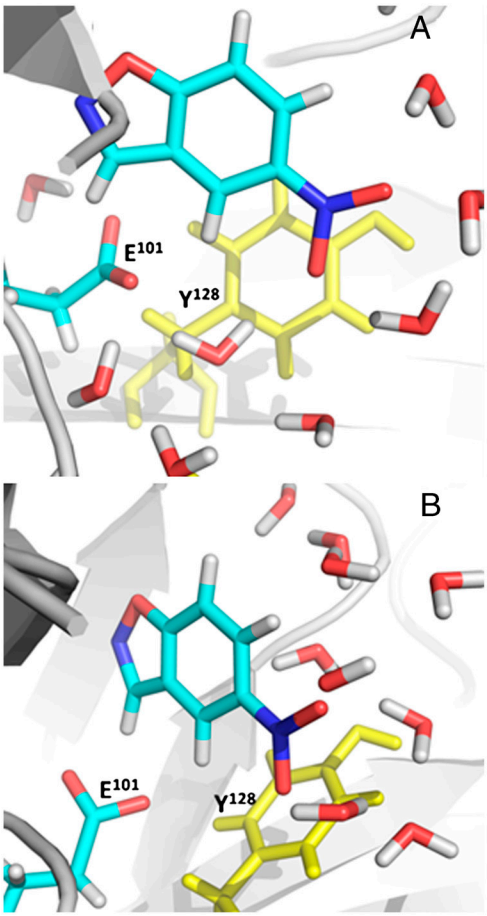


**Fig. 6.** Showing the water penetration near Glu101 in the wt and the R6 3/7 F mutant. As seen from the figure there are fewer water molecules near Glu101 in the mutant.

for the first time, to reproduce computationally the observed effect and thus have the tools to examine its origin. It was found that the mutant has an early TS (see Fig. S3), due to both small destabilization of the reactant state and stabilization of the product state. Apparently, whereas the water molecules play an important role in the wt, they provide less solvation of the reactant state charges of the mutant (see Table S2 and Fig. 6). However, even the directed evolution did not find a major way of TS stabilization (see below).

**1.5 What Can Be Learned from Related Systems.** The Kemp elimination reaction is formally similar to other reactions where a negative charge is being transferred from the ionized acid to the substrate. A good example is the reaction of haloalkane dehalogenase (DhlA) (19), where we have a negatively charged Asp group in the RS [(−) (0)] configuration transfers its negative charge to a leaving group (Cl) and form a [(−1/2)(−1/2)] configuration in the TS (here the first and second brackets correspond to the charge on Asp and on the Cl atom). In this case we have shown by a quantitative linear response approximation (LRA) analysis (19) that, whereas in solution the system loses a very large solvation energy upon moving to the TS, in the enzyme the loss of solvation energy is much smaller, resulting (see Table S2) in a major TS stabilization (relative to the solution reaction). This is accomplished by the preorganization of two polar hydrogen bonds (Trp125 and Trp175) that stabilizes the −1/2 charge of the leaving Cl atom. Unfortunately, in the case of the Kemp elimination, the charge that is being transferred to the substrate is much more delocalized and thus making it much harder to obtain larger preorganization effects (see also ref. 20). Here (see Table S2) it seems that the solution found by the directed evolution involves reducing the solvation of the reactant state by water molecules. This does not involve significant reactant state destabilization because $K_M$ increases by only a factor of 3 (which means that the binding energy is similar in the native and mutant enzyme). However, the destabilization of the ionized Glu101 chosen here can also be consider as a reactant state destabilization effect, but it is clearly not the standard avenue chosen by DhlA and other naturally evolved enzymes (see systematic analysis in Table S2). One key difference is the ability of the water molecules to approach the proton donor (see Fig. 6). Here a change of the enzyme scaffold to block entrance of water molecules near Glu101 can be useful. Perhaps replacing Asn103 by a larger residue will be effective in improving the wt enzyme.

**1.6 Prospect for Computer Aided Directed Evolution.** Our previous studies (e.g., refs. 4 and 21) have indicated that the effect of ionized residues at a distance of more than 6 Å can be approximated by using a relatively large effective dielectric constant for charge-charge interaction. Here we can use the approach of Eq. **S5** and obtain rather reliable prediction of the effect of ionized residues (see discussion in *SI Text* of Figs. S4 and S5 and Tables S3 and S4). Unfortunately, at present we do not have experimental information about single mutants of distanced ionized residues (see *SI Text*), and only actual evaluation of $k_{cat}$ will provide a validation of these predictions. Furthermore, unfortunately the dipole generated by the $\Delta Qs$ is relatively small and is not predicted to give substantial interaction with distanced ionized residues. Perhaps using the KE70 mutants where the proton acceptor is a histidine can lead to a larger dipole effect.

A further constraint on the design can be imposed by introducing mutations that compensate for the loss of stability during the refinement process (see discussion in ref. 2). This can be done by our recent focused dielectric approach (22) and the resulting optimized $q_j$, under the constraint of large folding energy, is (see *SI Text*)

$$(\Delta\overline{q_j}) \simeq -\partial G_{\text{fold}}/\partial\overline{q_j} - \alpha\partial\Delta\Delta g^{\neq}_{\text{elec}}/\partial\overline{q_j} \qquad [2]$$

where now (see SI Text) we grouped all the charges of each residue to one effective charge. It would be interesting to use this approach in an iterative way as a model for directed evolution.

## 2. Concluding Remarks.

The rational design of enzymes with native activity requires the ability to predict the proper TS stabilization, and this involves the challenge of predicting the overall preorganization effect. Attempts to estimate the catalytic effect by using gas phase models or even by looking at the electrostatic interaction between different residues and the TS are unlikely to reproduce the correct catalytic effect because it is impossible to assess the preorganization effect without including the protein and its reorganization in the simulations.

The challenge of evaluating the catalytic power of a given mutant is not different than that addressed in our early 1986 study of computer aided mutations (6). At this stage it seems to us that the potential of the EVB has been demonstrated in well defined cases (e.g. refs. 3 and 23), where it was found to reproduce the large effects of mutations that destroy the catalytic effect of evolved enzymes. Thus our main current challenge is to use this approach in improving nonefficient enzymes.

Attempting the advance computer aided enzyme design, we used first the EVB method in the study of the nature of the observed catalytic effect in the wild type (WT) and mutated enzymes. Obviously this major requirement cannot be accomplished by gas phase models, but more significantly it has not been accomplished by alternative MO-QM/MM studies (5), which yielded large discrepancies between the calculated and observed catalytic effects. Our ability to capture the observed trend established the effectiveness of the EVB in reliable enzyme modeling, even when the catalytic effects are small.

The next challenge is the understanding of the origin of the catalytic effect and hopefully helping in increasing this effect. In this respect it is useful to reemphasize that the initial constructs of the Kemp eliminase benefited mainly from the catalytic effect associated with placing the donor an acceptor at a closed proximity in the same cage, without the optimization of environmental preorganization effects. This cage effect has been understood almost quantitatively by the key early workers in the field (e.g., refs. 24 and 25), since the 70s, and it is a well known factor that has little to do with the secret of enzymatic reactions (4). As to other factors that were discussed in previous design efforts, we would like to clarify that TS stabilization by delocalization effects (16) is unlikely to provide a significant catalytic factor because the same effect exists in the reference solution reaction (see *SI Text*).

Using the hints from the group contribution obtained with the qualitative initial EVB charge set, we attempted to refine the electrostatic environment near O1. This attempt can be considered by some as an extension of the idea of placing an acid near O1 (2, 26), but having an acid in this site is a reminiscence of the so called low barrier hydrogen bond (LBHB) effect, whose anticatalitic effect is discussed elsewhere (4), or to the assumption that enzyme work by covalent catalysis, whose problems have been discussed in ref. 4. Furthermore, the support for this idea comes mainly from gas phase calculations (27), which tend to drastically overestimate LBHB effects. Thus, unless we deal with a new reaction path, with a proton transfer from the acid, we will not benefit from having an acid instead of a preorganized hydrogen bond. Thus we focused on the electrostatic stabilization of the TS by the nearby polar groups.

Surprisingly, focusing on the intuitive idea of increasing the polarity of residue 9 and the stabilization of the developing charge on O1 has not generated rate acceleration. Here we were forced by the results of the mutation experiments and by examining the ab initio TS charges to move to a more consistent EVB

charges that, in fact, consistently accounted for the absence of observed mutational effects.

In analyzing the above findings we noted that the Kemp reaction is not the best system for demonstrating the role of optimizing the active site environment, because the charge that is being transferred to the substrate upon proton transfer is delocalized in a way that does not lead to large group contributions and to simple hints about clear sites for possible effective TS stabilization (see Fig. 4). This type of problem could have been anticipated in fact in view of the study of ref. 20.

Furthermore, as we found out in this work, even the current directed evolution cycles have not exploited any major electrostatic stabilization of the TS, basically using the change in solvation by water plus small changes in "solvation" by the protein polar groups to provide small RS destabilization and small TS stabilization. At present is seems that the lack of clear sites with large change of charge distribution in the RS-TS transition (e.g., oxyanion type site) makes it hard for the enzyme to generate major catalytic effects. However, enzymes that have evolved during a long time found ways to exploit electrostatic effects, even when the charge changes are not localized; the best example is vitamin B12 enzymes, where the enzyme found a remarkable way to exploit electrostatic TS stabilization with no change in the charge distribution of the bond that is being broken (see ref. 28). Overall, it is not clear if directed evolution explores all options and it is clear the group contribution approach cannot be very effective with the TS charge distribution of our system (see, however, below). At any rate, while addressing the challenge of improving poor enzymes it should also be useful to take optimal and highly catalytic enzymes and then to predict which mutations will have the largest effect on reducing the catalysis.

Regardless of the small effect of distant residues we also see major potential in simple refinement of long-range electrostatic interactions. This strategy (*Results*, section 1.6) can be particularly instructive once it is connected with directed evolution experiments, and it can be used in a systematic refinement with small incremental increase in catalysis.

At present there are still many who assume that dynamical and other esoteric effects contribute to catalysis (for review see ref. 29). In many cases it is clearly suggested that improving such effects will be crucial for optimal enzyme design (e.g., ref. 30). However, it seems to us that by far the main factor that actually contributes to catalysis is the preorganization effect and thus we feel that there is no rational way for improving dynamics and related effects as these factors do not contribute to catalysis (29). We also suspect that factors such as π stacking stabilization are unlikely to be effective in providing significant TS stabilization, beyond the simple effect of induced dipoles. Thus the focus should be placed on the refining of the polar reorganization, as we attempted to do in this work.

Apparently the directed evolution has tried the "trivial" solution of desolvating the reactant state. Although, this type of catalysis is not used by naturally evolving enzymes (see Table S2) it might assumed by some that it provides a support for the long held desolvation hypothesis (see Discussion in ref. 4). However, the very large desolvation effect on the Kemp elimination reaction in say $CH_3CN$ (20) is not even mildly achieved in the Kemp eliminase. Here it is important to realize that the desolvation hypothesis reflects an incorrect thermodynamic cycle (see ref. 4), where one missed the free energy of moving the reactant state between water to the less polar environment. Thus it is actually more interesting to ask why the directed evolution used such an uneffective strategy, than to incorrectly assume that we have here a support to the desolvation proposal.

We would also like to emphasize that the present calculations were done with the assumption that the substrate is in the designed site with Glu101 as a base. This assumption has not been proven, but our calculations of the 34E4 catalytic antibody seem to establish our ability to reproduce the observed catalysis when the structure is certain. Finally, we would like to reclarify that we have demonstrated the ability to reproduce quantitatively the absolute catalytic effects and mutational effects in naturally evolved enzymes (4) and in designer enzymes (this work). This clearly indicates that the catalytic power of enzyme is not due to elusive effects (e.g., conformational dynamics), but to what is by now well understood; the electrostatic preorganization. Thus our difficulties in improving designer enzymes are not due to overlooking misunderstood factors, but to the difficulties in optimizing well understood factors. In other words, a method that reproduces the catalytic rate in known systems should be able to do so in any unknown sequence and the challenge is to find the unknown optimal sequence. At any rate, it seems to us that the present study provided a useful analysis of the reasons for the less than perfect performance of current designer enzymes.

## 3. Methods

The free energy surface of the reference solution reaction was estimated by ab initio calculations, in the same way described in our previous works (e.g., ref. 31), and the resulting surface is shown in Fig. S1. The ab initio effective charges of the RS, TS, and PS are given in Fig. S2. In converting the ab initio results to the corresponding EVB we have to take into account the complex nature of the reaction with a concerted proton transfer and C-N bond breaking. Usually this requires three diabatic states, but for the present purpose we decided to use two states with modified charges that reproduce the ab initio TS charges (see *SI Text*). The EVB calculations were carried by MOLARIS simulation program (32) using the ENZYMIX force field. The EVB activation barriers were calculated by the same free energy perturbation umbrella sampling (FEP/US) approach used in all previous studies. The simulation systems were solvated by the surface constrained all atom solvent (SCAAS) model (32) using a water sphere of 18 Å radius centered on the substrate and surrounded by 2 Å grid of langevin dipoles and then by a bulk solvent, whereas long-range electrostatic effects were treated by the local reaction field method (32). The EVB region consisted of the substrate and the carboxylic group of the glutamic amino acid that serves as a proton acceptor. Validation studies were done using 22 Å radius of inner sphere. The FEP mapping was evaluated by 21 frames of 20 ps each for moving along the reaction coordinate using SCAAS model. All the simulations were done at 300 K with a time step of 1 fs. To obtain reliable results the simulations were repeated 20 times with different initial conditions (obtained from arbitrary points in the relaxation trajectory). The mutant systems were generated from the systems listed in Table 1 via 100 ps relaxation runs.

**ACKNOWLEDGMENTS.** We thank Dan Tawfik, Olga Khersonsky, Don Hilvert, and Monica Fuxreiter for stimulating discussion. We thank the University of Southern California's High Performance Computing and Communication Center (HPCC) for computer time. This work was supported by National Institutes of Health Grant GM24492.

1. Toscano MD, Woycechowsky KJ, Hilvert D (2007) Minimalist active-site redesign: Teaching old enzymes new tricks. *Angew Chem Int Edit* 46:4468–4470.
2. Khersonsky O, et al. (2010) Evolutionary optimization of computationally designed enzymes: Kemp eliminases of the KE07 series. *J Mol Biol* 396:1025–1042.
3. Roca M, Vardi-Kilshtain A, Warshel A (2009) Toward accurate screening in computer-aided enzyme design. *Biochemistry* 48:3046–3056.
4. Warshel A, et al. (2006) Electrostatic basis for enzyme catalysis. *Chem Rev* 106:3210–3235.
5. Alexandrova AN, Rothlisberger D, Baker D, Jorgensen WL (2008) Catalytic mechanism and performance of computationally designed enzymes for Kemp elimination. *J Am Chem Soc* 130:15907–15915.
6. Warshel A, Sussman F (1986) Toward computer-aided site-directed mutagenesis of enzymes. *Proc Natl Acad Sci USA* 83:3806–3810.
7. Hwang JK, Warshel A (1987) Semiquantitative calculations of catalytic free energies in genetically modified enzymes. *Biochemistry* 26:2669–2673.
8. Liu H, Warshel A (2007) The catalytic effect of dihydrofolate reductase and its mutants is determined by reorganization energies. *Biochemistry* 46:6011–6025.
9. Warshel A (1991) Computer modeling of chemical reactions in enzymes and solutions. (Wiley Interscience, New York).
10. Grigorenko BL, Nemukhin AV, Topol IA, Cachau RE, Burt SK (2005) QM/MM modeling the Ras-GAP catalyzed hydrolysis of guanosine triphosphate. *Proteins* 60:495–503.
11. Marti S, et al. (2007) Computer-aided rational design of catalytic antibodies: The 1F7 case. *Angew Chem Int Edit* 46:286–290.

12. Marti S, et al. (2008) Predicting an improvement of secondary catalytic activity of promiscuos isochorismate pyruvate lyase by computational design. *J Am Chem Soc* 130:2894–2895.
13. Mulholland AJ (2008) Computational enzymology: Modelling the mechanisms of biological catalysts. *Biochem Soc T* 36:22–26.
14. Kemp DS, Casey ML (1973) Physical organic chemistry of benzisoxazoles II. Linearity of the bronsted free energy relationship for the base-catalyzed decomposition of benzisoxazoles. *J Am Chem Soc* 95:6670–6680.
15. Debler EW, et al. (2005) Structural origins of efficient proton abstraction from carbon by a catalytic antibody. *Proc Natl Acad Sci USA* 102:4984–4989.
16. Rothlisberger D, et al. (2008) Kemp elimination catalysts by computational enzyme design. *Nature* 453:190–195.
17. Thorn SN, Daniels RG, Auditor MT, Hilvert D (1995) Large rate accelerations in antibody catalysis by strategic use of haptenic charge. *Nature* 373:228–230.
18. Muegge I, Schweins T, Warshel A (1998) Electrostatic contributions to protein–protein binding affinities: Application to Rap/Raf interaction. *Proteins* 30:407–423.
19. Olsson MH, Warshel A (2004) Solute solvent dynamics and energetics in enzyme catalysis: The S(N)2 reaction of dehalogenase as a general benchmark. *J Am Chem Soc* 126:15167–15179.
20. Hollfelder F, Kirby AJ, Tawfik DS (2001) On the magnitude and specificity of medium effects in enzyme-like catalysts for proton transfer. *J Org Chem* 66:5866–5874.
21. Warshel A (1987) What about protein polarity? *Nature* 330:15–16.
22. Vicatos S, Roca M, Warshel A (2009) Effective approach for calculations of absolute stability of proteins using focused dielectric constants. *Proteins* 77:670–684.
23. Shurki A, Warshel A (2004) Why does the Ras switch "break" by oncogenic mutations? *Proteins* 55:1–10.
24. Rogers GA, Bruice TC (1974) Synthesis and evaluation of a model for the so-called "charge-relay" system of the serine esterases. *J Am Chem Soc* 96:2473–2481.
25. Schowen RL (1978) *Transition States in Biological Processes* (Plenum, New York).
26. Debler EW, Muller R, Hilvert D, Wilson IA (2009) An aspartate and a water molecule mediate efficient acid-base catalysis in a tailored antibody pocket. *Proc Natl Acad Sci USA* 106:18539–18544.
27. Na J, Houk KN, Hilvert D (1996) Transition state of the base-promoted ring-opening of isoxazoles. Theoretical prediction of catalytic functionalities and design of haptens for antibody production. *J Am Chem Soc* 118:6462–6471.
28. Sharma PK, Chu ZT, Olsson MH, Warshel A (2007) A new paradigm for electrostatic catalysis of radical reactions in vitamin B12 enzymes. *Proc Natl Acad Sci USA* 104:9661–9666.
29. Kamerlin SC, Warshel A (2010) At the dawn of the 21st century: Is dynamics the missing link for understanding enzyme catalysis? *Proteins* 78:1339–1375.
30. Nagel ZD, Klinman JP (2009) A 21st century revisionist's view at a turning point in enzymology. *Nat Chem Biol* 5:543–550.
31. Kamerlin SC, Warshel A (2009) On the energetics of ATP hydrolysis in solution. *J Phys Chem B* 113:15692–15698.
32. Lee FS, Chu ZT, Warshel A (1993) Microscopic and semimicroscopic calculations of electrostatic energies in proteins by the Polaris and Enzymix programs. *J Comput Chem* 14:161–185.