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Rapid estimation of catalytic efficiency by cumulative atomic multipole moments: application to ketosteroid isomerase mutants

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ABSTRACT
We propose a simple atomic multipole electrostatic model to rapidly evaluate the effects of
mutation on enzyme activity, and test its performance on wild-type and mutant ketosteroid
isomerase. The predictions of our atomic multipole model are similar to those obtained with
symmetry adapted perturbation theory (SAPT), at a fraction of the computational cost. We
further show that this approach is relatively insensitive to the precise amino acid side-chain
conformation in mutants, and may thus be useful in computational enzyme (re)design.

1. INTRODUCTION
In silico enzyme (re)design, which has become increasingly popular and successful in
recent years, ideally features a fast but accurate method to predict the influence of point
mutations on catalytic activity. To be practically useful, such a method should allow
computational procedures for enzyme (re)design that involve screening of multiple possible
variants (e.g. 100s). Due to the difficulty of predicting the structural perturbations caused by
amino acid mutations, an ideal method should be relatively insensitive to minor structure
deformations. To meet all these criteria is, however, an extremely difficult task, because
determining the catalytic activity of just one protein requires inclusion of many factors, from
substrate binding, through interactions in the active site, to conformational changes and large-
scale movements that enzyme undergoes during the process. Another factor that is difficult to evaluate is the effect of mutations on the enzyme-substrate ensemble, which
was found recently to differ significantly between some designed and in vitro evolved
enzymes. However, from the computational point of view, a critical challenge is the
calculation of the activation energy of the reaction (energy barrier): the relative activity of
mutants may depend centrally on the barrier to reaction. To calculate energy barriers for
reactions in enzymes, computational methods applying quantum mechanics to at least part of
the enzyme-reactant system can be used (e.g. the active site, through combined quantum
mechanical / molecular mechanical (QM/MM) methods\textsuperscript{11}. Another possibility is to use quantum chemical calculations to parametrize Empirical Valence Bond (EVB) model for reactants\textsuperscript{12}.

Evaluation of reaction barriers in mutant enzymes can be performed by treating the impact of mutation as a perturbation to the wild-type (WT) reaction profile. If the mutation does not influence the reaction mechanism and other factors considered above are (at least approximately) independent of this change, the difference in activation energy can be expressed in terms of intermolecular interactions within the active site. To quantify this effect we use the concept of Differential Stabilization energy\textsuperscript{13}, in which activation energy changes are expressed in terms defined within intermolecular interaction theory. If the activity of mutant enzymes depends on a single critical step, mutants can be compared in terms of these relative differences on an equal footing, avoiding the need for computationally expensive reoptimization of enzyme-substrate and enzyme-transition states. This would be a major advantage of such an approach, allowing rapid evaluation of many mutants. The general idea of ‘differential stabilization’ can further be used for optimizing relative stabilities of other important points along a reaction pathway as, for instance, ‘differential product stabilization’ can be used to describe the relative stabilities of different tautomers of adenine-thymine and guanine-cytosine base pairs in water\textsuperscript{14}.

‘Differential stabilization’ energies can be partitioned into components defined by either symmetry adapted perturbation theory (SAPT – more information in the computational details section 3)\textsuperscript{15,16} or hybrid variation-perturbation theory (HVPT) of intermolecular interactions\textsuperscript{17}. In the case of chorismate mutase\textsuperscript{17} and cAMP-dependent protein kinase (PKA)\textsuperscript{18}, this decomposition analysis indicated that interactions having positive impact on the catalytic activities could be solely represented by the electrostatic term (or its multipolar component) of the differential transition state stabilization energy (DTSS – more information
in section 2.1). This finding is in agreement with many other works indicating the importance of electrostatic interactions in enzymatic catalysis\(^1\)\(^{19}\). This electrostatic term seems to be a better estimator of relative stability of molecular complexes when distances shorter than expected are present in modeled structures (e.g. overlapping atomic radii), compared to high level MP2 and CCSD(T) results\(^2\)\(^{20}\). Nonempirical evaluation of the complete electrostatic interaction term \((E_{elst})\) including penetration requires significant computational effort scaling as \(O(N^6)\), but it can be considerably reduced by applying the cumulative atomic multiple moment (CAMM – see more details in section 2.2) expansion, which was recently found to possess the desired property of relative insensitivity of predictions to the structural perturbations, in particular for hydrogen-bonded complexes\(^2\)\(^{21}\). This gives the CAMM model a practical advantage over high-level \textit{ab initio} calculations in situations where equilibrium geometries may be distorted, for example due to the use of imperfect force fields, basis-set superposition error (BSSE) or crystal structure defects. The CAMM approach has previously been successfully applied to estimate binding affinities of inhibitors in leucine aminopeptidase (LAP)\(^2\)\(^{22}\) and fatty acid amide hydrolase (FAAH)\(^2\)\(^{23}\). In the latter case, the CAMM model was supplemented by a non-empirical dispersion term\(^2\)\(^{24}\) to improve the prediction of binding affinity, due to the non-polar hydrophobic character of the FAAH active site.

Here, we explore the use of the CAMM model for the first time for analysis and prediction of catalytic activity, by combining it with the concept of Differential Intermediate State Stabilization (DISS) energy, to evaluate differences in enzyme activity between a wild-type (WT) enzyme and mutants. We thus test whether CAMM, a simple, \textit{ab initio} derived model, can be used as a tool for rapid estimation of the influence of changing surrounding residues on enzyme activation barriers (with a focus on reproducing trends rather than absolute values). To evaluate the method in detail, we compare results obtained with CAMM.
to those from Symmetry-Adapted Perturbation Theory (SAPT) calculations of intermolecular interactions\textsuperscript{15,25,26}. As a test system we choose ketosteroid isomerase (KSI, EC 5.3.3.1) and several single-point mutants of this enzyme. KSI catalyzes the isomerization of 3-oxo-\(\Delta^5\) ketosteroids to their \(\Delta^4\)-conjugated isomers through two consecutive proton transfers, mediated by Asp38 (Figure 1; numbering for the \textit{C. testosteroni} enzyme is used throughout)\textsuperscript{27}. It is one of the fastest enzymes known, with a reaction rate near the diffusion limit for biologically relevant conversions\textsuperscript{28}. KSI is a central example in lively debates about the role of hydrogen bonds and electrostatic interactions in enzyme catalysis. The importance of electrostatics in this system was demonstrated by computational studies\textsuperscript{8,29} and more recently by spectroscopic experiments showing excellent correlation between the electric field in the active site and the apparent reaction barrier in a series of KSI variants\textsuperscript{30}, although the details of the source of the catalytic power of the enzyme are still a subject of discussion\textsuperscript{31,32}. In addition to its central role in debates on enzyme catalysis, KSI has also been shown to be a promising template for biocatalyst design, for example to catalyze Diels-Alder reactions\textsuperscript{33}.

![Figure 1. Mechanism of the isomerization reaction in KSI. Conversion of 5–androstene-3,7–dione into 4–androstene-3,7–dione occurs through two proton transfers. The first proton transfer is from C4 to Asp38 (proton abstraction by O\(\delta^2\))\textsuperscript{27,34}, and the second proton transfer is from Asp38 O\(\delta^2\) to C6 (protonation)\textsuperscript{35}. Residues in wild-type KSI that stabilize the dienolate oxygen are indicated.](image-url)
2. THEORY

2.1 Differential Stabilization

Here, the influence of catalytic environment is analyzed and quantified with a modified Differential Transition State Stabilization (DTSS) approach\(^\text{[13]}\). The model decomposes activation energy into internal reactant contribution, \(B_0\), and \(\Delta\) (DTSS energy), defined by:

\[
\Delta = B_{\text{enzyme}} - B_0 = E_{IE}(TS) - E_{IE}(S)
\]  

(1)

where \(E_{IE}(TS)\) and \(E_{IE}(S)\) denote enzyme interaction energies with transition state (TS) or substrate (S), respectively. The difference between activation energies of two variants of an enzyme (i.e. wild type (WT) or mutated one (mut) that share a common reaction path) can be approximated by the difference of their DTSS energies (Figure 2):

\[
B_{\text{mut}} - B_{\text{WT}} = [E_{IE}^\text{mut}(TS) - E_{IE}^\text{mut}(S)] - [E_{IE}^\text{WT}(TS) - E_{IE}^\text{WT}(S)] = \Delta_{\text{mut}} - \Delta_{\text{WT}}
\]  

(2)

The internal contribution or ‘vacuum’ (as it was called in previous works\(^\text{13,17,18}\)) reaction profile (see Figure 2) serves only as reference and is assumed to remain approximately constant among mutants. In the case of KSI, where the mechanism involves two proton transfer reactions with similar activation energies, it is the intermediate state that is specifically stabilized by the enzyme environment\(^\text{36–38}\) (similar to other enzymatic steps that involve proton or hydride transfer, such as citrate synthase\(^\text{39}\), aromatic amino dehydrogenase\(^\text{40}\) and methylamine dehydrogenase\(^\text{41}\)). It is thus relevant to consider the intermediate state instead of the transition state. We define the corresponding stabilization energy as the Differential Intermediate State Stabilization energy or DISS:

\[
\Delta^{\text{DISS}} = E_{IE}(I) - E_{IE}(S)
\]  

(3)
Figure 2. Differential Transition State Stabilization (DTSS) and Differential Intermediate State Stabilization (DISS). The difference between activation barrier of two enzyme variants sharing a common reaction path may be expressed by the difference in their differential stabilization energies (see equations 2 and 3).

2.2 Cumulative Atomic Multipole Moments

In macromolecular systems such as proteins, the electrostatic term is often modelled by a fixed-charge force field, with point charges placed on atom centers. This approach, however, may lead to errors due to the anisotropy of charge distributions and the arbitrariness of any atomic charge definition (including their dependence on the method and basis set employed in the charge derivation). These problems can be resolved by inclusion of higher atomic multipole moments. Here, we follow the Cumulative Atomic Multipole Moment (CAMM) formulation, in which molecular moments are partitioned into additive atomic contributions supplementing atomic charges from population analysis (Mulliken charges are used here;
other atomic charge definitions could be used) and subsequently transformed to a coordinate system centered on a corresponding atom:

\[
M_{klm,i} = \langle x^k y^l z^m \rangle_i = Z_i x_i^k y_i^l z_i^m - \sum_{l' \in \tilde{l}} \sum_{j \in \tilde{j}} P_{lj} \langle l | x^{k} y^{l} z^{m} | j \rangle
\]  

(4)

\[
M^\text{CAMM}_{klm,i} = M_{klm,i} - \sum_{k' \geq 0} \sum_{l' \geq 0} \sum_{m' \geq 0} \sum_{m'' \neq klm} \binom{k}{k'} \binom{l}{l'} \binom{m}{m'} \times x_i^{k-k'} y_i^{l-l'} z_i^{m-m'} M^\text{CAMM}_{k'l'm'i}
\]  

(5)

\[
E^{(10)}_{MTB}(\text{CAMM}) = \sum_{a \in \tilde{A}} \sum_{b \in \tilde{B}} \sum_{k_a} \sum_{k_b} M_{a}^{k_a}[k_a] T_{a}^{k_a+k_b} [k_b] M_{b}^{k_b}
\]  

(6)

In eq. 6, subscripts \(a\) and \(b\) denote atoms of molecule \(A\) and \(B\), respectively, \(M_{a}^{k_a}\) and \(M_{b}^{k_b}\) denote Cartesian multipole moments of rank \(k_a\) (\(k_b\)) and \(T_{a}^{k_a+k_b}\) is a tensor consisting of partial derivatives of \(r^{-l}\). CAMM expansion exhibits significantly improved convergence compared to one-center molecular multipole moments\(^{43,44}\) and considerably reduced basis set dependency\(^{43}\). In the present work, we truncate this expansion after the term that signifies quadrupole-quadrupole interactions: \(R^{-5}\). This is in agreement with our earlier work\(^{44}\) and the recent works of Jensen and coworkers\(^{45,46}\) which point out the importance of atomic quadrupoles and truncation of the expansion at \(R^{-5}\). At very short distances, including higher atomic moments can lead to divergent series (see figures S1-S5) which could be fixed by multipole shifting technique\(^{47}\).

3. COMPUTATIONAL DETAILS

Structures along the full reaction pathway of the wild-type enzyme were obtained from previously reported B3LYP/6-31+G*:CHARMM27 QM/MM optimizations\(^8\). In short, starting structures were sampled from AM1:CHARMM27 QM/MM stochastic boundary molecular dynamics simulations (within a 25 Å radius sphere) based on the crystal structure of KSI from *Comamonas testosteroni* complexed with the inhibitor 5α–trans-3,17–dione
(PDB ID: 1OHP). Prior to simulation, residue 38 was changed to the wild-type Asp and the inhibitor was converted into the dienolate intermediate obtained from 5–androstene-3,7-dione (see Figure 1). Two separate B3LYP:CHARMM27 pathways (one for each proton transfer) were then obtained by an adiabatic mapping procedure (which was found to provide a structure close to the ‘true’ TS structure in case of proton transfer in citrate synthase\textsuperscript{39}): consecutive QM/MM geometry optimizations were performed along a reaction coordinate, ensuring energy profiles representing a single continuous potential energy surface were obtained. The reaction coordinates for the first (RC1) and second (RC2) proton transfer were defined as: \( RC1 = d(O\delta_2\text{Asp}38–H) - d(C4–H) \) and \( RC2 = d(C6–H) - d(O\delta_2\text{Asp}38–H) \) (see Figure 1 for atom labels). B3LYP:CHARMM27 optimizations and SCS-MP2/aug-cc-pVDZ:CHARMM27 single point energy calculations were performed at 0.1 Å intervals.

All QM calculations in the current work were performed with the 6-311G** basis set. This choice is a compromise between reliability of results and the computational demand of SAPT calculations. For interaction energy (DTSS, DISS and similar) calculations, we used either a minimal system, consisting of part of the substrate (the two condensed rings that contain the carbonyl group and the carbon atoms to/from which protons are transferred; broken C-C bonds truncated by hydrogen atoms), the catalytic aspartate (Asp38) and the residues that donate hydrogen bonds to the reactant/TS (Tyr14 and Asp99 in the wild-type enzyme), or a larger system that includes the full substrate and Tyr55 (Figure 3). The small system (truncated substrate) was used in section 4.1 and the larger system (full substrate) was used elsewhere (sections 4.2 and 4.3). Asp38 was treated together with (part of) the substrate as the ‘reactive subsystem’, and the total interaction energy was expressed as a pairwise sum of contributions arising from the amino acids at positions 14 and 99 as well as 55 (for the larger system). Such a pairwise model was recently validated for the reaction of fatty-acid amide hydrolase with oleamide\textsuperscript{48}. 
Together with the WT enzyme, the four mutants considered here are Y14F, Y14S, D99N, and D99L. (Some calculations are also performed on Y55F and F30Y for comparison with previous works, see section 4.4.) Their structures were initially obtained by using minimal perturbations of the QM/MM optimized WT structures. For Y14F, the Tyr hydroxyl group was substituted by hydrogen; for D99N, the protonated carboxylic oxygen was replaced by an amine group; for D99L, the conformation of side chain was aligned so the distances between the Cδ1 and Cδ2 atoms from Leu and the Oδ1 and Oδ2 from Asp were minimal; finally, for the Y14S mutant, a water molecule ($W_{\text{TyrOH}}$) was inserted in the place of the Tyr14 hydroxyl group to maintain the hydrogen bond to reactant, consistent with the NMR studies of Kraut et al.\textsuperscript{49} and the Ser14 side chain was placed in a conformation with the hydroxyl group pointing towards reactant and $W_{\text{TyrOH}}$. To investigate the possibility of changes to the active site structure due to the mutations, we performed short molecular dynamics (MD) simulations of WT KSI and all mutants complexed with the intermediate state (see Supporting Information for full details). In brief, two independent 1 ns simulations were run of the full KSI dimer in explicit solvent with AMBER v. 14\textsuperscript{50}, using the CHARMM36\textsuperscript{51} force-field for the protein, TIP3P\textsuperscript{52} for water and CgenFF\textsuperscript{53} parameters with CHELPG charges (obtained with the RED Server\textsuperscript{54}) for the intermediate state. A one-sided harmonic restraint was applied to ensure that the distance between Asp38 Oδ2 and C4 of the substrate did not become larger than 2.65 Å, so that the conformations sampled are in line with the reaction mechanism. Hierarchical agglomerative clustering of the two trajectories (on the RMSD of the intermediate and active site residues) was performed using the AmberTools program cpptraj\textsuperscript{55}. The cluster centroid of the largest cluster was subsequently used as the representative structure for the mutant. Finally, the structure of substrate was fitted to the coordinates of intermediate by the last-squares procedure.
In order to analyze the nature of interactions between reactants and neighboring amino acids, we use Symmetry-Adapted Perturbation Theory (SAPT) for intermolecular interactions\textsuperscript{15}. Specifically, we chose the SAPT0 scheme\textsuperscript{16,56} where the interaction energy is decomposed according to following formula:\textsuperscript{15}

\[
E_{IE}^{\text{SAPT0}} = \left[ E_{\text{elst}}^{(10)} \right]_{\text{elst}} + \left[ E_{\text{exch}}^{(10)} \right]_{\text{exch}} + \left[ E_{\text{ind},r}^{(20)} + E_{\text{exch-ind},r}^{(20)} + \delta E_{HF}^{(2)} \right]_{\text{ind}}
\]

\[
+ \left[ E_{\text{disp}}^{(20)} + E_{\text{exch-disp}}^{(20)} \right]_{\text{disp}}
\]

where square brackets enclose terms that are taken into account together throughout this paper as electrostatic, exchange, induction and dispersion contributions, respectively. The first index in parenthesis denotes perturbation correction order in respect to interaction operator $V$. The second index corresponding correction in respect to electron fluctuation operator $W$ and its zero value indicates use of uncorrelated wavefunctions. Both first order contributions are in principle equivalent to those defined within our hybrid variation-perturbation theory (HVPT)\textsuperscript{17,57}, whereas the $E_{\text{ind},r}^{(20)} + E_{\text{exch-ind},r}^{(20)} + \delta E_{HF}^{(2)}$ terms correspond to a variationally determined delocalization term. The sum of first three brackets is equal to Hartree-Fock interaction energy, $E_{IE}^{HF}$. Note that throughout this work we drop the IE index in interaction energy terms (thus writing i.e. $E_{HF}$ instead of $E_{IE}^{HF}$). For DISS/DTSS energy contributions, we will precede the corresponding term with delta (i.e. $\Delta E_{HF}$ for DISS energy at Hartree-Fock level).

$\Delta E_{\text{elst}}^{(10)}$ is further decomposed into multipolar ($\Delta E_{\text{MTP}}^{(10)}$) and penetration ($\Delta E_{\text{PEN}}^{(10)}$) terms using dimer basis set for each monomer, consistently with the remaining terms defined within HVPT. Although our previous work\textsuperscript{17} indicated that quantities defined by differences between electrostatic interaction energies are well represented by just the $\Delta E_{\text{MTP}}^{(10)}$ term, the use of DMA atomic multipoles obtained in dimer basis set required significant computational
effort and sometimes resulted in divergence of multipole expansion. Therefore we use in this
contribution CAMMs obtained in monomer basis set, which is significantly more efficient.

CAMMs were calculated with the GAMESS(US) program\textsuperscript{58} with the Hartree-Fock method
and the interaction energies were obtained with our own Fortran code interfaced with Python.
We also calculated a non-empirical dispersion term $D_{\text{as}}$ as described in ref.\textsuperscript{24}. For comparison
of electrostatic models, a corresponding calculation with point charges only was performed
with atomic point charges for amino acids taken from the CHARMM27 force field. For the
androstenedione in the intermediate and reactant states, charges were obtained consistent with
the CHARMM27 force field through the RED server\textsuperscript{54} (method ESP-C1: ESP fitting with
charge equivalencing using the CHELPG algorithm, after optimization at the HF/6-31G(d)
level). SAPT0 contributions to interaction energies were calculated with the PSI4 software\textsuperscript{59},
which provides an algorithm enhanced by a density fitting procedure\textsuperscript{16,60}. The charge on O3
(see Figure 1) at all reaction coordinate points along the potential energy path was calculated
using the Merz-Singh-Kollman scheme\textsuperscript{61,62} with B3LYP/6-311G** with Gaussian09\textsuperscript{63} (for
comparison with QM/MM potential energies).

Calculated DISS values are compared to ‘apparent activation barriers’, $\Delta G_{\text{app}}^\ddagger$, related to
experimental $k_{\text{cat}}$ values by the simplified formula
\[ k_{\text{cat}} = \frac{k_b T}{h} \exp \left( -\frac{\Delta G_{\text{app}}^\ddagger}{RT} \right) \]
(similar as in the work of Fried and Boxer\textsuperscript{30}). Experimental data was taken from studies of Kraut and Choi\textsuperscript{49,64}.
In the case of the Y14S mutant, it was assumed that the ratio of the $k_{\text{cat}}$ for this mutant and
WT is the same for the 5-androstene-3,17-dione ($5$-\textit{AND}) and 5(10)-estrene-3,17-dione
($5(10)$-\textit{EST}) substrates (as experimental data for the 5-\textit{AND} substrate is not available).
Figure 3. The intermediate of the 5–androstene-3,7–dione to 4–androstene-3,7–dione isomerization as bound in the active site of ketosteroid isomerase. Colors indicate the system setup: the part treated as ‘reactive subsystem’ in the minimal system calculations is depicted with orange carbons (part of the substrate and the Asp38 side-chain); the additional part of the substrate included in the ‘larger region’ (corresponding to the QM region for the QM/MM geometry optimizations) is depicted with brown carbons. Cyan atoms depict the surrounding residues that were considered for interaction calculations (from top down: Asp99, Tyr14, Tyr55).

4. RESULTS AND DISCUSSION

4.1 Electrostatic multipole interaction energies along the reaction pathway

In this work, we evaluate the use of a new variant of the Differential Stabilization approach\textsuperscript{13,14,17} to predict the effect of four mutations on the catalytic efficiency of ketosteroid isomerase: Y14S, Y14F, D99L and D99N. Here, we first investigate how the electrostatic interaction (between the enzyme and the reacting species) changes during the reaction in wild-type KSI\textsuperscript{8} and the four mutants using the multipolar part of this contribution,
\(E_{el,mtp}^{(10)}\), as calculated with the CAMM expansion truncated at \(R^{-5}\) for all available points along the reaction path (52 in total, Figure 4a), where DISS(CAMM) converges approximately after the \(R^{-3}\) level for all mutants (see Figures S1-S5 in Supporting Information).

The electrostatic interaction energy is attractive throughout the reaction, indicating favorable interactions with the active site. Analogous results have been obtained for chorismate mutase\(^{65}\). The interaction energy is lowest (most attractive) at the intermediate state for all five enzyme variants (Figure 4a): this indicates that the stabilizing influence of the enzyme environment is maximal at the intermediate state in all cases. This is expected (because the electron density on the enolate oxygen is largest in this intermediate state, Figure 4c) and in line with previous results for the wild-type system\(^{8,29}\). Individual contributions to stabilization by the residues that donate hydrogen bonds to this enolate oxygen (O3 in Figure 1) follow the same trend (Figure 4b). Large contributions come from Tyr14 (−7.7 kcal/mol) and Asp99 (−5.4 kcal/mol), and from the water molecule that replaces the Tyr hydroxyl in the Y14S mutant (−6.4 kcal/mol; hereafter named \(W_{\text{TyrOH}}\)). The contribution of Asn99 in the D99N mutant is the smallest of the hydrogen-bond donors analyzed, but still offers significant stabilization (−4.6 kcal/mol).

The residues that do not donate hydrogen bonds to the enolate oxygen exhibit no or minor changes in interaction energy with the reactive region along the reaction pathway. Among these residues, the most significant change in interaction energy along the path is observed for Leu99 (from the D99L mutant) in the vicinity of the first transition state. This is most likely a result of the approximated conformation of the Leu99 side-chain (see further below).

The results clearly demonstrate that the total interaction energy profiles are dominated by the residues donating hydrogen bonds to the enolate oxygen. They also present a trend consistent with experimentally determined enzyme activities (see Figure 6d); only the D99N...
and Y14S profiles lie closer to each other than expected based on their apparent free energy barriers. The results further demonstrate that the differences between mutants are largest at the point where the overall stabilization is largest, i.e. at the intermediate state. This argues for using the intermediate state (instead of one of the transition states) as the reference for comparison between enzyme variants, i.e. the use of DISS instead of DTSS (see Theory and Figure 2). An obvious difference between the DTSS and DISS approaches is the slope of the best fit line (Figure 4d). This difference can be explained by the fact that the charge redistribution in the transition state (TS) is ‘partially developed’ compared to the intermediate state (see Figure 4c). Since both differential stabilization energies lead to the same order of predicted activities and similar correlation, further analysis will consider DISS only.
Figure 4. Overview of electrostatic stabilization along the reaction path in KSI. The reaction coordinate is defined as in ref. 8 (see Computational details). a) Total relative electrostatic multipole ($E_{\text{mipm}}^{(\text{ele})}$) interaction energy profiles along the reaction pathway for wild-type KSI and mutants; b) contributions of individual amino acids to the interaction energy profile; c) relative QM/MM energy for the reaction calculated at the SCS-MP2:CHARMM27 level from ref. 8, together with the ESP charge on O3 (see Computational details); d) Comparison of CAMM Differential Transition State and Intermediate State Stabilization energies (DTSS and DISS, respectively) with experimental data ($\Delta G_{\text{app}}^\ddagger$) from the works of Kraut and Choi 49,64 (see Computational details).

4.2 Reliability of side-chain conformations in the mutant structures

Atomic positions of the mutated residues were initially based on 'minimal perturbations' of the wild-type QM/MM optimized conformations (see Computational details). In order to check whether such conformations are reliable, brief molecular dynamics (MD) simulations of mutant complexes with the intermediate bound were performed (see Supporting...
Information for details). For each mutant, the conformations obtained by minimal perturbation were compared to a representative structure from simulation (obtained by clustering of two trajectories; see Figure 5b). The minimal perturbation conformations for Y14S, Y14F and D99N are in acceptable agreement with the MD simulations, but a significant discrepancy is observed for the D99L mutant. The main cluster from MD simulations of this mutant shows a rotamer of Leu99 with a minimal distance from O3 of 2.57 Å, about one angstrom more than in the initial guess (1.51 Å). This makes the contribution of Leu99 to the interaction energy significantly lower in the more realistic conformation observed in MD simulation. (In the ‘minimal perturbation’ conformation, the higher interaction energy terms are particularly affected due to the unphysical short-range interactions, as shown in Figure S8 in Supporting Information).

Comparison of DISS(CAMM) results obtained with minimal perturbation structures and clusters from MD simulations (Figure 5) shows that the difference between mutant DISS energies obtained with these two approaches differs by about 1 kcal/mol, whereas the global trend is preserved. Thus ‘the minimal perturbation approach’ could be useful, although the separation of DISS energies by ~1 kcal/mol is likely caused by the difference in the structures obtained through minimal perturbation and MD. The relative independence on structural differences of the DISS(CAMM) trend is a useful practical advantage of this method and could be useful in fast enzyme (re)design protocols. It is also a feature quite specific to CAMM in comparison to higher-level methods (\( \Delta E^{(10)}_{\text{elst}}, \Delta E_{\text{HF}} \) and \( \Delta E_{\text{SAPT0}} \)), which are affected by more or less mutually cancelling short-range terms, especially visible in the case of Leu99 in the D99L mutant (see Figures S7 and S8). However, to compare DISS(CAMM) with SAPT results without a bias of the structural perturbation affecting the latter, optimized representative structures from MD simulations are used in the remainder of this work.
Truncation of the substrate to the system containing only two main rings (see Computational details, Figure 3) leads to DISS(CAMM) contributions shifted by less than 0.1 kcal/mol per residue, whereas the corresponding differences for DISS values calculated with SAPT0 are less than 0.16 kcal/mol per residue (Table S7). The contribution of Tyr55 is constant by definition in the ‘minimal perturbation’ set of structures (as its position is unchanged) and nearly constant among minimized cluster centroids from MD simulations. Corresponding DISS(CAMM) contributions are −1.7 kcal/mol for the ‘minimal perturbation’ structures and between −1.3 kcal/mol and −1.5 kcal/mol among MD cluster centroids. The nonadditivity error (arising from pairwise treatment of interaction energies), discussed in earlier work, has been also estimated here by additional calculation treating the residues 14 and 55 as one subsystem. It remains approximately constant among MD cluster centroids (between 0.4 and 0.6 kcal/mol), with the exception of Y14S mutant (1.2 kcal/mol; the larger difference in the latter case may be attributed to high water polarization, which is discussed later in this work). All those shifts are nearly systematic and thus don’t influence the conclusions of the work nor the predictions of the model (see Supporting Information for details).
Figure 5. a) Comparison of the ‘minimal perturbation’ conformations (red; based on the wild-type QM/MM optimized intermediate structure) and representative conformations from MD simulations (blue; centroid of the main cluster from two 1 ns MD simulations; see Supporting Information) for the four mutants. b) Comparison of DISS(CAMM) energies for structures calculated with ‘minimal perturbation’ approach and minimized clusters from MD simulations against experimental data ($\Delta G_{app}^{\dagger}$).\textsuperscript{49,64}

4.3 The electrostatic nature of Differential Intermediate State Stabilization (DISS) in KSI

To test the influence of the type of calculation used to determine the DISS energy of the different KSI variants, calculations were performed with different models: atomic point charges (taken from the CHARMM27 force field for protein atoms and CHELPG charges for androstenedione, see Supporting Information) and atomic multipoles (CAMM). For further comparison, CAMM interaction energies ($E_{MTP}^{(10)}$(CAMM)) where supplemented by addition of a non-empirical ($D_{ab}$)\textsuperscript{24} or SAPT ($E_{disp}$) dispersion term (including exchange-dispersion correction). Finally, complete electrostatic ($\Delta E_{elst}^{(10)}$), Hartree-Fock ($\Delta E_{HF}$) and SAPT0
(ΔE_{SAPT0}) were obtained. All these models were then compared to the apparent free energy of activation, obtained from experimental data ΔG_{app,exp}^+(Figure 6).

All models exhibit a similar trend, as reflected by their correlation coefficients (Table S1). Leaving out the Y14S mutant (which is expected to have a significantly different activation entropy than the other variants, see next paragraph) led to a correlation coefficient close to 1. Similar results to the CAMM+D_{as} model are obtained with CAMM+ΔE_{disp} (a dispersion correction from SAPT calculations), which justifies using the computationally efficient fitted formula used for D_{as}^{24}. It is also clear that in this system, the dispersion interaction contribution to DISS is both small and approximately systematic.

Considering polarization effect (responsible for the most of the difference between electrostatic and Hartree-Fock level, Figure 6), its absolute value is quite significant, which may be a reason for the disagreement between experimentally measured electric field and the value obtained by MD simulations, as reported by Fried and Boxer^{30}. On the other hand, it seems to cancel out with other energy contributions (see Figure S8 in Supplementary Information) or at least sum up to an approximately systematic value (see global trends in Figure 6 and Tables S1-S2 in Supporting Information). This is in accordance with our previous work^{17} where we concluded the dominant role of electrostatics comparing to polarization.

DISS values calculated from Force Field point charges (ΔE_{elst}(FF)) are in reasonable agreement with those calculated from CAMMs (ΔE_{MTP}^{(10)}) and full electrostatic term (ΔE_{elst}^{(10)}).

Although closer to ΔE_{elst}^{(10)} than ΔE_{MTP}^{(10)}(CAMM), their deviation from expected value of the electrostatic interaction is not systematic, compared to CAMM. Moreover, despite point charge approximation works well in the case of reactive system with non-zero charge (as in present one), CAMM was found to reproduce electrostatic properties along reaction paths more accurately than point charges^{66}. From the values in Supplementary Table S3 it can be
appreciated that the difference between these two models is not systematic and there is thus no simple correction term that can be applied to the point-charge calculation in order to improve the correlation to experiment. Deviations of the point charge model from $\Delta E_{MTT}^{(10)}$ (CAMM) interaction energies in the intermediate state are significantly smaller than those for the reactant state. This is likely because in the intermediate state, the negative charge in the reactive region is largely concentrated on the enolate oxygen, near the mutated residues; it is therefore reasonably well-described at point charge level. In general, a key difference between the CAMM approach and a atomic point charge approach is that the CAMM approach can be applied anywhere along the reaction (including at the transition states), whereas a atomic charge approach is only clearly defined for intermediate states where consistent force-field charges can be assigned. The CAMM approach is thus more generally applicable, e.g. in the many enzymes where the transition state is the key species stabilized by the enzyme environment (instead of the intermediate state in KSI).

The Y14S mutant is the main outlier from the general trend. In this case, the DISS energy (dominated by contributions of −5.4 kcal/mol from Asp99 and −6.4 kcal/mol from $W_{TyrOH}$, the water placed based on the Tyr14 hydroxyl in WT KSI) predicts a lower activation barrier than is observed experimentally. One possible explanation for this (aside from the fact that the apparent free energy barrier was estimated based on kinetic data obtained with a different substrate, see Computational details) can be a smaller contribution of $\Delta S^\ddagger$ to the activation barrier in this mutant compared to WT KSI and other mutants. It should be noted that our DISS approach here, based on single structures, does not take into account differences in entropic contributions (i.e. the assumption is that entropic contributions are approximately similar between WT and mutants). In the case of Y14S, where $W_{TyrOH}$ provides a hydrogen bond to O3, we can expect a larger reduction in mobility of the $W_{TyrOH}$ hydrogen bond donor in the intermediate state (compared to the reactant state, due to a stronger hydrogen bond
interaction) than is observed for Tyr14 (present in WT KSI and mutants D99L and D99N, and held in place by interactions with surrounding residues, e.g. a hydrogen bond with Tyr55 and hydrophobic interactions with Leu18, Ile26, Phe80 and Phe101). Experimental kinetic measurements\textsuperscript{67} indicate $T\Delta S^\ddagger$ contributions for the enzyme and acetate catalyzed reactions of $-4.2$ kcal/mol and $-7.5$ kcal/mol, respectively. It can thus be expected that the corresponding value for Y14S moves closer to the latter, decreasing by up to $\sim 3$ kcal/mol (but probably smaller, as $W_{\text{TyrOH}}$ is confined by the enzyme environment) compared to WT KSI and other mutants. Such a change may thus explain the underestimation of the activation barrier calculated using DISS (e.g. the Y14S mutant lies $\sim 1$ kcal/mol above the least-squares fitted line for DISS(CAMM)). Another effect possibly important in this case could be polarization of the water molecule\textsuperscript{68,69}. The smaller separation of Y14S mutant from the main trend in HF and SAPT0 calculation (Figure 6) shows that this may indeed play some role in this mutant. Nevertheless, our results for Y14S support the deduction in the work of Kraut \textit{et al.}\textsuperscript{49}, who argued for the presence of a water molecule in the cavity left by the Y14S mutation. The substrate (reactant) is unlikely to shift significantly towards Tyr55 in the Y14S mutant (e.g. the O3-Tyr55 distance for Y14S/D38N is 4.1 Å in PDB ID 3IPT vs. 4.7 Å in 1OHP), we can estimate (by subtracting the contribution from the water molecule) that without the water molecule present, the overall DISS would be $-5.8$ kcal/mol, which would lead to a predicted activation barrier that is higher than that of the Y14F mutant (the mutant with the lowest activity). Thus, a water molecule interacting with the dienolate oxygen must be present to explain an activation barrier for Y14S KSI that is closer to the barrier in WT KSI than in Y14F KSI.
Figure 6. Differential Intermediate State Stabilization (DISS) energies in KSI mutants, calculated with various models, compared to experimental apparent free energies of activation, \( \Delta g_{app}^+ \) (taken from the works of Kraut and Choi\(^{49,64} \), see Computational details).

\( \Delta E_{\text{MTP}}^{(10)} \), multipolar contribution to electrostatic energy calculated with CAMM; \( \Delta E_{\text{D}} \), non-empirical dispersion correction (analytical formula fitted to reproduce SAPT data\(^{24} \));

\( \Delta E_{\text{elst}}(FF) \), Coulomb electrostatic energy using CHARMM27 force field charges; \( \Delta E_{\text{disp}} \), dispersion (total) interaction from SAPT; \( \Delta E_{HF} \) and \( \Delta E_{SAPT0} \), Hartree-Fock and SAPT0 DISS energies, respectively.

4.4 Comparison with other works

Our evaluation of the different contributions to the changes in activation barrier upon mutation indicates that electrostatics based on atomic multipole expansion (i.e. \( E_{\text{MTP}}^{(10)} \)) is a relatively good estimator of the differences in stabilization in enzyme variants (at least in the case of mutants evaluated). Our results thus support the previously reported importance of electrostatic preorganization in KSI\(^{37} \). We can further compare our work to the spectroscopic study of Fried \textit{et al.}\(^{30} \), which indicated that the magnitude of the electric field in the enzyme active site correlates with the magnitude of the rate enhancement achieved by the enzyme.

Specifically, the theoretical ‘zero-field barrier’ reported by Fried \textit{et al.} compares well to a
‘zero-DISS barrier’ as extrapolated from the CAMM results (y value at x=0 in Figure 5b as well as Figure 6), i.e. ~18.8 kcal/mol (from Fried et al. work\textsuperscript{30} vs. 18.8 kcal/mole (see Table S2). The high correlation between electrostatic (both $\Delta E_{elst}^{(10)}$ and $\Delta E_{MTP}^{(10)} (CAMM)$) DISS energies with experimental barriers supports the dominating role of electric field interactions in KSI as obtained from spectroscopic Stark effect measurements conducted by Fried and Boxer, as well as more recent analysis with unnatural aminoacids\textsuperscript{70}. However, our current work cannot further help specify the degree of the ‘electrostatic catalysis’ contribution to the overall catalytic effect in this system, as was discussed in response to Fried and Boxer’s work\textsuperscript{31,32}.

The two least active mutants considered here (D99L and Y14F), together with WT KSI, were also analyzed by Chakravorty and Hammes-Schif\textsuperscript{29}. Their work, in agreement with our SAPT DISS analysis (Figure S8), indicated that dispersion (or Van der Waals) interactions provide a negligible contribution to the activation barrier in these variants. Their activation barriers agree qualitatively with experiment, while intermediate electrostatic stabilization energies (both absolute values as well as those taken with respect to substrate) do not reproduce the trend (see Tables S4 and S5)\textsuperscript{29}. On the other hand, our DTSS(CAMM) and DISS(CAMM) results show the correct trend and are thus in (near) quantitative agreement with experiment. The likely reason for this difference is that the interaction energies reported by Chakravorty and Hammes-Schiffer are calculated between the environment (enzyme & solution) and the reactant, whereas here, the substrate is treated \textit{together} with Asp38 as a one subsystem. (treatment similar to that in QM/MM calculations).

Our CAMM approach to prediction of mutant catalytic activities of KSI shares some similarities with a recent Fragment Molecular Orbital (FMO) study on the same system\textsuperscript{71}, but direct comparison is complicated because the reactions are modeled with a different substrate and a different protein starting structure (PDB code 1QJG). Nevertheless, conclusions from
the FMO study are consistent with the results presented here: the differences between wild
type KSI and variants D99L and Y14F in DISS energy (as calculated by CAMM), i.e. the
predicted difference in activation barriers, are 1.4 and 2.3 kcal/mol, respectively, which differ
from the corresponding results from the FMO study: 4.3 and 5.1 kcal/mol by about 3
kcal/mol.

Residues involved in a hydrogen bonding network with Tyr14 have been investigated in
some detail. Tyr55 (present in KSI from both *P. putida* and *C. testosteroni*) directly
hydrogen bonds to Tyr14 (see Fig. 3), and can thus be expected to assist in stabilizing the
reaction intermediates (as confirmed by the aforementioned FMO study). We can quantify
this stabilizing influence by considering the Y55F mutant: when using the ‘minimal
perturbation’ strategy (see computational details), we find a DISS(CAMM) energy of −11.6
kcal/mol (compared to −13.1 kcal/mol for WT KSI). This corresponds to a predicted
activation barrier of 12.8 kcal/mol (using the equation of the ‘minimal perturbation’ model
best fit line in Fig. 5b), which is in excellent agreement with the apparent free energy barrier
from experiment on Y55F KSI from *P. putida* (12.7 kcal/mol). In KSI from *P. putida*,
Tyr30 donates a hydrogen bond to Tyr55, and the Y30F mutation led to a minor increase in
barrier (about 0.3 kcal/mol). In KSI from *C. testosteroni* studied here, Phe30 is the
equivalent residue, for which DISS(CAMM) detects a very minor stabilizing contribution
(0.03 kcal/mol). Mutation to Tyr does not lead to (further) stabilization, in line with the
occurrence of Phe30 in KSI from *C. testosteroni*.

4.5 Electrostatic multipole differential stabilization energy as rapid predictor of
enzyme catalytic activity

In this work, we show that the atomic multipole component of DTSS/DISS acts as a
reasonably accurate predictor of relative activity in a typical enzyme where catalysis is
dominated by electrostatic stabilization. By applying the cumulative atomic multipole
moment (CAMM) expansion, the atomic multipole component can be calculated rapidly. We show that the CAMM approach for calculating differential stabilization energies is less sensitive to structural perturbation compared to a fully quantum-mechanical electrostatic component ($E_{elst}^{(10)}$, see Supplementary Figure S7), and more general than a point-charge model (see ref.66). It is worth emphasizing that CAMM energy ($E_{MTP}^{(10)}(CAMM)$) scales as $O(A^2)$ (with $A$ the number of atoms), whereas the evaluation of the exact $E_{elst}^{(10)}$ would scale as $O(N^4)$ (with $N$ the number of basis set functions), which is impractical in the case of large molecular systems. Other interaction energy terms (e.g. exchange, induction and dispersion terms) are even more computationally demanding.

The CAMM model (with optional dispersion correction) proposed here for evaluation of enzyme activities has several advantages over full QM/MM or empirical models (e.g. EVB). CAMM and the $D_{as}$ dispersion correction do not rely on empirical parameters, since both are obtained from ab initio calculations: $E_{MTP}^{(10)}(CAMM)$ as an multipolar representation of electrostatic interaction energy $E_{elst}^{(10)}$ (eq. 9) and $D_{as}$ as a formula fitted to SAPT calculations24. Both scale as $O(A^2)$) with $A$ denoting number of atoms. This means that with our approach, evaluation of the change in reaction barrier between WT and mutant enzymes involves only single SCF calculations for WT reactant and transition (or intermediate) states (to obtain corresponding CAMMs), followed by interaction energy calculations for each mutant that are on the order of seconds (which is significantly faster than the time required for calculation of Hartree-Fock or MP2 activation energies for each mutant, see further Supporting Information, Figure S9). Moreover, the CAMM-based differential stabilization estimate of the change in activation barrier is less sensitive to geometry of mutants than high-level QM calculations, whilst exhibiting a similar correlation to experimental data.
Application of our CAMM approach for prediction of mutant activation energies does have some caveats: DISS (CAMM) energies are associated only with the enthalpic contribution to the free energy of activation and assumes that the same mechanism is followed between the variants compared. The approach is therefore not suitable to predict the catalytic activity of mutations that significantly change the activation entropy or the mechanism of the reaction. Keeping in mind these limitations, our computationally efficient CAMM approach could be applied to determine a subspace in combinatorial space to which more sophisticated screening of mutants could be applied. Given a database of atomic multipoles for amino acid residues (which we are currently developing), the DISS(CAMM) approach requires only the knowledge of transition / intermediate and reactant state structures in one variant (e.g. the wild type) and one subsequent SCF calculation for each structure for obtaining corresponding CAMMs. Subsequent calculations take no more than a second, without the need for any empirical parameters. Thus, such CAMM calculations are less (human and computer) time consuming than other approaches, such as FMO analysis\textsuperscript{71} or semi-empirical quantum chemical calculations involving full geometry optimization of mutant conformations\textsuperscript{10}, with the additional benefit of relatively low sensitivity to uncertainties in the structure of mutants.

5. CONCLUSIONS
To summarize, we show that differential stabilization energies based on rapidly calculated cumulative atomic multiple moments (CAMMs) correlate well with experimental apparent free energy barriers for KSI variants, without the need of any empirical parameters. This approach can thus be used to make quick and reasonable initial predictions of the impact of mutations on the catalytic activity of an enzyme, which may be a part of computational design protocol.
Associated content
Additional details, including CAMM convergence with respect to exponent truncation, rationalization of the use of DISS to evaluate the catalytic efficiency of the KSI isomerization reaction and MD simulations of intermediate state complexes of the mutant can be found in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Rapid estimation of catalytic efficiency by cumulative atomic multipole moments: application to ketosteroid isomerase mutants

Wiktor Beker, Marc van der Kamp, Adrian Mulholland, W. Andrzej Sokalski
Figure 1 / Mechanism of the isomerization reaction in KSI. Conversion of 5–androstene-3,7–dione into 4–androstene-3,7–dione occurs through two proton transfers. The first proton transfer is from C4 to Asp38 (proton abstraction by Oδ2)27,34, and the second proton transfer is from Asp38 Oδ2 to C6 (protonation)35. Residues in wild-type KSI that stabilize the dienolate oxygen are indicated.

40x10mm (300 x 300 DPI)
Figure 2 / Differential Transition State Stabilization (DTSS) and Differential Intermediate State Stabilization (DISS). The difference between activation barrier of two enzyme variants sharing a common reaction path may be expressed by the difference in their differential stabilization energies (see equations 2 and 3).

152x101mm (300 x 300 DPI)
Figure 3 / The intermediate of the 5–androstene-3,7–dione to 4–androstene-3,7–dione isomerization as bound in the active site of ketosteroid isomerase. Colors indicate the system setup: the part treated as ‘reactive subsystem’ in the minimal system calculations is depicted with orange carbons (part of the substrate and the Asp38 side-chain); the additional part of the substrate included in the ‘larger region’ (corresponding to the QM region for the QM/MM geometry optimizations) is depicted with brown carbons. Cyan atoms depict the surrounding residues that were considered for interaction calculations (from top down: Asp99, Tyr14, Tyr55).

70x44mm (300 x 300 DPI)
Figure 4. Overview of electrostatic stabilization along the reaction path in KSI. The reaction coordinate is defined as in ref.8 (see Computational details). a) Total relative electrostatic multipole ($E_{\text{mtp}}^{(10)}$) interaction energy profiles along the reaction pathway for wild-type KSI and mutants; b) contributions of individual amino acids to the interaction energy profile; c) relative QM/MM energy for the reaction calculated at the SCS-MP2:CHARMM27 level from ref. 8, together with the ESP charge on O3 (see methods); d) Comparison of CAMM Differential Transition State and Intermediate State Stabilization energies (DTSS and DISS, respectively) with experimental data ($\Delta G_{\text{app}}^{\ddagger}$) from the works of Kraut and Choi49,64 (see Computational details)
Figure 5 / . a) Comparison of the 'minimal perturbation' conformations (red; based on the wild-type QM/MM optimized intermediate structure) and representative conformations from MD simulations (blue; centroid of the main cluster from two 1 ns MD simulations; see Supporting Information) for the four mutants. b) Comparison of DISS(CAMM) energies for structures calculated with 'minimal perturbation' approach and minimized clusters from MD simulations against experimental data ($\Delta G_{\text{app}}^{\ddagger}$).49,64

$\Delta G_{\text{app}}^{\ddagger} = 0.52 \Delta E_{IS} + 18.78$
$\Delta G_{\text{app}}^{\ddagger} = 0.47 \Delta E_{IS} + 17.91$

177x101mm (300 x 300 DPI)
Figure 6 / Differential Intermediate State Stabilization (DISS) energies in KSI mutants, calculated with various models, compared to experimental apparent free energies of activation, $\Delta G_{app}^{\ddagger}$ (taken from the works of Kraut and Choi49,64, see Computational details). $\Delta E_{MTP}^{(10)}$, multipolar contribution to electrostatic energy calculated with CAMM; $\Delta E_{\text{disp}}$, non-empirical dispersion correction (analytical formula fitted to reproduce SAPT data24); $\Delta E_{\text{elst}}$ (FF), Coulomb electrostatic energy using CHARMM27 force field charges; $\Delta E_{\text{disp}}$, dispersion (total) interaction from SAPT; $\Delta E_{HF}$ and $\Delta E_{SAPT0}$, Hartree-Fock and SAPT0 DISS energies, respectively.

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