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AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Perspective

Designed Local Electric Fields—Promising Tools for Enzyme Engineering

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Cite This: *JACS Au* 2023, 3, 3259−3269

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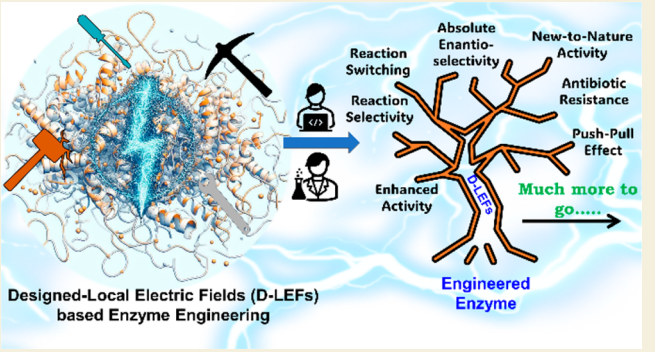
ABSTRACT: Designing efficient catalysts is one of the ultimate goals of chemists. In this Perspective, we discuss how local electric fields (LEFs) can be exploited to improve the catalytic performance of supramolecular catalysts, such as enzymes. More specifically, this Perspective starts by laying out the fundamentals of how local electric fields affect chemical reactivity and review the computational tools available to study electric fields in various settings. Subsequently, the advances made so far in optimizing enzymatic electric fields through targeted mutations are discussed critically and concisely. The Perspective ends with an outlook on some anticipated evolutions of the field in the near future. Among others, we offer some pointers on how the recent data science/machine learning revolution, engulfing all science disciplines, could potentially provide robust and principled tools to facilitate rapid inference of electric field effects, as well as the translation between optimal electrostatic environments and corresponding chemical modifications.



KEYWORDS: *Designed-Local Electric Fields, Catalysis, Enzyme Engineering, Machine Learning, De Novo Enzyme Design*

1. INTRODUCTION

Despite the impressive progress made in synthetic chemistry and catalysis over the past century, the catalytic properties of enzymes still remain unrivaled in many ways.[1,2] Not only do these versatile macromolecules facilitate a rich repertoire of highly efficient and selective chemical transformations, but they do so under ambient—and arguably sustainable/green[3,4]—conditions. Consequently, it should be no wonder that scientists have long aimed to gain an understanding of the underlying catalytic mechanism of these molecular machines.

Various competing explanations have been put forward over time,[2,5,6] but ever since the 1980s, Warshel's hypothesis of electrostatic interactions at the active site of an enzyme being the main contributors to enzyme catalysis, has become increasingly accepted by the community.[7] Despite the overwhelming computational support for the electrostatic model, it was not until 2014 that an unequivocal experimental validation, under the form of an electric field quantification at the active site of ketosteroid isomerase through vibrational Stark effect spectroscopy, was provided by Boxer and co-workers.[8] Around the same time—and inspired by more than decade-old theoretical predictions by Shaik et al.[9,10]—Coote and co-workers demonstrated that electrostatic catalysis could also be performed on apolar Diels−Alder reactions in a scanning tunneling microscopy break-junction approach,[11] underscoring the universality of the electrostatic model of catalysis.

Ever since these seminal contributions, the interest in the effects of (local) electric fields, both those exerted by the protein matrix in enzymes, as well as by molecular constituents in other catalytic systems and set-ups, has soared.[12−23] A virtuous cycle of theoretical predictions being confirmed by ingenious experiments, which in their turn sparked new theoretical insights, has been triggered and is resulting in exciting advances across the field.[24−35] Of particular interest in recent years is the effect of electrostatics on the function of the water, a pivotal solvent for biological systems. For example, a theoretical study by Cassone showed that the presence of an electric field causes delocalization of the electron density of a water molecule, which lowers the ionization threshold resulting in altered kinetics of proton transfer.[36] In fact, the presence of electric fields under the form of different ions significantly increased the hydrogen-bonding pattern of water clusters and induces electro-freezing, which has been validated both

Received: September 12, 2023
Revised: October 11, 2023
Accepted: October 12, 2023
Published: December 1, 2023

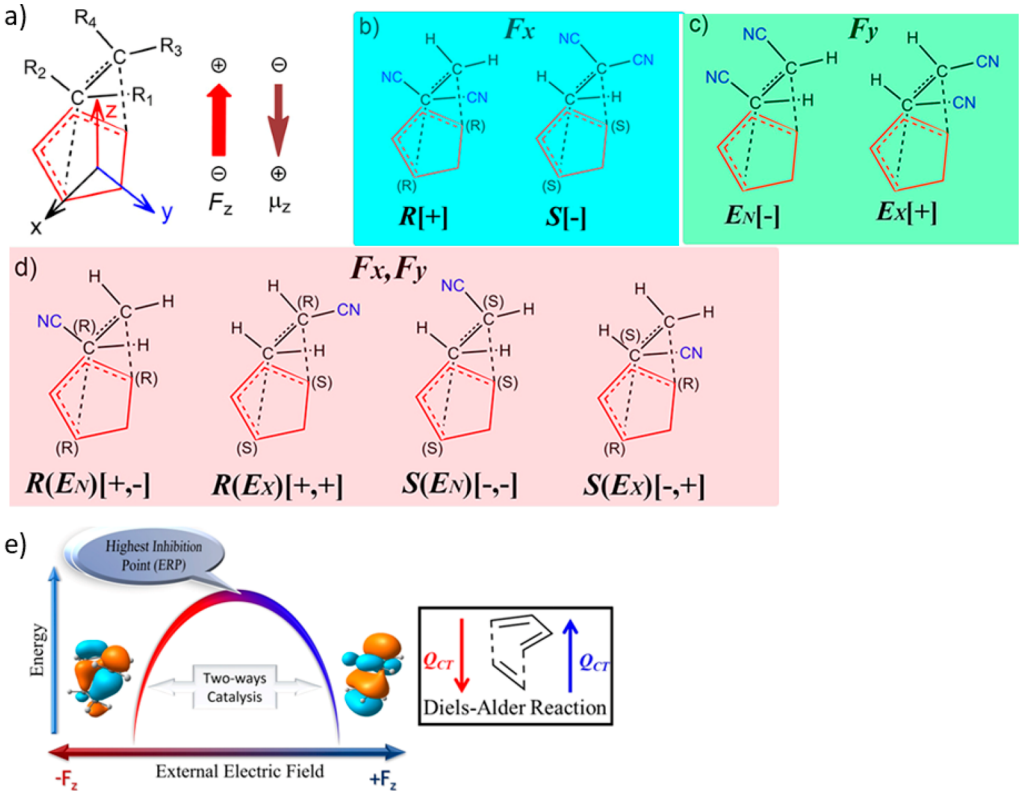


Figure 1. (a) Generic Diels–Alder reaction with *x*-, *y*-, *z*-axes defined. The direction of the dipole moment and the electric field direction that will result in catalysis are shown in the Gaussian convention. (b) Enantiomeric transition state structures for another Diels–Alder reaction. The individual structures can be selectively stabilized with the help of an EEF oriented along the *x*-axis (positive and negative signs indicate the respective stabilizing field direction). (c) *Exo*/*endo* transition states for a similar Diels–Alder reaction. Here, and EEF oriented along the *y*-axis controls the relative stability. (d) *Endo*/*exo* as well as *R* and *S* enantiomeric transition state complexes for a final example Diels–Alder reaction. Here, positive and negative signs show the directions of the x and y-aligned electric fields that result in selective stabilization. Adapted with permission from ref 15. Copyright 2020 American Chemical Society. (e) Generally, reaction rates increase in one direction of EEF and decrease in another. However, as the magnitude of the EEF rises, the inhibitory direction changes to a rate-enhancing one after the highest inhibition point has been reached.[10] Adapted with permission from ref 10. Copyright 2023 Wiley.

theoretically and experimentally.[37−39] A very recent experimental study by Coote and co-workers[40] demonstrates, in accord with observations of Zare et al,[41] that surface O–H groups of water microdroplets induce electric fields that catalyze cleavage of a dihalogen by pyrimidine.

An outstanding challenge, that could potentially have major societal ramifications, is how to productively exploit our understanding of the catalytic effect of local electric fields to inversely design *de novo* enzymes, as well as other catalytic systems,[42−44] or to modify existing enzymes (through targeted mutations) to modulate their efficiency/selectivity, or even to change the specific transformation being catalyzed altogether.[45−47] Some pioneering work has already been performed in this area, among others by Head-Gordon and co-workers,[23,23] but major challenges still lie ahead, particularly in translating a desired fine-tuning of the electrostatic environment into actionable molecular modifications.

In this Perspective, we will consider the current status of the field, with a special focus on the challenges ahead. More specifically, we will start by laying out the fundamentals of how local electric fields affect chemical reactivity and review the computational tools available to study electric fields in various settings. Subsequently, we will critically and concisely discuss the advances made so far in optimizing enzymatic electric fields through targeted mutations. We will end with an outlook on some anticipated evolutions of the field in the near future. Among others, we will offer some pointers on how the recent data science/machine learning revolution, engulfing all science disciplines, could potentially provide robust and principled tools to facilitate rapid inference of electric field effects, as well as the translation between electrostatic environments and actual chemical modifications.[28,48]

2. LOCAL ELECTRIC FIELDS (LEFs) AND THEIR IMPACT ON REACTIVITY

At the most fundamental level, chemical reactivity can be characterized as bonding rearrangements, i.e., the movement and/or (re)pairing of (valence) electrons. Since electrons inherently carry a negative charge, electric fields can be expected to affect their dynamics, and hence reactivity.

Macromolecules such as enzymes and supramolecular catalysts usually contain plenty of charged/polar functional groups, which tend to be distributed in a fairly rigid and ordered arrangement around the (re)active site. Consequently, these molecules can induce islands of local polarization/charge separation, i.e., local electric fields, which can alter the reactivity taking place at these sites.

To facilitate the qualitative discussion of electrostatic effects exerted by the environment around active sites, it is helpful to approximate local electric fields as uniformly oriented ones, also known as "oriented external electric fields" (OEEFs), and to focus on the main direction of charge transfer associated

https://doi.org/10.1021/jacsau.3c00536
JACS Au 2023, 3, 3259−3269

with the reaction to which this OEEF is applied, the so-called "reaction axis".[22] Both OEEFs and the change in dipole moment, i.e., the charge transfer along the reaction axis, can conveniently be represented as vectors, and the change in energy difference between any two points along the reaction pathway resulting from their interaction can then simply be expressed as follows,

$$\Delta\Delta E = 4.8\vec{F}\cdot\Delta\vec{\mu} \tag{1}$$

where $\Delta\Delta E$ (kcal mol^{-1}) is the change in energy difference between the two reference points, $\vec{F}$ is the OEEF vector (expressed in V Å^{-1}), and $\Delta\vec{\mu}$ is the difference in the dipole moment vector between the two points (expressed in Debye). For polar reactions, the amount of charge transfer tends to increase gradually and uniformly throughout the reaction, and hence the energetic effect increases uniformly as well: the energy difference between reactants and products, i.e., the thermodynamic driving force associated with the reaction, will be affected the most by the electric field,

$$\Delta\Delta E_{RP} = 4.8\vec{F}\cdot(\overrightarrow{\mu_P} - \overrightarrow{\mu_R}) \tag{2}$$

where $\Delta\Delta E_{RP}$ corresponds to the change in thermodynamic driving force, and $\vec{\mu}_R$ and $\vec{\mu}_P$ correspond to the dipole moments of the reactant and product (complexes) respectively. For apolar reactions, e.g., Diels–Alder reactions,[9,11] on the other hand, charge separation tends to be low in both reactants and products, but can become more significant in the region around the transition state.[22] Consequently, the thermodynamic driving force tends to be affected to a limited extent by electric fields, but the kinetics, i.e., the energy difference between reactants and TS or the reaction barrier, tends to change significantly more. As such, for this type of reaction, the true impact of the electric field on the reactivity is best reflected when the reactants and TS are taken as reference points,

$$\Delta\Delta E_{TS} = 4.8\vec{F}\cdot(\overrightarrow{\mu_{TS}} - \overrightarrow{\mu_R}) \tag{3}$$

where $\Delta\Delta E_{TS}$ corresponds to the change in barrier height and $\vec{\mu}_{TS}$ corresponds to the dipole moment of the transition state.

From expressions 2 and 3, it is straightforward to understand that, depending on the magnitude and direction of the electric field, chemical transformations can be accelerated or inhibited (either kinetically or thermodynamically), and, when multiple competing reaction pathways associated with different charge transfer directions/magnitudes can be defined, the electric field can tip the balance between them. The latter provides a handle to control chemo-, regio-, and enantioselectivity, and even induces mechanistic crossover. In Figure 1, a graphical overview of the main interaction scenarios is provided. For a more in-depth discussion of the interactions between OEEFs and electric fields, we refer to some recent reviews on this topic: refs 15, 18, 21, and 22.

Despite the conceptually appealing abstraction of approximating the impact of local electric fields as OEEFs operating on rigid dipole moment vectors, it is important to stress that this is only a crude, zeroth-order, approximation. First and foremost, it is important to note that the dipole moment of a reacting system is not independent of the electrostatic environment itself: the charge distribution within a reacting system can change significantly in the presence of an electric field compared to the field-free species (electric fields are said to "wake up" dormant ionicity/charge separation in molecules, cf. ref 18). The impact of an electric field on the dipole moment, $\vec{\mu}$, of a chemical system can be expressed in terms of the field-free dipole moment, $\vec{\mu}$, and the polarizability tensor, α, as follows (a higher accuracy can be reached by introducing additional terms to the Taylor expansion):

$$\vec{\mu}\cdot\vec{F} = \overrightarrow{\mu_0} + \alpha\cdot\vec{F} \tag{4}$$

Furthermore, local electric fields can be very heterogeneous, so that not all parts of the reacting system experience the same field strength,[49] and the dynamics of both substrates at the active site, as well as of the protein matrix, can change the nature of the electrostatic interactions significantly throughout the reaction.[50,51]

3. QUANTIFYING THE MAGNITUDE OF LOCAL ELECTRIC FIELDS AND SIMULATING THEIR ENERGETIC EFFECT ON CHEMICAL REACTIONS

As already indicated above, despite its limitations, electric field quantification is a powerful tool to gain insights into the environmental effects within (supramolecular) catalysts, both because of its predictive power with regard to reactivity modulation, as well as its conceptual straightforwardness and low computational cost. Within the context of QM/MM calculations, the focus usually lies on the electrostatic effects of the MM region on the reaction being modeled in the QM region.[52,53] Alternatively, the magnitude of the electric field exerted by the entire enzyme/catalyst aligned with a specific bond of the substrate is also often selected as a target,[51] since these computed values can be compared directly with experimental values determined from vibrational Stark effect spectroscopy.[8]

Quantification of electric fields exerted by a protein environment at active sites is usually performed based on Coulomb's law,

$$\vec{F} = \frac{1}{4\pi\varepsilon_0}\sum_i^N \frac{q_i}{r_i^2} \tag{5}$$

where ε_0 is the permittivity of vacuum, N is the number of atoms included in the simulation, q_i is the magnitude of the point charge at atom i, and $\vec{r}_i$ is the distance vector from this atom to the point of measurement. Despite the inherent classical point charge approximation made in eq 5, the quality of the computed electric field value will mainly depend on the quality of the provided input charges and positions. In principle, atomic charges extracted from a polarizable force field, e.g., the AMOEBA force field,[54] are more accurate than those extracted from traditional force fields with fixed atomic charge parameters.[51] It should, however, be noted that the true extent by which taking polarization into account improves the description of various types of simulations is not yet fully established, and particularly for QM/MM simulations, the available evidence so far suggests almost negligible advantages when polarizable force fields are used (so that their additional computational cost is not necessarily justified).[55] For example, Ganguly et al. observed only marginal effects on the computed activation and reaction energies upon switching from a fixed point-charge to a polarizable force field description in their QM/MM studies on the Claisen rearrangement in chorismate mutase and the hydroxylation reaction in p-hydroxybenzoate hydrolase.[56]

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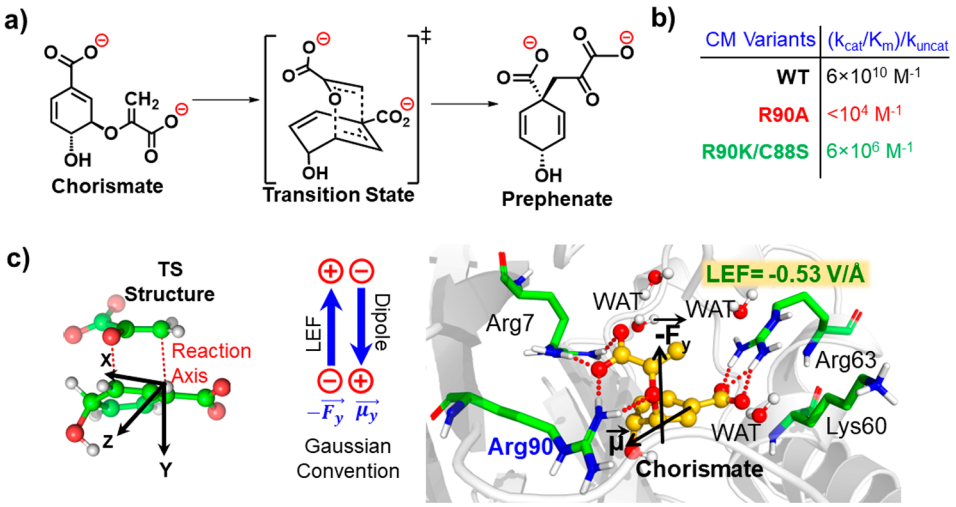

Figure 2. (a) Schematic representation of the Claisen rearrangement of chorismate to prephenate through a chairlike TS. (b) Kinetic parameters for WT chorismate mutase and its three variants. Red and green indicate loss and gain in the reactivity of enzyme, respectively. (c) Reaction axis (C−O bond axis) overlaid on the TS structure, and local electric field quantification in the WT enzyme along the reaction axis.

Various codes to automatically compute local electric fields based on the output of (QM/MM) calculations, as well as their alignment with specific bonds and/or the reaction axis have been developed over the years. Two open-source and user-friendly Python implementations are TITAN[53] and TUPA.[57] TITAN takes as input either an abstract point charge distribution, an AMBER-[58] or CHARMM-compatible[59] PDB file, or a GAUSSIAN09 log file[60] containing a natural bond order (NBO) analysis,[61] as well as an additional input file specifying the charges/atoms/residues of interest as well as the evaluation point and reaction/bond axis, and outputs the intensity, as well as the components along the specified axes, of the electric field.[53] Additionally, it can also automatically generate abstract charge distributions mimicking uniform and chiral electric fields, which can be visualized in VMD, PyMol, Chimera, Avogadro, etc. TUPA has similar capabilities but is more tailored toward MD analyses. It enables among others the automated calculation of an electric field across all timeframes of an MD simulation, facilitating an analysis of the temporal evolution of the field exerted at the (active) site of interest.[57]

4. LEFs IN ENZYMES TO OPTIMIZE REACTIVITY

In this section, we concisely discuss naturally occurring LEFs in enzymes and their impact on the selectivity and reactivity of native biochemical reactions through consideration of some selected examples.

4.1. LEF in Chorismate Mutase

Chorismate mutase (CM) is a prototypical example where nature has harnessed LEFs, exerted by the protein matrix, to enhance the electrostatic catalysis in enzymatic systems.[62]

This enzyme performs the Claisen rearrangement reaction from chorismate reactant to prephenate product via a chairlike dianion transition state (see Figure 2a). In 1996, Hilvert et al. explored the active site of *Bacillus subtilis* Chorismate mutase and highlighted the importance of electrostatic catalysis in the enzyme's function using combinatorial mutagenesis and selection techniques.[63] The study shows that the residue Arg90, which lies near the reaction center, plays an electrostatic-driven moderator in the function of CM. This was substantiated by the mutations of this charge residue Arg90 to uncharged Ala90 and Gly90 that dramatically diminished the catalysis of the reactions. In contrast, reinstating matching charged or polar groups, such as Lys90, partially restored the catalytic functions[64] (see Figure 2b and c). Using computational tools, we recently quantified the LEF of the CM and established a direct correlation between enzymatic LEF and the reactivity of this enzyme.[62]

4.2. LEF in CYP450 Peroxygenases

The cytochrome P450 peroxygenases family, particularly its two members, i.e., the $CYP450_{OleT}$ and $CYP450_{BS\beta}$ enzymes, provide another interesting example where nature harnesses the LEF to diversify the catalytic function.[65] These two members share identical active sites with the exception of a residue present at the 85th position. In $CYP450_{BS\beta,}$ which catalyzes (predominantly) the hydroxylation reaction, this residue is Glu85, while in $CYP450_{OleT,}$ which performs the decarboxylation, it is His85.[65,66] In a recent study, using computational tools, we demonstrated that the presence of charged residue His85 is the root cause of the diversification in the function in these two enzymes (see Figure 3).[65] The enhanced polar and electrostatic environment in the first catalytic shell of the $CYP450_{OleT}$ stabilizes the Cpd II/substrate radical, which, in turn, attacks the C_n-C_{n-1} of the fatty acid and triggers the decarboxylation. In contrast, the catalytic shell of $CYP450_{BS\beta}$ is less polar, and thus the Cpd II/substrate radical is less stabilized in $CYP450_{BS\beta,}$ which quickly goes for the typical rebound mechanism to perform hydroxylation similar to other CYP450 monooxygenases. These results underscore how mutation of selected residues facilitate diversification of enzymatic functions.

4.3. Modulating Push−Pull Effect in Heme-Containing Enzymes

The push−pull effect of the axial ligand of heme-porphyrin-based enzymes is supposed to be a key factor for the versatility of reactions.[67−69] In a recent study, Alexandrova and Bim found that it is, actually, the local electric fields (LEFs) along the axial ligation that modulates the different functions in various Heme proteins containing different axial ligands[29] (see Figure 4). Notably, the study shows that the LEFs of the enzymes aligned along the Fe−O bond of Compound I (Cpd

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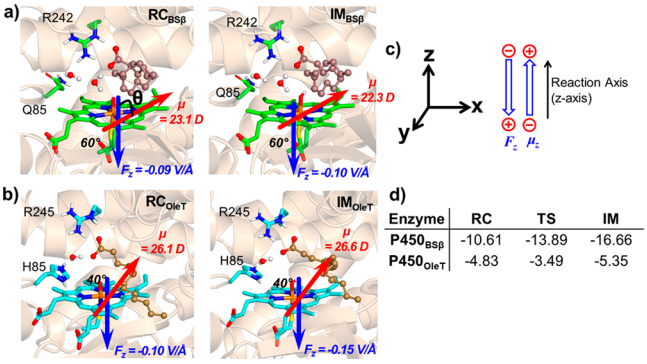

Figure 3. (a) Quantified net dipole moment and enzyme's LEF along the Fe−O axis of the reactant and intermediate in CYP450$_{BS\beta}$. (b) Quantified net dipole moment and enzyme's LEF along the Fe−O axis of the reactant and intermediate in CYP450$_{OleT}$. (c) Cartesian axis and the Gaussian convention used for electric field and dipole moment vectors throughout this perspective. (d) Electrostatic stabilization energy in kcal/mol obtained from interaction of the LEFs with the dipole moment of the substrate. Note that RC, TS, and IM stand for reactant, transition state, and intermediate, respectively.

I) have different magnitudes for different enzyme types. More specifically, they found that the Cysteine-ligated heme enzymes exhibit a high LEF which is correlated with H-abstraction capacity. Peroxidases with histidine ligands had a moderate LEF, preferring electron transfer. Catalases, with tyrosine ligands, exhibited the lowest LEF and relatively lower activity compared to Cys-ligated P450 enzymes. This study shed light on the unique characteristics and diverse capabilities of naturally designed LEFs in various enzyme classes.[45]

4.4. LEF-Induced Selectivity in TyrH

Another stimulating example has been studied by Wang et al.[31] In this work, the authors demonstrated that the LEF can exert opposing effects in two different reaction steps in tyrosine hydroxylase (TyrH). The catalytic step of TyrH encompasses two vital stages: stage I involves the activation of H_2O_2, leading to the formation of the active species compound I (Cpd I), while stage II entails Cpd I-mediated hydroxylation of L-tyrosine to L-DOPA as shown in Figure 5. The study by Wang et al. shows that initially, the LEF of the enzyme reorganized in such a way that it promotes the Cpd I formation while in the next step of the catalytic step, it inhibits the substrate oxidation. Since Cpd I formation is the rate-determining step, the overall effect of the LEF is to catalyze the reaction.

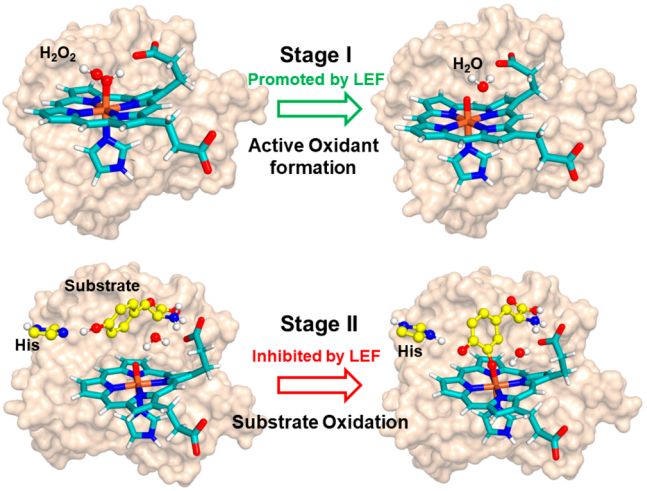

Figure 5. Promotion and inhibition of the state I and II (active oxidant Fe═O formation and substrate oxidation) by IEF, respectively.

4.5. Changes in the LEFs Drive the Emergence of Antibiotic Resistance in β-Lactamases

We end this discussion with a series of recent studies by Boxer and co-workers on the interplay between electrostatics, chemical positioning, and the emergence of antibiotic resistance.[70,71] Resistance to penicillin G, as well as other types of β-lactam antibiotics, arises from the ability of β-lactamases to hydrolyze the ester moiety in the β-lactam rings of these drugs.[70,72] β-lactamases evolved from penicillin-binding proteins (PBP), which dissociate the covalent bond, formed between penicillin G and its active site, at a much slower rate, resulting in inhibition. Through a focus on the prototypical TEM-1 β-lactamase enzyme, Boxer and co-workers demonstrated that the impressive rate enhancement of the hydrolysis reaction in β-lactamases relative to the original PBPs is caused by a couple of strategic mutations around the active site which dramatically alter the electric field exerted along the β-lactam ester moiety. To this end, the authors reverse engineered the evolutionary process by introducing targeted mutations in a TEM-1 β-lactamase, which gradually diminished the electric field from −171 to −138 MV/cm. These electric field diminishing mutations resulted in a significant rate deceleration, i.e., an increase of the inhibiting power of penicillin G (Figure 6). Furthermore, the

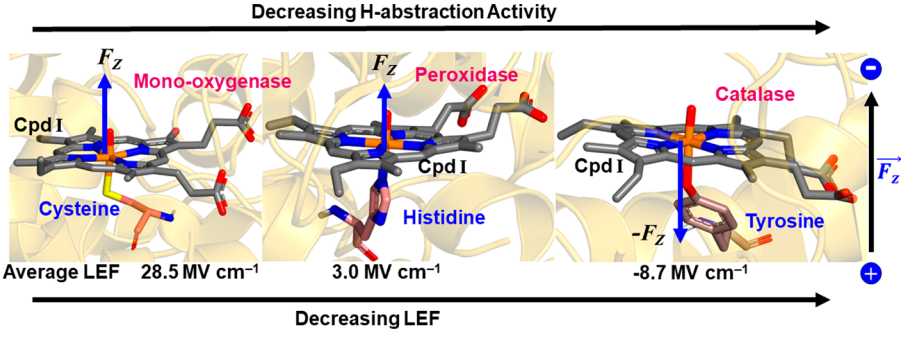

Figure 4. Average local electric field (LEF) gradually attenuates along the Fe−O axis within different categories of heme-iron proteins, specifically mono-oxygenase, peroxidase, and catalase (from left to right). This observed trend is complemented by the illustration of the electric field convention employed in the study, showcased on the right side of the figure. Note that 1 MV cm^{-1} = 10^{-2} V Å^{-1}. For the electric field vector, the physics convention was used.

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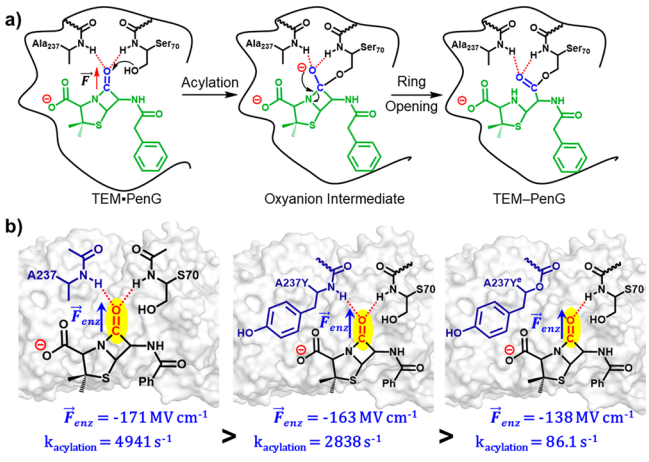


Figure 6. Reaction and pivotal electrostatic interactions within the active site of TEM β-lactamases. (a) Illustration of the PenG acylation process. The hydroxyl group of S70 functions as a nucleophile, engaging the β-lactam C═O bond of PenG. This leads to the creation of an oxyanion intermediate, which subsequently transforms into an acyl-enzyme complex. (b) Main three mutants, WT, A237Y, and A237Y^e, each accompanied by its corresponding electric field vector, F, along the C═O reaction axis. The magnitudes of the electric fields are indicated for all three mutants along with their respective acylation rates. Herein, note that 1 MV cm^{-1} = 10^{-2} V Å^{-1}.

authors demonstrated that avibactam, a next generation antibiotic, counteracts the electric field in TEM-1 β-lactamase by positioning its ester linkage to the enzyme differently, causing the alignment between the bond to be hydrolyzed and the electric field to be broken.

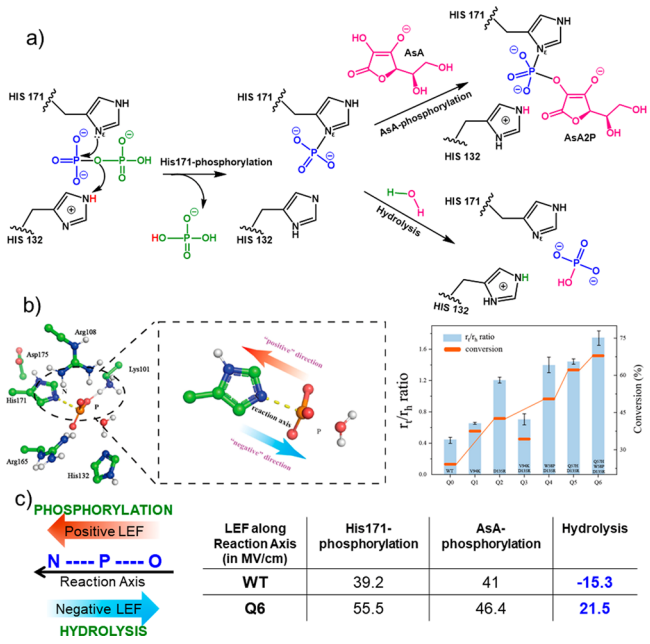
In this section, we have shown through five representative examples that, by strategically installing charge and polar amino acids, nature is able to tune the enzyme-LEFs to optimize the reaction selectivity and controls a wide range of biochemical transformations. In the next section, we will discuss some examples where researchers have adopted this strategy of LEF tuning to modify reactivity characteristics of enzymes and supramolecular catalysts.

5. DESIGNED-LEF (D-LEF) TO ACHIEVE THE DESIRED ACTIVITY THROUGH BIOENGINEERING

5.1. Switching between Hydrolysis vs Phosphorylation in PaAPase Guided by D-LEF

In a recent work, Wang et al. used designed a LEF to modulate the reactivity in PaAPase in a combined experimental and computational investigation.[73]

As can be seen in Figure 7a, the enzyme PaApase can perform the Asa-Phosphorylation as well as hydrolysis involving the formation and breaking of the His171N–P bond, respectively. The authors performed strategic mutations to tweak the LEF along the reaction axis in order to enhance the desired phosphorylation activity. In so doing, they chose the direction of His171Nε → P as the negative reaction axis (Figure 7b,c) and calculated the local electric fields along the His171Nε → P reaction axis. By strategically engineering charged residues near the enzyme's active site, the local electric field along the reaction axis was manipulated. As a result, the competition between hydrolysis and phosphorylation was significantly influenced, favoring the latter. In doing so, a series of variants Q1–Q6 were studied computationally followed by experimental validation. Figure 7b shows transferase/hydrolase (r_t/r_h) ratio for different variants created, based on designed local electric field. Remarkably, the Q6 variant, which underwent the Asp135 → Arg135 mutation, showed an impressive 2.9-fold enhancement in phosphorylation/hydrolysis ratios compared to the enzyme's wild-type form. This mutation resulted in a reversal of the LEF for hydrolysis, shifting it from −15.3 to +21.5 MV/cm, consequently promoting the phosphorylation reaction.



LEF along Reaction Axis (in MV/cm)	His171-phosphorylation	AsA-phosphorylation	Hydrolysis
WT	39.2	41	-15.3
Q6	55.5	46.4	21.5

Figure 7. (a) Outlined are the conjectured catalytic processes underpinning the function of PaAPase. These proposed mechanisms delve into the intricate steps and interactions that likely drive the enzyme's catalytic activity: His171-phosphorylation, AsA-phosphorylation, and phospho-His171 hydrolysis. (b) Catalytic residues, the reaction axis in the active site and employing directed evolution techniques to refine the performance of the original enzyme Q0 (PaAPase) with the aim of augmenting its ability to phosphorylate AsA along with their r_t/r_h ratio and percentage conversion. (c) N–P–O negative reaction axis. Direction of positive and negative LEF for achieving phosphorylation vs hydrolysis, respectively. And the LEF exerted by WT and Q6. Figure (b) is adapted with permission from ref 73. Copyright 2021 American Chemical Society.

5.2. Enhancing the Catalytic Rate of Hydride Transfer in LADH by D-LEF

In a very recent study, Boxer et al. presented significant advancements in designing the local electric field within the active site of horse liver alcohol dehydrogenase (LADH) (see Figure 8a).[78] Combining the computational and experimental methods, the authors focused on the concept of electrostatic catalysis by tweaking the LEF generated by the charged and polar chemical groups at the active site of the enzyme (see Figure 8b).

To alter the electric fields in the active site of LADH, the authors modified the enzyme in the following two ways: (1) they replaced the serine hydrogen bond donor with threonine (Ser48Thr) and (2) they replaced the catalytic Zn^{2+} with Co^{2+} (Figure 8c). The study demonstrated that both modifications induced an increase in the electric field along the carbonyl bond of the aldehyde substrate, which in turn led to a reduction in the free energy barrier for hydride transfer. Remarkably, the experimentally observed rate acceleration (by

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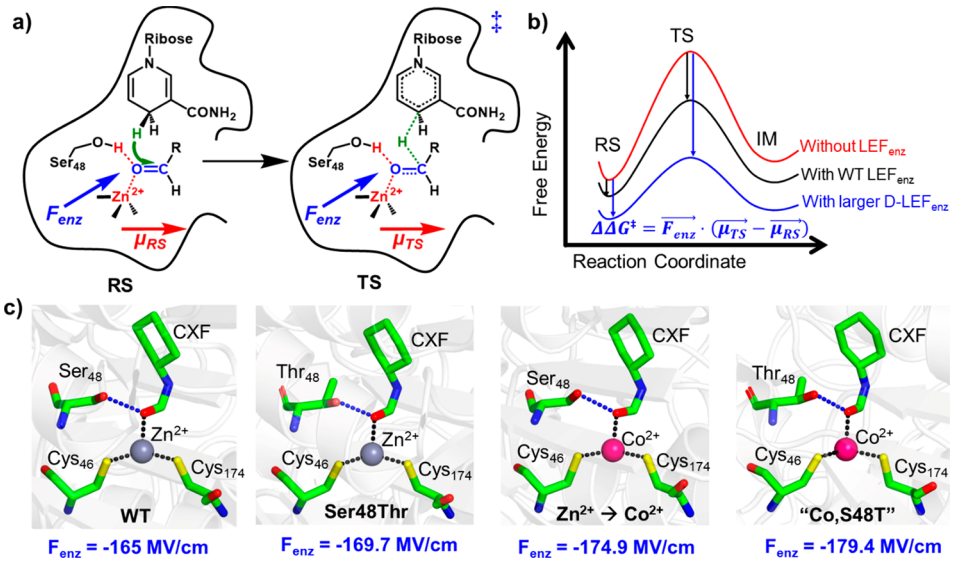
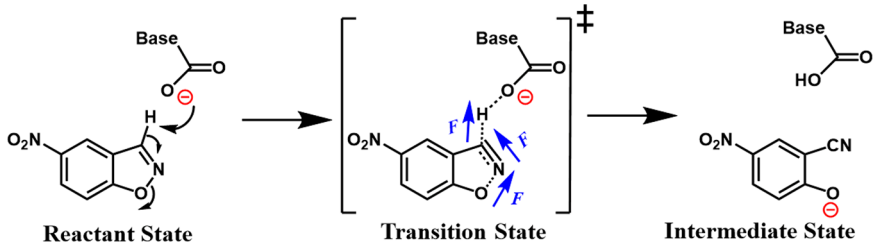


Figure 8. (a) Operational mechanism underlying aldehyde hydrogenation, facilitated by LADH and utilizing NADH as the cofactor. RS and TS stand for reactant state and transition state, respectively. Electric field and dipole moment directions are shown by F_{enz} and μ vectors. (b) The electric field F_{enz} at the active site reduces the activation energy barrier by $\Delta\Delta G^{\ddagger}$, achieved by selectively stabilizing the transition state (μ_{TS}) over the reactant state (μ_{RS}). It is essential to recognize that these fields result from charges and dipoles arranged within the protein structure, distinct from externally applied electric fields. (c) The different variants of the WT enzyme created: WT, Ser48Thr, $Zn^{2+} \rightarrow Co^{2+}$, and Co,S48T. Their enzymatic LEF is also shown at the bottom of each variant.

Scheme 1. Kemp Elimination Reaction, Involving a Singular Proton Transfer from the 5-Nitrobenzisoxazole Substrate by a Catalytic Base Leading to the Rupture of the 5-Membered Ring and the Formation of the End Product, α-Cyanophenol[a]



[a] *F* vectors showing the projection of the Enzymatic LEFs.

a factor 50) closely matched the prediction made based on the electric field enhancement, underscoring the predictiveness of electric fields in catalyst design.

5.3. D-LEF Unleashes a 43-fold Catalytic Boost: Designed Kemp Eliminase

Head-Gordon et al. used a computational method to optimize the local electric field around the substrate in a *de novo* designed Kemp Eliminase enzyme, KE15, to improve catalysis (Scheme 1).[23] They identified regions in the enzyme's active site where the electric field was not optimal for catalysis and introduced mutations around the enzyme's active site to optimize the electric field exerted on the substrate.

The mutations were initially selected based on a computational analysis, after which experimental validation was performed with the help of site-directed mutagenesis. The catalytic activity of the mutated enzymes were then measured and compared to the original enzyme, experimentally. The four mutations together resulted in a decrease in the energy barrier of 2.5 kcal/mol and an increase in k_{cat} from 0.007 to 0.31 s^{-1} which is a 43-fold boost in the catalytic efficiency of the designed kemp eliminase.

5.4. D-LEF in Supramolecular Cages for Absolute Selectivity

In a very recent study, we have extended the scope of tailored local electric fields to supramolecular cages, and we have shown that such D-LEFs enable precise and tunable control over the catalytic environment, providing a highly flexible and adjustable platform for enhancing reactivity and selectivity.[74] Here, a computational approach was used to engineer a porphyrin-based cage (PB-1)[75] and to encapsulate the active oxidant (HM1)[76] and substrate (tetralin, TLN)[77] inside it. Subsequently, to enhance the catalysis, we engineered the cage so as to orient the local electric field along the reaction axis (i.e., the Fe−O axis). It was found that the engineered cage possesses a significant dipole moment along *y*-axis which interact with the LEF along the *y*-direction and slightly lowers the barrier for *R*-selective reaction.[74] Inspired by this finding, strong *R*/*S* switchable cages were computationally engineered as well, as shown in Figure 9. When a charged substituent (two COO^- groups) was placed in the negative *y*-direction, the reaction resulted in *R*-enantioselectivity, while placing the same charged substitution on the positive *y*-direction resulted in *S*-enantioselectivity.

https://doi.org/10.1021/jacsau.3c00536
JACS Au 2023, 3, 3259−3269

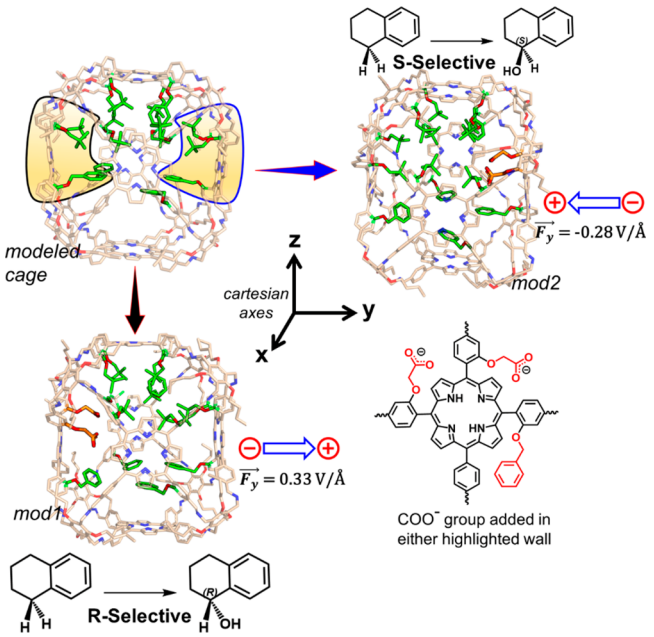


Figure 9. Further modifications done in the model cage to achieve absolute *R*/*S*-selective porphyrin boxes: mod1 and mod2. The *y*-LEF is also shown for each cage. The substitutions introduced are present at the bottom right.

Overall, this work demonstrates a new promising strategy for the design of asymmetric catalysts, based on a directed and tunable electric field on supramolecular cages.

6. OUTLOOK AND CONCLUSIONS

It should be clear that the field of designed and engineered local electric fields has the potential to revolutionize biocatalysis and catalysis in general. By strategically manipulating the LEFs within the active sites of enzymes, a whole new realm of functionalities can in principle be unlocked. For example, it is possible to envision the generation of enzymes and supramolecular catalysts with enhanced catalytic activity, selectivity, and efficiency, and tailor them to suit specific industrial processes and applications. This level of precision in enzyme design opens doors to more sustainable and eco-friendly chemical processes, reducing the need for harsh conditions and toxic reagents, thereby minimizing environmental impact. Additionally, LEF analysis can also be expected to become a significant aspect of the development of new (small molecule) antibiotics—as well as other drugs and therapies—where inverse design of ideal positionings of inhibitors, that minimize the alignment between the exerted LEFs and their covalent linkages to the active site, can be envisioned. Furthermore, critical mutations that could alter this alignment can potentially be anticipated as well, providing an indication about the robustness of the developed inhibition strategy.[70,71,78]

Further advances in computational methods, molecular modeling, and quantum mechanical calculations are instrumental in the progress of this research area. In particular, we anticipate an increasing importance of data science and machine learning techniques in the design of *de novo* enzymes and supramolecular catalysts. Up to this point, most successful attempts at electrostatic-based enzyme engineering have started from an initial template, i.e., a naturally occurring enzyme, which was rationally altered by modifying a limited number of residues.[20,23,43,73,78] Deviating significantly from the initial amino acid sequence increases the likelihood of dramatic changes in protein folding—and hence the structure of the enzyme and its active site—so that caution is usually required when proposing mutations that ought to fine-tune enzymatic LEFs. In recent years, however, various deep learning models have been developed that are able to predict, with reasonable accuracy, the folding of a protein based on its amino acid sequence, e.g., AlphaFold[79] and RoseTTAFold,[80] so that excursions further into the uncharted space of mutant enzymes are becoming increasingly realistic and viable.

We also envision the development of machine learning tools to directly predict changes in LEFs upon potential mutations. Considering the vastness of the space of potential mutations—with 20 potential amino acids and typically dozens or even hundreds of amino acids, billions upon billions of possibilities that can be distinguished—such tools could facilitate a more systematic screening of promising mutation sites than the current manual, trial-and-error, approach. As the flip side of this coin, the first examples of deep learning tools to predict the impact of electric fields on the energetics and structure of molecules/substrates on-the-fly have already been released in recent years; see, for example, refs 27 and 41.

An even more recent evolution is the emergence of generative deep learning models to directly design protein structures around an initial active site blueprint, e.g., RFdiffusion.[80] These new tools open the door toward protein design from scratch, i.e., without an initial template enzyme. Generative models for protein sequences could potentially enlarge the scope of reactions amenable to enzymatic catalysis, since enzymes could be engineered for synthetic reactions that are not typically catalyzed *in vivo*.

In summary, to realize the full potential of designed local electric field-based enzyme engineering, cross-disciplinary collaborations are essential. Experts from biochemistry, computational biology, physics, materials, and data science must join forces to tackle the challenges and explore the vast opportunities that lie ahead. By mimicking Nature's designs, we can expect these methods to facilitate the development of enzymes with tailor-made functions, pushing the boundaries of what biotechnology can achieve and resulting in real-world applications, ranging from greener industrial processes to accelerated drug development and beyond.

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https://doi.org/10.1021/jacsau.3c00536
JACS Au 2023, 3, 3259−3269

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

K.D.D. acknowledges Department of Biotechnology, Ministry of Science and Technology, Govt. of India for Ramalingaswami re-entry research grant (BT/RLF/Re-entry/10/2017). S.S. is supported by Israel Science Foundation (ISF grant 520/18). T.S. thanks the French National Agency for Research (ANR) and the French National Center for Scientific Research (CNRS) for a CPJ grant.

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https://doi.org/10.1021/jacsau.3c00536
JACS Au 2023, 3, 3259−3269