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The role of reorganization energy in rational enzyme design☆

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Computational design is becoming an integral component in developing novel enzymatic activities. Catalytic efficiencies of man-made enzymes however are far behind their natural counterparts. The discrepancy between laboratory and naturally evolved enzymes suggests that a major catalytic factor is still missing in the computational process. Reorganization energy, which is the origin of catalytic power of natural enzymes, has not been exploited yet for design. As exemplified in case of KE07 Kemp eliminase, this quantity is optimized by directed evolution. Mutations beneficial for evolution, but without direct impact on catalysis can be identified based on contributions to reorganization energy. We propose to incorporate the reorganization energy in scaffold selection to provide highly evolvable initial designs.

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Dedicated to Arieh Warshel to congratulate his Nobel prize in Chemistry, 2013. He provided a seminal contribution to use reorganization energy for interpretation of enzymatic catalysis.

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Tailoring activities of biomolecules is a dream for both computational and experimental biochemists. Enzymes that catalyze nonbiological reactions are awaited and utilized in biomedicine and biotechnology. De novo enzyme design comprises two main steps. First a computational process [1,2] provides a model with the desired function, albeit with moderate activity. This is followed by experimental optimization of the initial model by repeated rounds of random mutagenesis and natural selection [3,4]. In general, directed evolution increases k_{cat} by 10^2 to 10^3 fold. Currently, owing to the synergistic effort of computational design and laboratory optimization, artificial enzymes with efficiencies close to that of catalytic antibodies could be engineered, but reaction rates are still far from what has been optimized by Nature [5]. Although the success of a recently evolved Kemp eliminase is promising [6••], enzyme designs still seem to lack major catalytic factors.

Computer-assisted model generation requires an in-depth understanding of structure–function relationships of enzymes. Albeit it has been debated for long, the stabilization of the transition state (TS) is the origin of enzymatic catalysis [7]. The modes and mechanisms of how this is actually achieved however, remain to be clarified [8,9]. Various factors, such as proximity effects [10], acid–base catalysis, near attack conformation [11], strain [12], dynamics [13], desolvation [14] etc. contribute to lowering the activation barrier as compared to solution reactions. The individual effect of these factors is moderate and results in a rate acceleration $< 10^4$ fold. The only factor with major impact on catalysis is the electrostatic preorganization [15••], which can provide 10^7 to 10^{10} fold rate acceleration [16]. On the basis of the Marcus theory electrostatic preorganization can be quantified by the reorganization energy (λ) [17]. This expresses the work of the protein while it responds to changing charge distribution of the reactant along the reaction pathway (Figure 1). Although reorganization energy is the concerted effect of all enzyme dipoles, group contributions could be approximated (see Box 1).

Current design approaches aim to maximize the binding energy of the TS, but do not evaluate the free energy profile of the catalyzed reaction [18]. Thus response of the enzymatic environment to changes in charge distribution from ground state to TS is not correctly represented. Furthermore, steric strain is ignored, if significant deformations between the ground and TS geometries occur. All these effects are critical for the energetics of the reaction and are influenced by the interplay between the active site groups and the enzymatic environment. Hence considering only key interactions in the TS can result in different mechanism in the design and the real enzyme. Catalytic antibodies might provide a misleading impression that a few residues, which contact or located in the proximity of the reactants are sufficient for catalytic activity [19]. Indeed, the efficiencies of enzyme designs with complex scaffolds are comparable that of simple models [20•] or even re-engineered cavities [21]. This suggests that design strategies mostly optimize *proximity* or *medium* effects, which can be exerted by simply

Figure 1

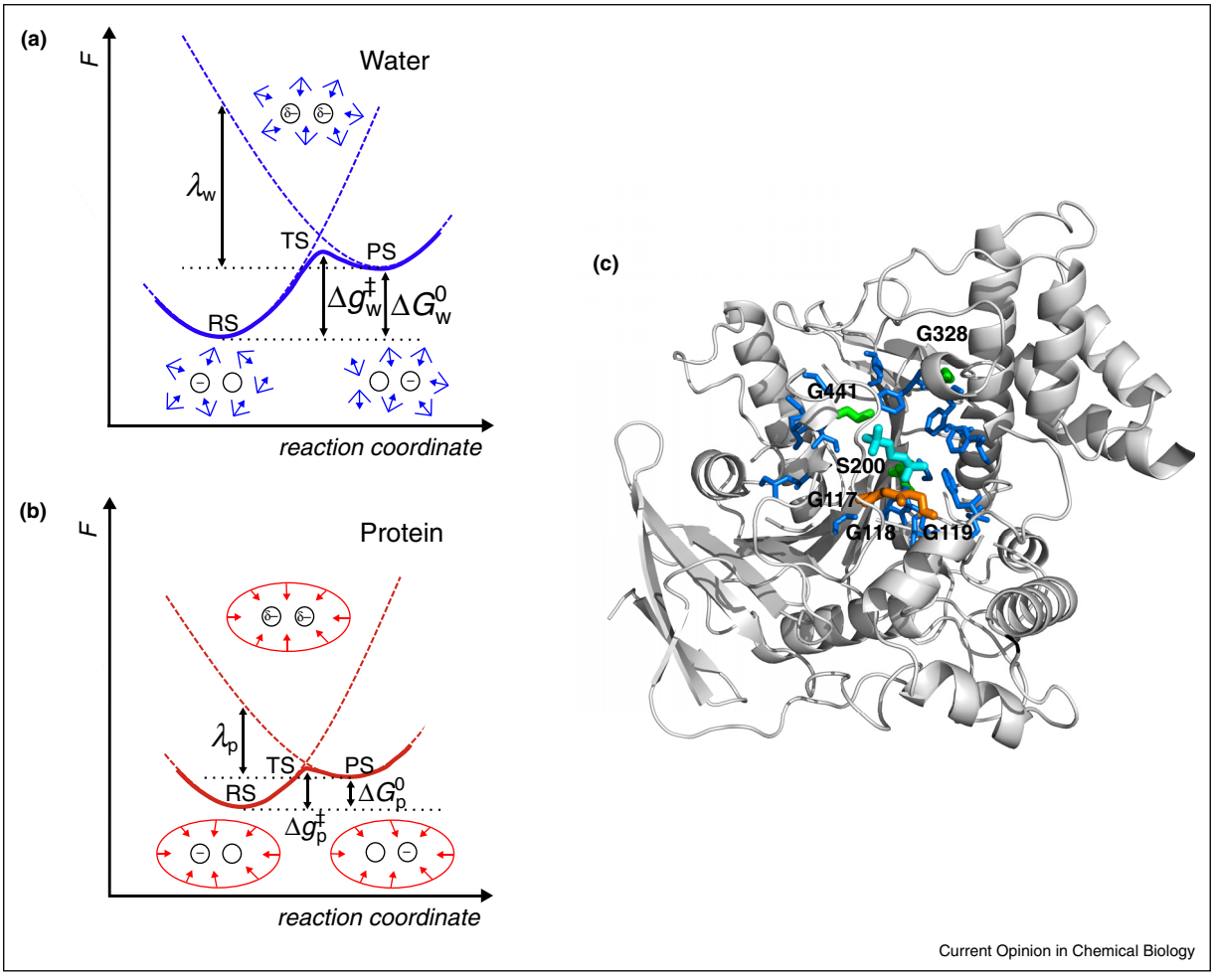


Illustration of the reorganization effect. General scheme of the adiabatic (solid lines) and the diabatic (dashed lines) free energy profiles as functions of a hypothetical reaction coordinate in **(a)** water solution and **(b)** enzymatic environment. The relationship between the activation barrier ($\Delta g^{\ddagger}$), reaction free energy (ΔG°) and reorganization energy (λ) is shown. Schematic pictures at reactant (RS), transition (TS) and product (PS) states represent that reorganization of dipoles is considerably smaller in protein than in solution. **(c)** In acetylcholinesterase (PDB: 1ace) reduction in reorganization energy accounts for 10 kcal/mol out of 15 kcal/mol decrease in the activation barrier [16]. This is mostly due to main chain dipoles of Gly117, Gly118 and Gly119 (orange), which establish hydrogen bonds with the substrate (cyan). Other residues with favorable contributions to catalysis are displayed by green. Although the active site mostly consists of hydrophobic residues (blue), desolvation is destabilizing for the TS.

changing the macroscopic dielectric properties of the system. Activities of enzyme designs are also lowered by structural instabilities (floppiness) [22,23]. Inclusion of flexibility [24] or molecular dynamics (MD) thus significantly improves the efficiency of computed variants [25,26,27•] (see below).

Here we overview the basic concepts, which are implemented in computer-aided enzyme design and assess their performance in directed evolution. We find that electrostatic preorganization is significantly optimized in laboratory as it was quantified in case of KE07 Kemp eliminase [28•]. We exemplify how contributions to reorganization energy could be exploited for screening. We propose that reorganization energy is a missing key catalytic factor in computational design, incorporation of which can be a promising approach to yield highly evolvable enzyme variants.

Design strategies

Computer-aided enzyme design is comprised of three main steps [29]: (i) determination of the TS geometry and optimal arrangement of the key functional groups (theozyme) [30]; (ii) scaffold selection and optimization of the active site environment; (iii) ranking the candidates. De novo design normally utilizes three to four functional groups for catalysis [18] as more complex theozymes can be prohibitory in scaffold selection.

Box 1 The reorganization energy (λ) was introduced by Marcus for electron transfer reactions [17] and establishes relationship between the reaction free energy (ΔG°) and the activation barrier ($\Delta g^{\ddagger}$). It can be approximated as:

$$\Delta g_{ij}^{\ddagger} \cong \frac{(\Delta G_{ij}^{0} + \lambda_{ij})^2}{4\lambda_{ij}} \quad (1)$$

It refers to intersection of free energy functionals of two states (*i,j*), corresponding to reactants and products of an elementary reaction step. In enzymes reorganization energy expresses the effect of pre-oriented dipoles, which upon charging the TS costs significantly less to reorganize than corresponding solvent dipoles [45]. Reorganization energy decrease by enzymes originates in two factors (Figure 4): (i) decreasing ΔG°, (ii) shifting the diabatic free energy functions as compared to each other.

Reorganization energy is computed as the vertical difference between the free energies of the system at reactant and product equilibrium geometries on the diabatic product free energy curve (Figure 1):

$$\lambda = F_{PS}(\xi_{RS}) - F_{PS}(\xi_{PS}) \quad (2)$$

where ξ_{RS} and ξ_{PS} are the values of the reaction coordinate at the reactant and product states and $F_{PS}(\xi)$ is the diabatic product state free energy function.

Computing reorganization energy requires the reactant and product potential energy surfaces, which are available within the framework of the Empirical Valence Bond (EVB) method [46]. According to Eqn (2) reorganization energy can be obtained by moving the system from the reactant to the product states using for example Free Energy Perturbation method and then the diabatic product state can be calculated by the Umbrella Sampling technique. Instead of constructing a full free energy profile, reorganization energy can be obtained using linear response approximation (LRA) [47] that requires configurations only at the reactant and product diabatic states:

$$\lambda \cong \langle \Delta E \rangle_{RS} - \frac{1}{2}(\langle \Delta E \rangle_{RS} + \langle \Delta E \rangle_{PS}) \quad (3)$$

where $\Delta E = E_{PS} - E_{RS}$ is the energy difference between the product and reactant diabatic states and averaged over configurations at the respective states. This approach has been successfully applied to many systems [48,49]. LRA can be employed to determine contributions to the reorganization energy using individual energy terms in Eqn (3) [28•]. Reorganization energy can also be evaluated using hybrid QM/MM methods, where QM is applicable to diabatic potential energy surfaces of reactant and product states [50,51].

Design strategies prioritize shape and charge complementarity. Tight binding of the substrate and optimal orientation of the functional groups constrained for catalysis can be achieved by increasing the *packing* at the active site. In the most active design for stereoselective bimolecular Diels-Alder reaction, the theozyme was grafted on a six bladed β-propeller scaffold (PDB id: 1E1A), the active site pocket of which was tightly filled by hydrophobic residues [31]. As nonspecific hydrophobic pockets did not catalyze the reaction, activity was not due to *medium* effect. Instead, close *packing* ensured the right orientation of the functional groups, in accord with their sensitivity to mutations back to the original scaffold.

An active retro-aldolase design employed a TIM barrel scaffold, where a hydrophobic pocket interacted with the aromatic part of the substrate [32••]. Applying a more diverse rotamer library for screening optimized the *packing* at the active site, which resulted in ~10 fold improvement in k_{cat} [33]. Hydrophobic residues contributed to only ~10 fold rate acceleration in RA61 retro-aldolase design via *medium effect*, by shifting the pKa of the Schiff-base lysine residue [34]. *Packing* also influenced the hydrogen-bonding network, which positioned the active site water molecules [32••]. In accord, simultaneous mutation of water coordinating residues caused almost 10^3 fold drop in catalytic activity [23]. In under-packed cases these water molecules remain rather mobile and decrease the *preorganization* of the enzymatic environment. Hence including a water-mediated hydrogen bond in retro-aldolase designs with a catalytic His-Asp dyad increased the number of active variants [32••]. These observations illustrate that tighter *packing* is not necessarily required for *desolvation*, instead it optimizes polar, *preorganized* environment.

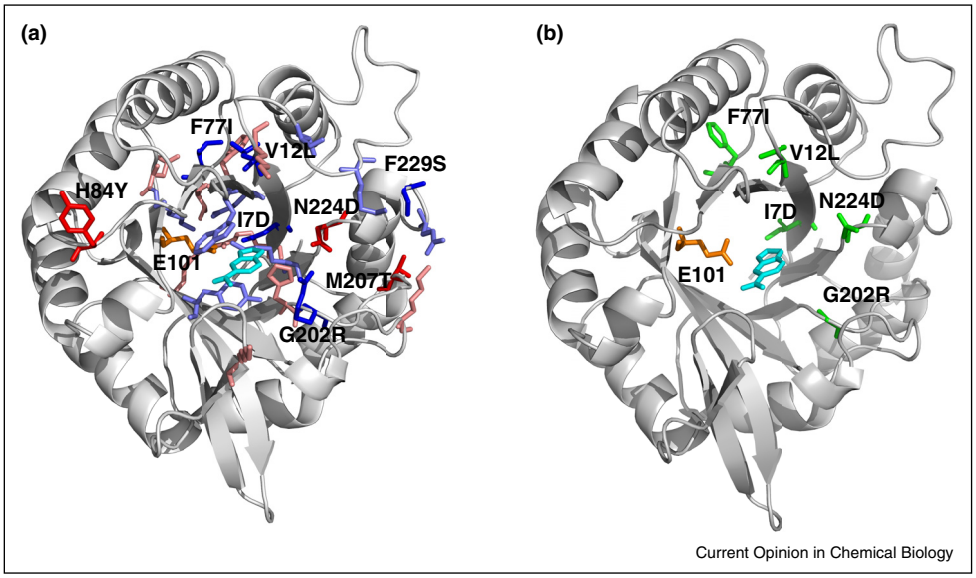
The low activity of the enzyme designs in various cases is due to *dynamical* rearrangements in the real enzyme, which deviate from the ideal catalytic configuration in small models. MD simulations on a retro-aldolase (RA22) found that nearly iso-energetic conformations in *ab initio* calculations significantly changed preference in heterogeneous protein environment [35]. An altered substrate conformation for example, rearranged the hydrogen-bonding network at the active site, which hampered the formation of the catalytic His233-Asp53 dyad. Another covalent retro-aldolase complex showed that wobbling of a catalytic lysine residue is compromising for activity by reducing efficiency of a proton transfer [23].

Dynamics can also distinguish between active and inactive designs. In MD simulations, the active KE70 Kemp eliminase exhibited minor deviations from the designed structure [26], while the catalytic dyad of the inactive KE38 adopted a significantly different geometry. Such instabilities, similarly to that of retro-aldolases [35] alter hydrogen-bonding geometry and perturb proton shuttling. Hence considering *dynamic effects* is critical in maintaining polar networks. To underscore this point, an MD-based approach resulted in an efficient catalyst for Kemp elimination (HG-3) [27•], which could be evolved to a variant with activity close to that of natural enzymes [6••].

What can be improved by directed evolution?

Directed evolution [4,36] is an efficient way to improve initial designs by mimicking natural optimization. Despite several magnitude increase in reaction rates [22,37,38••], experimental optimization is limited by the selected scaffold or an ill-defined target effect. For

Figure 2



Group contributions to catalysis in the designed and the evolved KE07 Kemp eliminase with Glu101 as the general base (orange) and the substrate (cyan) included. **(a)** Effect of directed evolution on the electrostatic contributions of residues with large catalytic effects: favorable (decreases, light blue) and unfavorable (increases, light red). Changes in contributions of mutated residues are shown by dark colors. **(b)** Mutations, which were predicted based on their contributions to the reorganization energy (green) were in accord with those observed in libraries of active variants in directed evolution [28•].

example, improving ground state destabilization [39] is not efficient to improve catalysis [40].

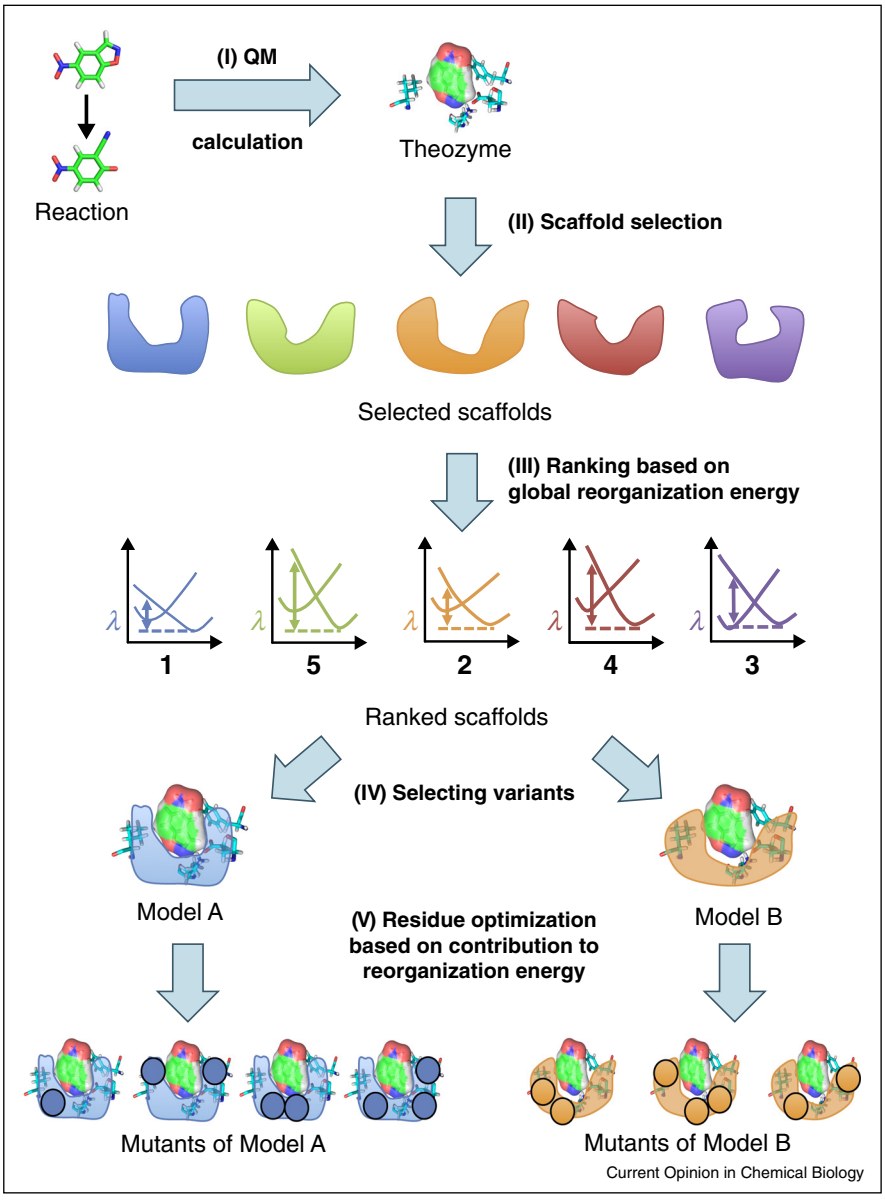
The most successful example of computer-aided enzyme design is the Kemp eliminase [6••], which carries out a conversion 5-nitrobenzisoxazole to cyanophenol (Figure 2). The reaction requires a general base to induce ring-opening, a hydrogen bond to stabilize the negative charge on the phenolic oxygen and a π stacking with the aromatic part of the substrate. This reaction is particularly challenging, owing to the limited charge transfer to the substrate, which also decreases the *preorganization* effect [39]. Indeed, this reaction can be catalyzed by serum albumins with comparable efficiency to those of specific antibodies [41]. Thus it has been argued that catalysis is due to *medium* effect instead of specific positioning of functional groups.

Employing computational design, different series of Kemp eliminases were generated depending on the identity of these functional groups [27•,42]. KE07 contains a glutamate (E101) as a general base, a lysine (K222) as a hydrogen bond donor and a tryptophane (W50) to interact with the benzene ring. In KE70 the His-Asp dyad (H17-D45) serves as a general base, a serine (S138) is the hydrogen bonding donor, and a tyrosine (Y48) is involved in π stacking. KE59 was designed to have a tight hydrophobic pocket, with glutamate (E230) as a general base, utilizes a tryptophane (W109) for π stacking and two serines (S179 an S210) establish hydrogen bonds with the nitro group. The structure of the KE07 and KE70 enzymes was based on the TIM barrel scaffold (PDB codes: 1THF and 1JCL, respectively) while KE59 was designed on α/β barrel scaffold (PDB code: 1A53). The efficiencies of the original designs were comparable to an off-the-shelf catalyst, but they could be optimized further in the laboratory [6••,22,37,38••].

Introducing eight mutations into the KE07 design improved k_{cat} by 10^2 [37]. Replacement of hydrophobic residues by polar ones rearranged the hydrogen- bonding network in the active site and elevated the pKa of the general base (Figure 2). The evolved active site was better *preorganized* for catalysis, which was also reflected by the decreased stability of the evolved variant. Similarly to KE07, rearranging the interaction pattern in KE70 via considering multiple conformations in loop redesign increased k_{cat} by 400 fold [38••]. Changes in the polar network fine-tuned *electrostatics* around the catalytic His-Asp dyad. Although KE70 was more redesignable and evolvable than KE07, modifying *electrostatic interactions* was the key factor in optimizing both designs.

Directed evolution of KE59 required to introduce stabilizing mutations and resulted in 2000 fold increase in catalytic activity [22]. Optimization increased *hydrophobicity* of the active site and raised the pKa of the catalytic base by *desolvation*. Orientation of the functional groups was adjusted by mutations at the rim, which affected active site geometry via changing *dynamics* [26]. An *alternative*

Figure 3



Flowchart of the computational enzyme design process including reorganization energy calculations. The theozyme (I), which is determined by QM methods is docked into scaffold library (II) and a set of scaffolds is filtered based on steric and electrostatic complementarity (III). Reorganization energy is computed for the selected scaffolds, using short MD simulations within the LRA framework (IV). Top-ranked candidates are subjected to further refinements. In addition to TS binding energy and electrostatic complementarity, residue contributions to reorganization energy are also evaluated and used for scoring (V).

rotamer of Trp-109 resulted in a stabilizing interaction with the general base, which contributed to improving activity.

The HG-3 design was based on the catalytic antibody 34E4 and was optimized by a combination of crystallography and MD [27•]. It employed an aspartate (D127) as the general base, aromatic residues to provide π-stacking for substrate interactions and polar residues (serine, threonine, glutamine) to donate a hydrogen bond to the isoxazolic oxygen of the 5-nitrobenzisoxazole. This Kemp eliminase design was evolved to the most efficient artificial catalyst, with k_{cat} of 700 s^{-1}, which provided 6×10^8 fold rate acceleration as compared to the uncatalyzed reaction [6••]. Activity of the HG3.17 variant originated in the extremely tight fit of the substrate, which was also enabled by a shortened hydrogen bond to the general base Asp127. It is often believed that tight *packing*, which was also observed in evolution of other

designs [31,33], contributes to catalysis by *desolvating* the substrate. In case of HG-3 however, similar pH profiles of the original design and the evolved variant argue against *medium* effect. *Hydrophobic* contacts on the other hand can also optimize the arrangement of the functional groups and result in better *preorganization*. In the evolved HG3.17 Kemp eliminase the network of hydrogen- bonding interactions, which was enabled by the alternative substrate conformation, provided better stabilization of the negatively charged TS.

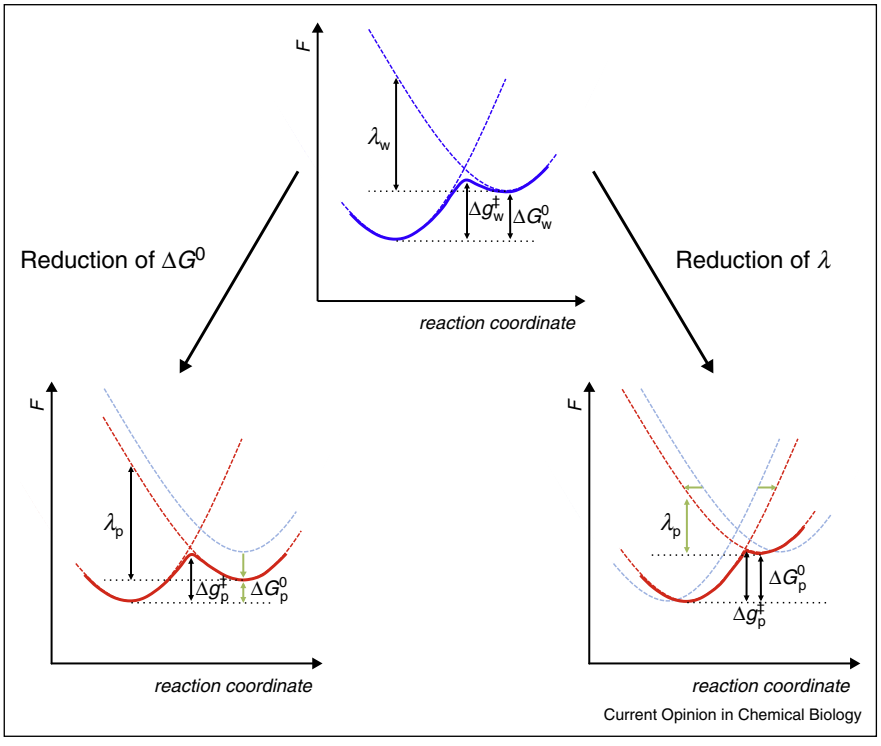
Reorganization energy in optimizing KE07 catalysis

Although the original KE07 design was optimized for ground state *desolvation*, its laboratory evolution improved electrostatic *preorganization* around the TS [39,43]. To assess how this effect improves in enzyme evolution, reorganization energies of the original and the evolved KE07 variants were determined [28•].

Free energy profiles of the designed and the evolved KE07 variants were calculated by Free Energy Perturbation/Umbrella Sampling techniques resulting in activation barriers in good agreement with the experiments [37]. Although the reorganization energy of the KE07 design was less favorable than that of the corresponding reaction in water, it decreased significantly in directed evolution (by 27.4 kcal mol^{-1}). Analyzing different contributions to the catalytic effect in the original and the evolved KE07 enzyme indicated that the reorganization energy was the most sensitive component of the catalytic effect, which was also amenable to optimization by directed evolution.

It is also important to follow how contributions of individual residues to the catalytic effect change during enzyme evolution. Interestingly, in the evolved KE07 variant some mutated residues destabilized the transition state (Figure 2). Residue contributions to the reorganization energy were used to screen for mutations that facilitate evolution of the original KE07 design. Residues, which did not compromise the reorganization energy were selected [28•]. The predicted mutations were in agreement with libraries of active variants from different rounds of directed evolution [37]. This indicates that screening should also allow those residues, which are not involved in catalysis directly, but enable structural changes required along the

Figure 4



Schematic representation of the two extreme cases of the reduction of the activation barrier in enzymatic environment (red) as compared to the uncatalyzed reference reaction (blue) based on the Marcus theory. In this approximation the diabatic curves are either shifted vertically (reduction of reaction free energy; lower left) or horizontally (reduction of reorganization energy; lower right). In actual enzyme catalysis the activation free energy is a combination of the two extrema.

pathway. KE07 analysis also demonstrates that reorganization energy can be optimized during evolution via small rotamer changes and smaller scale rearrangements in the electrostatic interaction pattern.

Towards implementing reorganization energy in design

Besides KE07 Kemp eliminase, further examples indicate that electrostatic preorganization could be tuned in directed evolution [6••,31,32••]. This implies that variants, where the preorganization effect was maximized, could serve as promising starting points for further laboratory optimization. As reorganization energy is invested upon protein folding [44], so evaluating it could affect scaffold ranking and selection.

The proposed flowchart of the computer aided design complemented by reorganization energy calculations is shown in Figure 3. First, *ab initio* calculations are employed to determine the reaction mechanism, the TS geometry and the parameters for the energy functionals for the reactant and the product state. Second, a high-throughput scaffold search is performed based on shape complementarity and TS binding energy. Third, global reorganization energy is computed for top-ranked scaffolds, and will serve as a basis of filtering. Selected variants will be further optimized based on comparing individual reorganization energy contributions of the original and mutated residues.

Conclusion

Successful enzyme designs provide insights into how catalysis can be evoked. The performance of artificial enzymes varies in a wide range, but even with the assistance of directed evolution remains inferior to natural enzymes. Moderate efficiency of man-made constructs indicates the absence of a major catalytic factor, which can also be optimized in laboratory. Electrostatic preorganization has dominant contribution to the catalytic effect and it can also be significantly improved by directed evolution. On the basis of the reactant and product energy functions, reorganization energy can be computed in an economical manner and individual contributions can be determined. We propose to utilize global reorganization energy for refinement and final evaluation of top-ranked scaffolds. Screening based on individual contributions can result in variants similar to evolved libraries, which also include stabilizing or compensatory mutations in addition to those, which have direct impact on catalysis. Reorganization energy-assisted designs can serve as promising starting points for directed evolution experiments towards achieving efficiencies comparable to natural enzymes.

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