



High throughput and quantitative enzymology in the genomic era

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Abstract

Accurate predictions from models based on physical principles are the ultimate metric of our biophysical understanding. Although there has been stunning progress toward structure prediction, quantitative prediction of enzyme function has remained challenging. Realizing this goal will require large numbers of quantitative measurements of rate and binding constants and the use of these ground-truth data sets to guide the development and testing of these quantitative models. Ground truth data more closely linked to the underlying physical forces are also desired. Here, we describe technological advances that enable both types of ground truth measurements. These advances allow classic models to be tested, provide novel mechanistic insights, and place us on the path toward a predictive understanding of enzyme structure and function.

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[1,2]. In this perspective, we describe why the approaches that have been so successful in protein design are unlikely to lead to analogously predictive models for protein function, and we introduce concepts and experimental approaches that address these limitations and move us toward the ultimate goals of accurately and quantitatively predicting and designing function.

Structure broadly and deeply informs our understanding of function — consider the striking visualizations of motor proteins that have revealed the lever arms of myosin, dynein, and kinesin and their ATP-dependent power strokes [3], and the myriad of proteins whose shape is integral to their function, such as the β -clamp that encircles DNA to enhance polymerase processivity [4]. Nevertheless, more than structure is needed to describe, understand, and quantitatively predict function. Indeed, many proteins with the same fold differ in function, quantitatively and even qualitatively [5].

Function involves a series of states, such as the conformations through the myosin reaction cycle or the states in chemical reactions catalyzed by enzymes (substrate binding ($E \bullet S$), transition state ($E \bullet S^\ddagger$), product complex ($E \bullet P$), and release ($E + P$) to regenerate free enzyme ready for another round of catalysis). A minimal description of protein function therefore requires describing these states and determining the rate and equilibrium constants that define their transition probabilities and relative populations, respectively.

But still, more is required to understand and ultimately predict and design new functions — an ability to specify the functional consequences of sequence changes. Enzymes are large, with residues beyond the active site required for function, minimally to fold into and stabilize the correct binding and active site configurations [6]. But regions far from the active site can also have considerable functional consequences [7], as evidenced by allosteric modulation [8,9] and remote mutational effects frequently identified in high-throughput screens [10–12]. To find and describe which residues, sets of residues, and substructures affect function, as well as the particular aspects of function that are affected, we need approaches to systematically interrogate all residues and to determine the effects of perturbing them

Current Opinion in Structural Biology 2021, **71**:259–273

This review comes from a themed issue on **Catalysis and Regulation**

Edited by **Ruth Nussinov** and **Mikako Shirouzu**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 27 September 2021

<https://doi.org/10.1016/j.sbi.2021.07.010>

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Introduction

Scientists can now predict and design protein structure with ångström accuracy, a triumph culminating from decades of experimental and computational efforts

through each step of the enzyme's reaction cycle. In other words, we need to measure many rate and equilibrium constants for many mutants. Here, we highlight a breakthrough approach that allows these measurements to be made.

In principle, with sufficient empirical data, machine and deep learning approaches could be used to provide accurate, predictive models of enzyme function. However, sequence space is vast — so vast that nature has only sampled a miniscule corner of it [13]. And whereas pairwise residue information is largely sufficient to predict protein structure [14], enzyme function is much more complex, with multiple distal residues exerting functional effects on one another. These effects correspond mathematically to higher-order terms to account for the effects from combinations of multiple residues and are thus terms that are needed to quantitatively describe function. Because of this complexity, vast amounts of data would likely be required to define the relationship between sequence and function, and we suspect that the scale of data needed to predict enzyme function via machine learning and deep learning approaches may greatly exceed what is measurable, even with recent breakthroughs.

In contrast, physics-based approaches are scalable, so that simple rules can be used to describe the behavior of arbitrarily complex systems [15,16]. These models relate atomic forces and motions, captured by the preferred substates in conformational ensembles, to thermodynamic and kinetic constants. From the perspective of statistical mechanics, the constants that define function arise from the energy landscapes that define an ensemble of enzyme substates and the transition and reaction probabilities for each substate, represented mathematically in Figure 1 [17,18].

Thus, we need to go beyond structures to ensembles, and beyond structure–function relationships to ensemble–function relationships, and we will need to use experimental determinations of these relationships to test and build the physics-based models needed to quantitatively and accurately predict enzyme function. Here, we describe emerging X-ray crystallographic approaches that can provide this needed ensemble information.

Current design efforts yield enzymes that require cycles of randomization and selection to begin to approach natural enzymes. Perhaps the tortoise rather than the hare is needed to win this race [19], wherein large-scale quantitative and in-depth data are first collected and used to test and build models that will ultimately have the accuracy to predict and design enzyme function. We expect accurate enzyme functional prediction to remain a grand challenge of 21st-century biophysics — it is a still distant goal. Systematic blind tests of models built

from large-scale quantitative data provide a promising, and perhaps necessary, path forward.

Ground truths are needed for model development

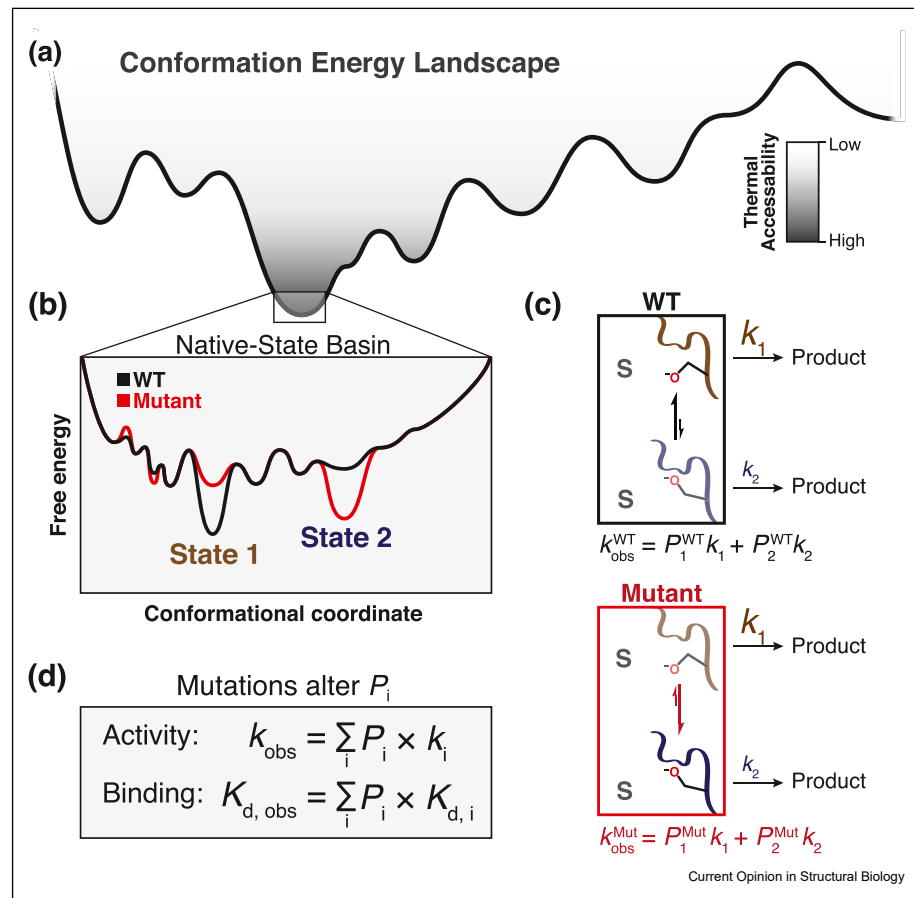
To develop and establish a model, 'ground truths' are needed. Ground truths are experimental data in a form that can be predicted by and thus compared to a model; without ground truths, there is no way to definitively test a model.

The most sophisticated models in enzymology combine quantum mechanics and molecular mechanics (QM/MM) and have been used to predict reaction rate constants [20,21]. However, in nearly all instances, the rate constants predicted by QM/MM were already measured and thus do not represent actual predictions that foretell a future event and that are incontrovertibly independent of the existing experimental findings [22]. The importance of predictions before measurement is underscored by the fact that the inability of protein folding models of the 1970s–1990s to predict structures was not apparent till they were challenged with truly blinded predictions (CASP, Critical Assessment of Structure Prediction); [1,23,24]). Ultimately, the algorithms and models that accurately predict structure were built using information from the large number of solved structures in the Protein Data Bank (PDB) [25], mining this information, and combining it with vast information from sequence conservation along with simplified energy potentials or rules derived via machine or deep learning [14,26–29,2].

Analogously, we need many measurements of kinetic and thermodynamic constants as ground truths to build and test predictive models of enzyme function, but current approaches are severely limited in their ability to deliver these essential data. Changes in residues throughout an enzyme can affect kinetic and thermodynamic constants, and combined changes will often not give additive effects; from a mathematical perspective, this property corresponds to a need for many terms in a model that can predict the effects from all sequence changes. With only a handful of measurements, there will not be enough data to constrain the model — from a simple algebraic standpoint, one needs the number of measurements to equal or exceed the number of variables in an equation to solve for those variables. Although we are unlikely to ever obtain sufficient measurements to fully define all of the variables of a master equation for function, we need many measurements to guide model development and then many predictions from these models — followed by many additional quantitative measurements — to provide a robust test of the models.

Structural ensembles can provide orthogonal ground truths. The relative occupancy of different

Figure 1



Enzymes function on a high-dimensional energy landscape. (a) Enzymes form a set of states specified by energy wells on a free energy landscape, with dimensionality defined by the thousands of degrees of freedom from each rotatable bond of each residue. (b) An example ensemble of near-energy substates in which State 1 (brown) and State 2 (blue) lie within the lowest energy basin ('native state basin') and equilibrate at room temperature. These substates have different intrinsic reactivities, reflecting barrier heights along the reaction coordinate that differ when projected from different conformational coordinates. (c) A mutation that does not affect intrinsic reactivity (red) can nevertheless diminish catalysis by redistributing equilibrium populations in the native state basin to favor less-active conformers (i.e. with higher free energy barriers to reaction; State 2 vs State 1). Mathematically, the apparent rate of the wild type ($k_{\text{obs}}^{\text{WT}}$) and mutant ($k_{\text{obs}}^{\text{Mut}}$) toward a substrate (S) is the probability-weighted (occupancy-weighted) sum of the intrinsic rate constants of each microscopic substate. In this two-state example, $k_1 > k_2$, so $k_{\text{obs}}^{\text{WT}} > k_{\text{obs}}^{\text{Mut}}$. (d) Observed enzymatic activity is the summed activity of each microscopic substate (k_i) weighted by its fractional occupancy (P_i). Mutations can alter the free energy landscape and change apparent activity (k_{obs}), or binding (K_d), by altering P_i , as shown in the figure, or by altering the reaction barrier (not shown).

conformational states reflects a balance of physical forces and thus provides ground truths that can be used as tests of models that account for these forces. Of further value, each ensemble provides a wealth of data — the distribution of states for each residue and around each backbone and sidechain bond, as well as information about their hydrogen bonds and electrostatic and van der Waals interactions. In contrast, average structures can be predicted without these 'details' being accurate, as evidenced by the rather simple force models present in successful Rosetta structural prediction algorithms [26,30].

The sections that follow describe recent advances in obtaining these ground truths for enzyme function.

The need for quantitative enzymology at scale

Recognizing the need for an immense amount of data to describe and understand enzymes and their function, high-throughput approaches have been used to interrogate up to $\sim 10^6$ sequence variants in parallel. In particular, deep mutational scanning (DMS) approaches have been applied to all possible single mutants for dozens of unique proteins [31,32].

Some DMS studies report the effects of mutations in a particular protein on organismal fitness, a convolution of multiple factors [33,34]. These experiments can also be designed to report more specific aspects of function, including catalytic efficiency, substrate specificity, stability, and interaction with binding partners [10,11,35–39].

Although valuable and sometimes of immediate practical benefit, these functional readouts still represent a convolution of contributing factors. For example, for observed catalytic function, a mutant enzyme down 100-fold in catalysis can be 99% unfolded, 99% partitioned to an alternative misfolded state, have a misaligned catalytic residue, have a binding interaction removed, or exhibit some combination of these factors. Thus, these functional readouts fall short of delivering the needed ground truths.

At the other end of the spectrum, traditional enzymology provides kinetic and thermodynamic constants that describe the catalytic cycle and have been combined with incisive mechanistic probes (e.g. alternative substrates, isotope effects, and so on) to provide deep mechanistic insights. However, these approaches are only feasible for a small number of variants of each enzyme. Past efforts to quantify properties of many variants in T4 lysozyme, pyruvate kinase, and β -glucosidase B underscore that data for many mutants can be collected when heroic means are used [40–43]. But even in these cases, the time and cost to carry out the additional measurements required to probe the mechanistic origins of the observed effects would be prohibitive.

It is hard to identify a discipline that has not been transformed at one time or another by a breakthrough technology. Here, technology was needed to efficiently provide the rich and quantitative data of traditional enzymology at a much larger scale for many variants and multiple enzymes.

Quantitative enzymology on a chip

Advances in microfluidics provided the opportunity to marry the strengths of traditional enzymology with automated high-throughput data collection and bring enzymology into the genomic age [44–46]. High-throughput microfluidic enzyme kinetics (HT-MEK; Figure 2) allows 1500 enzyme variants to be produced, purified, and subjected to multiple quantitative assays in days, at a miniscule fraction of the cost of traditional approaches [47].

Figure 2 outlines how HT-MEK experiments are performed. HT-MEK uses a microfluidic device with chambers aligned to a DNA microarray of 1500 isolated variant plasmids (Figure 2a). Expression and purification of enzyme variants are carried out in parallel, so that all 1500 enzymes can be purified, quantified, recruited to antibody-patterned surfaces, and ready for assay in hours (Figure 2b). Pneumatically controlled valves allow the user to protect the enzyme from flow-induced shear forces, while the expression solution is removed and an assay solution containing substrate is added to the chambers, followed by opening of the valves to simultaneously initiate reactions in all chambers (Figure 2c).

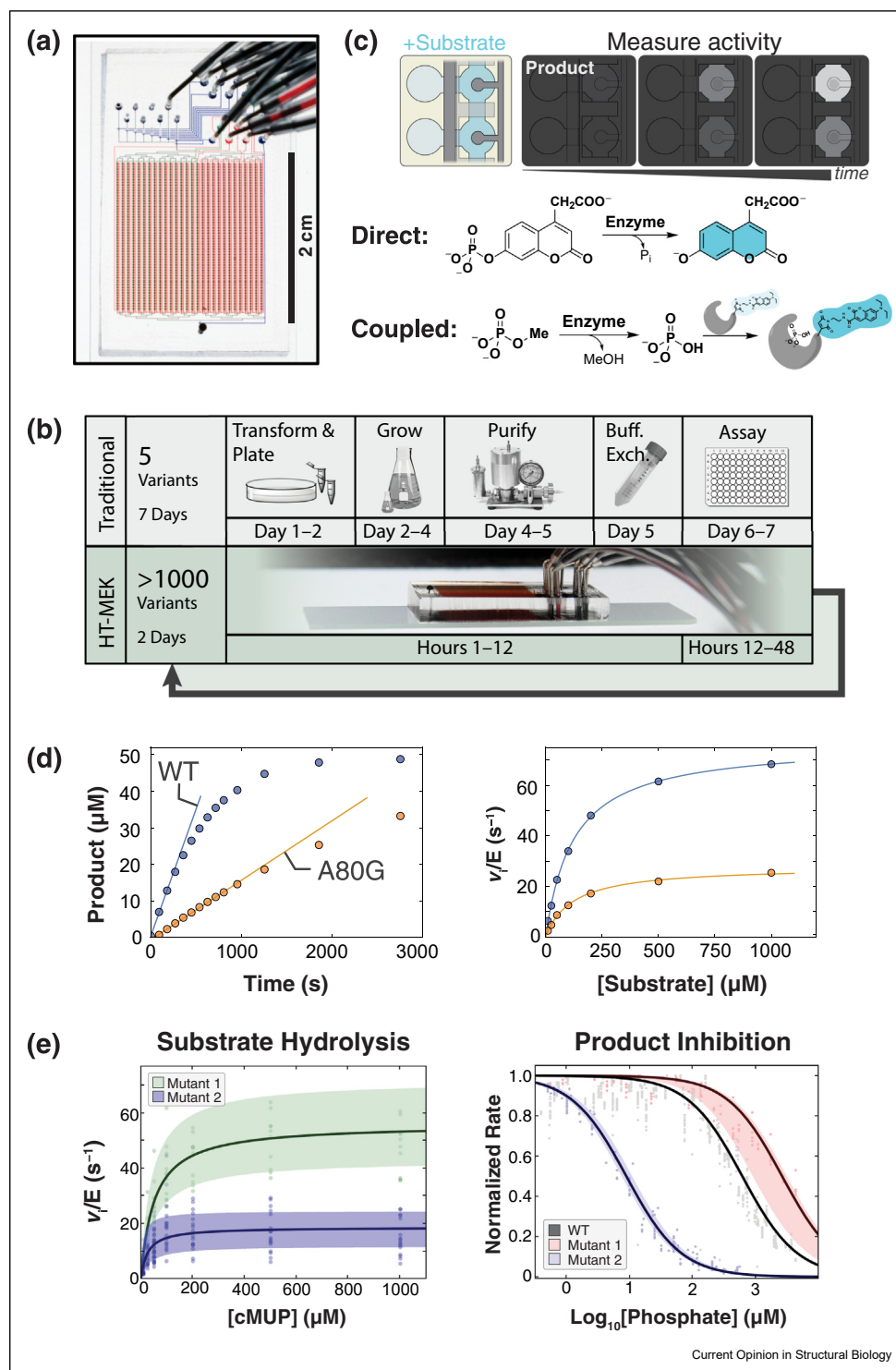
Product formation is quantified over time via fluorescence, either directly using a fluorogenic substrate or indirectly using a coupled assay (Figure 2c). Once complete, reaction product is flowed out, and a new substrate stock is flowed in so that a series of assays can be performed iteratively. Figure 2d shows example Michaelis–Menten and inhibition curves obtained in HT-MEK experiments.

Each HT-MEK device can be used to carry out tens of reactions, and a single researcher can fabricate tens of devices in a day. These properties make it possible to carry out hundreds of assays — with multiple substrates, inhibitors, and solution conditions, as used traditionally in mechanistic enzymology — but to do so across thousands of enzyme variants and to do so in a few weeks. Thus, the properties of HT-MEK allow measurements of many kinetic and thermodynamic constants that provide valuable information about an enzyme and can serve as ground truths for model testing.

For PafA [48], a phosphate monoesterase and our test case, we obtained >6000 kinetic and thermodynamic constants from >650,000 kinetic measurements for 1036 mutants. HT-MEK provides a wide dynamic range, $\sim 10^5$ in rate for PafA, which allows measurement of large active site effects and reaction rates for noncognate substrates. High measurement precision is obtained with rigorous error estimates using bootstrapping, which is possible because of the many replicates acquired within each HT-MEK assay.

In addition to providing a large number of ground truth measurements that can be used to evaluate and guide the development of quantitative models, the initial PafA data provided extensive mechanistic information not previously accessible. The observation that mutations at most of the 526 PafA positions altered one or more kinetic and thermodynamic parameters underscores the need for measurements throughout an enzyme to map its function. Further revealing and displaying the intricacy of enzyme function, different sets of residues affected different reaction steps and underlying catalytic mechanisms as well as folding, as illustrated in the functional maps of Figure 3. The largest mutational effects were seen at the active site and directly around it, as expected, but effects extended from the active site all the way to the enzyme surface, with large effects many ångströms from the active site and different remote regions affecting different aspects of function (Figure 3a). We do not think that these effects could have been predicted *a priori* using current approaches. Regardless, researchers with predictive algorithms can now use those algorithms on any of the multiple enzymes amenable to HT-MEK, so that we can determine the algorithm's predictive power.

Figure 2

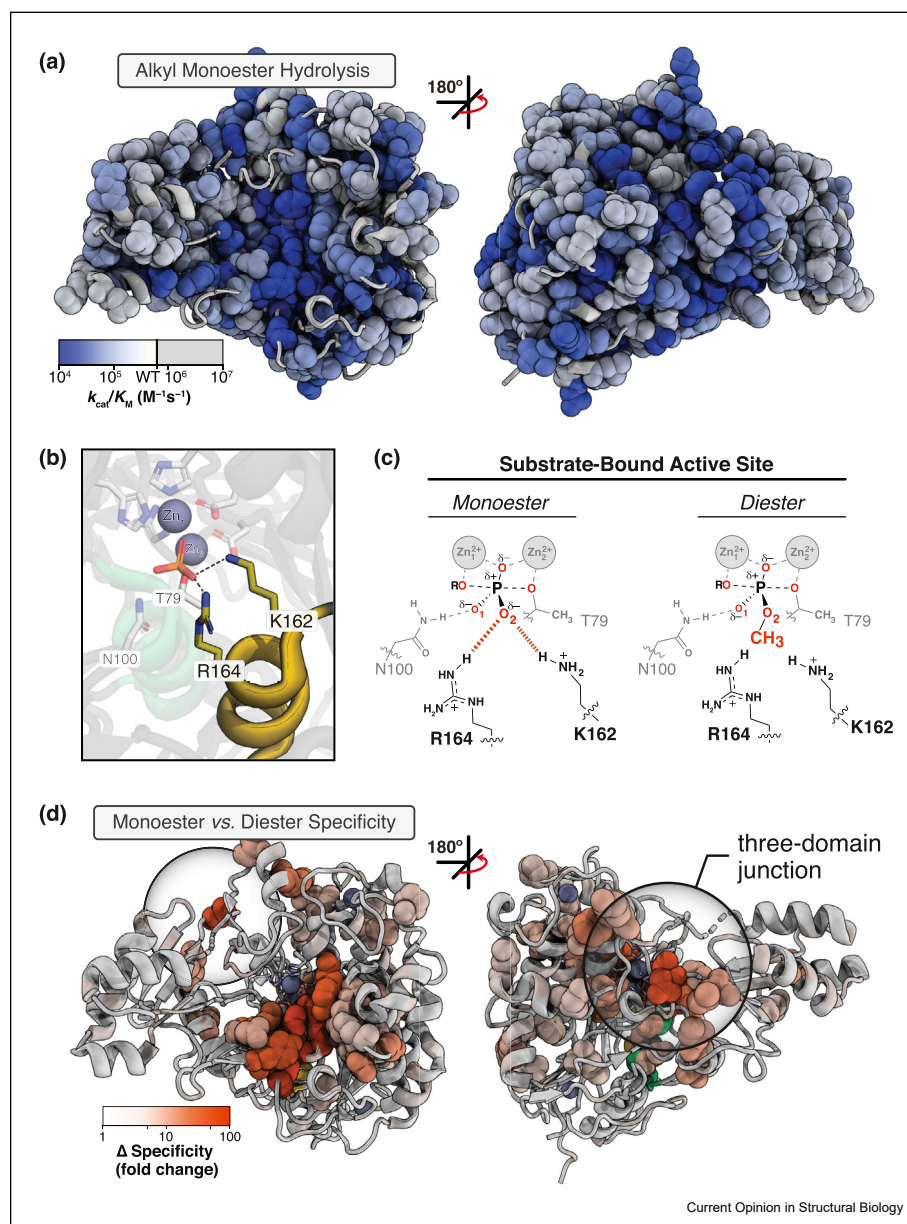


Application of HT-MEK to study catalysis and inhibition. (a) HT-MEK uses a valved microfluidic device containing >1500 chambers to (b) in vitro express, purify, and assay 1500 enzyme variants in days. Iteratively varying substrates, inhibitors, and conditions give kinetic and thermodynamic parameters of function. (c) Reactions are performed by introducing fluorogenic substrate (light blue) into reaction chambers and synchronously exposing surface-immobilized enzyme to the substrate with pneumatic valving. Product (darker blue) is quantified by fluorescence over time, using direct (fluorogenic) or indirect (coupled) assays. The assays shown are for phosphatase activity or for any enzyme that directly or indirectly generates inorganic phosphate. (d) Example on-chip fluorogenic substrate turnover curves for the PafA phosphatase with fit initial rates (left) and Michaelis–Menten curves (right) for wild type (blue, WT) and mutant (orange) PafA variants. A per chamber standard curve is used to convert fluorescence to product concentration. (e) Many replicates (within separate chambers) for each variant (Mutant 1: Y74V, Mutant 2: Y112G) over multiple chips are used to calculate bootstrap errors on fit Michaelis–Menten (left) and competitive inhibition (right) parameters (reproduced from Markin, Mokhtari, et al. (2021) [47]). HT-MEK, high-throughput microfluidic enzyme kinetics.

Consider, as an example, the active site arginine and lysine residues that contact one of the substrate phosphoryl oxygen atoms and are responsible for reaction specificity for phosphate monoesters over diesters (which are substrates of related superfamily members; [48–52]) (Figure 3b and c). Although mutation of most residues contacting these active site residues

diminished specificity, a majority of the affecting residues were remote, including residues at the junction of three auxiliary domains (Figure 3d; auxiliary domains are structural regions present in subsets of Alkaline Phosphatase superfamily members [52]). The auxiliary domain junction sits ~ 20 Å from the active site and on the opposite side of the enzyme from the catalytic

Figure 3



Spatial patterns of PafA mutational effects on specific functions. (a) Alkyl phosphate hydrolysis (k_{cat}/K_M) for glycine and valine substitutions at each position in PafA. Significant effects are shown as spheres, color-coded by the size of the effect. (b) The PafA-active site (PDBID: 5TJ3) showing contacts between covalently bound phosphate (T79) and active sites K162 and R164 (yellow sticks). (c) Schematic of PafA-active site residue contacts to bound phosphate monoester (left) versus diester (right) substrates. (d) Substrate specificity effects upon valine and glycine mutation of PafA positions, visualized as in (a). Large mutational effects (spheres) cluster primarily around R164 and K162, extending distally to a three-domain junction (circled) more than 20 Å away. Panels (a), (c), and (d) are adapted from Markin, Mokhtari, et al. (2021) [47].

pocket but nevertheless exhibits mutational effects of up to 60-fold [47]. These observations suggest that the auxiliary domains and their positioning are critical for catalytic function by the active site arginine and lysine, but we would not have predicted these or other remote effects for mutations throughout PafA.

One would be tempted to conclude, in the absence of data to the contrary, that most distal functional effects arose from destabilizing the active enzyme. With HT-MEK (and related approaches under development), we can independently assay folding [47,53]. We found that none of the PafA effects arose from equilibrium unfolding (PafA is a secreted enzyme and is highly stable). Nevertheless, our ability to assay PafA with multiple substrates and under multiple expression and reaction conditions allowed us to uncover the presence of an unanticipated long-lived misfolded state of the enzyme. Without accounting for the effects of misfolding and unfolding on observed reaction rates, functional models cannot be unambiguously made or tested.

As noted previously, an immediate challenge is to predict distal effects for multiple enzymes and to use HT-MEK to determine what is correctly predicted, quantitatively or qualitatively. We can also directly use data from HT-MEK to aid enzyme engineering at a practical level. Functional maps generated by HT-MEK can inform where mutations should be made to enhance the chance of altering and tuning specific functional parameters. In addition, HT-MEK can rapidly assess human alleles to reveal the biophysical bases of mutations associated with disease and to inspire new and precise therapeutic strategies. For instance, the discovery of surface residues (through mutation) allosterically linked to function may allow druggable enzyme activation as well as inhibition [54].

Conformational ensembles for evaluating and building physical and catalytic models

Ultimately, we want to predict kinetic and thermodynamic constants for a particular reaction with a specified sequence. We can measure many of these values via HT-MEK as ground truths and compare them to values predicted by models, but these functional constants are emergent properties that result from the enzyme's underlying physical properties. We would like to have ground truths more closely connected to the enzyme's physical properties; these measurements would provide more direct tests to evaluate and improve physics-based models. In particular, we want to know an enzyme's conformational landscape and how this varies with bound ligands and through the enzyme's reaction, and we want to determine the affinities and reactivities of the states that constitute the landscape (Figure 1a–c).

The value of and need for ensemble information to understand protein folding and function have been recognized for decades [17,55,56]. For catalysis, the clear evidence for remote effects — from allosteric ligands and post-translational modifications — and efforts to understand how enzymes so efficiently navigate their reaction paths have led to a panoply of functional models that invoke dynamics (e.g. studies reported by Hammes *et al.*, Schwartz, Hanoien *et al.*, Kochen, and Warshel and Bora [57–61]). Experimentally, NMR provides relevant information about the rates of transitions between conformational states and information (e.g. order parameters) on the relative conformational freedom of residues (e.g. studies reported by Palmer and Alderson and Kay [62,63]). However, detailed atomic information that provides more direct tests of models, such as the extent and direction of motion and which motions are coupled or independent, is difficult to obtain via NMR. Fortunately, emerging X-ray crystallographic approaches can provide extensive and detailed information about conformational ensembles that can be more directly related to predictions from physics-based models.

A key technological breakthrough in X-ray crystallography was cryofreezing crystals to reduce their susceptibility to radiation damage and make crystal handling more reliable. Indeed, at least 90% of more than 150,000 protein X-ray structural models in the PDB were obtained under cryogenic conditions (diffraction source temperature ≤ 125 K) [25]. Nevertheless, X-ray structures can be obtained at ambient temperatures as well, conditions that do not quench a protein's inherent dynamic motions [64]. Ambient or 'room-temperature' (RT) X-ray crystallography requires high resolution (typically sub-1.5 Å) to provide reliable and extensive information about conformational heterogeneity at the atomic level and requires larger-than-average crystals to limit X-ray damage during data collection. Fortunately, many enzymes of interest yield crystals of the desired size and quality, and recent methodological improvements allow RT X-ray crystallography to be broadly implemented [65].

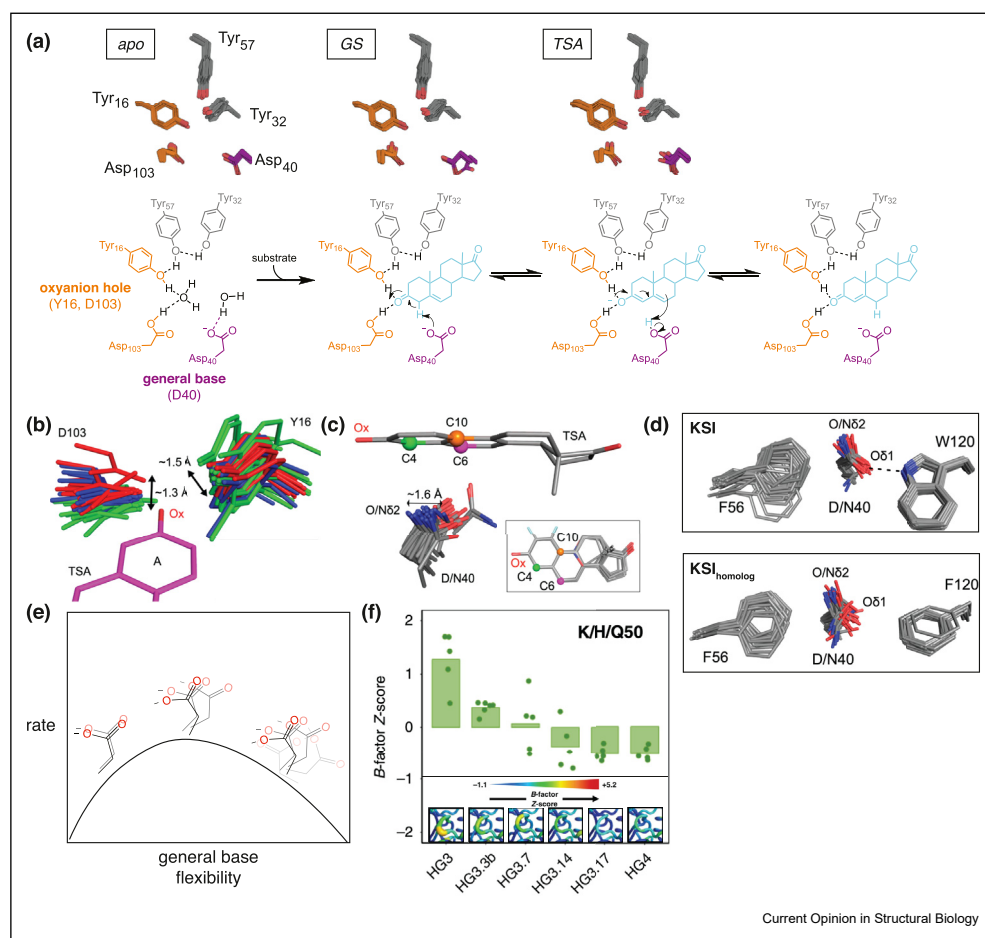
Conformational ensembles can also be generated from cryo X-ray structures, by combining multiple static structures into a so-called pseudoensemble [66,67]. In brief, it is assumed that individual cryogenic X-ray structures of proteins sharing the same or highly similar sequences (e.g. with one or a few mutations) provide conformers trapped in different low-energy wells on the protein's energy landscape, so that combining many cryo-structures can approximate the protein's accessible ensemble of states (for pseudoensemble computational tools, see studies reported by Monzon *et al.*, Li *et al.*, Zhang *et al.*, and Grant *et al.* [68–71]). Although motions are restricted, and some are changed on freezing,

several lines of evidence and direct comparisons support a close correspondence of the flexibility within pseudoensembles and the motions present at ambient temperatures [67,72].

Pseudoensembles and RT data are complementary — the latter providing the most reliable information about conformational heterogeneity and the former retaining information about correlated motions within the constituent conformers. These approaches have provided insights into multiple systems, including HRas GTPase,

protein tyrosine phosphatase, proline isomerase, soybean lipoxygenase, β -lactamase, dihydrofolate reductase, isocyanide hydratase, herpes virus protease, and designed and laboratory-evolved Kemp eliminases [73–83]. The most extensive X-ray ensemble data to be collected and analyzed is for ketosteroid isomerase (KSI; Figure 4a). For KSI, pseudoensembles and high-resolution RT ensembles have been obtained for complexes representing the states in the enzyme's reaction cycle, for KSIs from two different species, and for WT and mutant KSIs [72].

Figure 4



Ensemble crystallography provides mechanistic insights toward enzyme catalysis and engineering. (a) KSI reaction mechanism and ensemble crystallography of multiple states in the reaction cycle. Active-site residues from experimental RT multiconformer models are shown for apo, ground-state (GS) bound, and transition state analog (TSA) bound KSI. (b, c) Pseudoensembles for KSI oxyanion hole residues Y16 and D103 (b) and the general base (D40, wild type, or mutant D40N) (c), derived from cryo-structures containing bound transition state analogs (TSA, equilenin and phenols). Bound TSAs were superimposed, aligned on phenol ('A') rings and shown as a single structure for simplicity. (d) Pseudoensemble for the KSI general base (D40 or mutant D40N) and neighboring residues for KSI (F56, W120) and a KSI_{homolog} (F56, F120). KSI and KSI_{homolog} k_{cat} measurements for both the WT and the position 120 reciprocal mutants (KSI W120F; KSI_{homolog} F120W) demonstrate a 4-fold (KSI) and 8-fold (KSI_{homolog}) rate increase for phenylalanine versus tryptophan-containing variants. (e) Cartoon depiction of the relationship between KSI rate and general base (D40) flexibility. (f) Increase in active-site rigidity during directed evolution of a designed Kemp eliminase (from HG3 to HG4). B-factor Z-scores are plotted for each heavy atom of the position 50 side chain (K, H, or Q). Structural views of the Kemp eliminase residues 87–90 loop from room-temperature crystallography, with B-factor Z-scores colored as per legend and increasing with sausage plot thickness (inset). Panels (b) to (e) adapted from Yabukarski et al. (2020) [72]; panel (f) adapted with changes from Broom et al. [83] (<http://creativecommons.org/licenses/by/4.0/>). KI, ketosteroid isomerase; RT, room temperature.

Insights from KSI ensembles

The function of a catalytic residue depends not only on its presence in the vicinity of the substrate but also on the adoption of conformational states with the correct distance and orientation to the substrate and/or other residues. Indeed, positioning is universally invoked or assumed in descriptions of enzyme catalysis, but without ensemble information, we cannot know the nature and extent of this positioning. Ensemble information is also needed to understand the motion inherent in all chemical reactions, minimally to go from van der Waals distance to form a bond, a change on the order of 1 Å, and how or whether this is affected by the enzyme environment. Furthermore, many enzymes catalyze multistep reactions and use the same functional groups in different poses to carry out successive reaction steps. Again, ensemble information is needed to understand how enzymes navigate these challenges.

KSI, a steroid isomerase, abstracts a proton from its steroid substrate with a general base, transferring the proton to a different position of the resulting intermediate to give the more stable conjugated product, using an oxyanion hole to stabilize negative charge accumulation on the intermediate (Figure 4a). Oxyanion holes for serine proteases have been suggested to contribute catalytically via ground-state destabilization, by forming suboptimal, geometrically constrained hydrogen bonds that sit out of the plane of the ground state sp^2 oxygen [84–87]. The KSI ensembles reveal motions of the oxyanion hole hydrogen bond donors on the scale of ~ 1 Å and an absence of discrimination between the sp^2 and sp^3 oxygen electronic configurations (Figure 4b). Instead, the oxyanion hole seems to provide catalysis by forming hydrogen bonds that are stronger than those to water in solution [6,72,88–90].

Extensive site-directed mutagenesis studies revealed an astounding effective molarity of 10^3 – 10^5 M for the KSI aspartate general base [91]. Although the simplest explanation for this large catalytic effect is precise positioning, positioning at multiple sites would be required to accommodate KSI's multiple substrates and successive reaction steps. As previously mentioned, KSI ensembles allowed this model to be tested, revealing a broad distribution of general base positions (Figure 4c), as needed to abstract and donate protons at multiple positions and indicating that alternative models are needed to account for the highly efficient observed general base catalysis [91–93]. In addition, the flexibility in the oxyanion hole, while precluding ground-state destabilization, contributes to the conformational plasticity of the general base and substrate with respect to one another (Figure 4b and c).

One might expect there to be a balance between positioning and flexibility: clearly, too much flexibility of the

KSI general base would hamper catalysis while too-restricted positioning could as well, by limiting access to the multiple states needed to carry out the full reaction cycle. Remarkably, functional results and ensembles for wild type and mutant KSIs provide evidence for the balance: an aromatic–anion interaction provides greater flexibility of the general base than a hydrogen bond and faster reaction, whereas mutations in the general base loop that disorder it substantially impair catalysis (Figure 4d and e).

In addition, comparisons of how conformational ensembles of KSI side chains change from mutations in nearby residues provide information about the balance of forces, including conformational entropy, that determine where and how much the oxyanion hole tyrosine is positioned [72] (Figure 4b). Effects such as these will provide rich testing grounds for force fields in physics-based models. Finally, the observation that, at least in this case, ensemble rearrangements remain local suggests at least some limitation to the complexity of models needed to account for energy landscapes of enzymes (Figure 1a and b; see also 'The complexity of functional models' in the following).

Rules for enzyme design from ensemble crystallography

A major challenge is to understand why the performance of de novo enzyme designs falls short of natural enzymes [94–96], and how to rationally engineer solutions that nature (or researchers) discover through evolution. Early de novo design of Kemp eliminases (KE) yielded some success [97], but the same fold rate enhancement, or more, for the eliminase reaction is achieved in the active site of KSI, an enzyme evolved to carry out different chemistry with different substrates. This result suggests that designed KEs accomplish only coarse positioning against a general base within a binding pocket [98]. With laboratory-evolved improvements (17 substitutions), a $\sim 10^5$ -fold increase in k_{cat}/K_M was achieved [99]. Recent work from the Chica and Fraser groups sought to understand the mechanistic bases for these improvements, interrogating four variants along the mutational trajectory via RT crystallography [83,100]. This effort revealed that apocatalytic elements rigidified along the mutational trajectory, favoring catalytically preorganized poses, consistent with classical proposals for origins of catalysis from positioning of substrates and catalytic groups (Figure 4f) [93,101,102].

More generally, crystallographic ensembles can be used to test models of catalysis that attempt to link motions or positioning to function, identifying the types and scales of motions that may be relevant to progress along the reaction coordinate. This ability allows the structure–function paradigm to be supplanted by ensemble–function analyses.

Ensemble measurements versus the reaction coordinate

The X-ray ensemble approaches described previously are needed to relate structure to energetics and function but also have limitations. Most centrally, they provide information about the ‘lower levels’ of the enzyme’s conformational landscape. States that are uphill by $> \sim 2$ kcal/mol, representing $< 5\%$ of the total population, are unlikely to be observed. This limitation alone is not severe, as one can decipher much of the underlying energetics by having lots of data — as is provided by X-ray ensembles — in the $\sim 0\text{--}2$ kcal/mol energy range. But what is missing is information about the conformations and motions as one climbs further toward the reaction’s transition state. Transition states are by definition fleeting states, lasting < 1 ps, and highly improbable. To assess what happens at these rarified regions of the energy landscape and whether the data obtained closer to the base of these mountains are adequate to model the reaction coordinate, we need additional ground truths for these transient, high-energy states. Although such information cannot (yet) be obtained in high throughput, several methods exist to provide this critical information.

The highest time resolution structural data use laser pulses to initiate a process and serial high-resolution X-ray data collection. X-ray free-electron lasers and cutting-edge synchrotron sources capture crystallographic snapshots at ambient temperatures by supplying intense femtosecond X-ray pulses [103,104]. Tenboer et al. [105] used nanosecond laser pulses in conjunction with X-ray free-electron laser crystallography to isomerize the double bond of the photoactive yellow protein chromophore and to measure protein and chromophore conformational changes after time delays of 10 ns and 1 μ s. These experiments revealed the significant side-chain displacements associated with photocycle transients at high resolution. Schlichting et al. [106] used a 150 fs laser pulse to dissociate CO from myoglobin and were able to follow in real-time the synchronous nonequilibrium picosecond oscillations of the heme ring that arise from the CO dissociation energy and dissipate on the order of 10–100 ps. Vibrational spectroscopy, although not directly measuring atomic positions, is particularly powerful because frequencies can be assigned to specific bonds located within proteins or bound reactants and can provide information about changes in the strength and properties of those interactions. Dyer and Callender [107] used temperature-jump infrared spectroscopy on the microsecond timescale to identify multiple distinct and noninterconverting substrate-binding conformations with different reactivities in lactate dehydrogenase, providing a detailed energetic map of reaction trajectories unavailable with traditional methods. It may also be possible to carry out time-resolved vibrational studies on enzyme libraries in high throughput. A critical next step will be to apply these synchronized approaches more

broadly so that motions and transitions are not averaged among the population of molecules in the crystal [82].

The complexity of functional models

Residues are functionally, and thus energetically, interdependent. This interconnection is most simply appreciated by recognizing that without the ‘right’ residues surrounding the catalytic and binding residues those active site residues do not yield significant catalysis and vice versa [108]. Consequently, descriptions of ‘residue function’ cannot be made from single-mutant variants alone. The extent of residue connectivity — how many residues affect the function of a particular residue and by how much — defines the complexity of the model that is needed to mathematically describe an enzyme’s function [109,110]. Although this complexity is likely to vary — for enzymes with different folds, with allostery, and so on — we want to know the scale and range of this complexity as it will dictate the form of models and how they are developed and tested [111,112].

We also know that residues are not fully interdependent (epistatic), as if this were the case any single mutation would shatter the active site and fully abolish activity. Classical mutational studies have found some regions, including active sites, with limited energetic dependencies among sets of three or fewer residues [113–115]. In one striking case, a single residue change was predominantly responsible for improved activity in psychrophiles versus improved stability in thermophiles [116]. Phylogenetic comparisons, verified experimentally, identified two nearby residue changes needed to fully shift stability and activity but with much smaller effects.

Phylogenetic comparisons across many enzyme families containing psychrophilic, mesophilic, and thermophilic variants suggested instances of limited epistasis in temperature adaptation [116]. Most of these covarying sets of residues corresponded primarily to pairs of residues that correlate with temperature adaptation and likely confer function within divergent sequence backgrounds of a given enzyme family. These observations suggest substantial simplifications in residue interdependences and model complexity in many cases. Nevertheless, there are also larger co-occurring sets, and our initial HT-MEK experiments in PafA have also identified functional interconnections among tens of residues in regions extending from the active site to the surface.

Experimentally, even measuring effects of all possible triple-mutant substitutions within a single small (100 residue) enzyme is intractable as it would require $> 10^9$ variants. Instead, it will be necessary to prioritize higher-order mutants most likely to be informative, guided by

maps of enzyme architecture identified in initial HT-MEK surveys and additional phylogenetic information such as that described to understand temperature adaptation [117]. This is an area that will likely require innovative ideas and rigorous tests to identify paths toward predictive models. The lengths of those paths and the difficulty in traversing them will be determined by how rapidly partially predictive physics-based models can be developed and used as guides.

Conclusion and outlook

Accurate quantitative prediction of protein and enzyme functions from amino acid sequence is the ultimate litmus test of our biophysical understanding. But as other disciplines have experienced, breakthroughs frequently arrive later than hoped, and only with sustained effort at the nexus of a deep need, technological development, and an empirical or theoretical framework.

The need for accurate and general quantitative models of functional prediction is clear — they would transform medicine, industry, and biotechnology. But what remains uncertain is whether we possess the requisite theoretical and technological foundation. Where we stand is likely to be clarified only through careful tests of current general predictive models and algorithms, particularly physics-based ones, using blinded comparisons to large-scale empirical measurements. With HT-MEK and crystallographic ensembles, we are starting to acquire the needed quality and quantity of data to compare with predictions.

Most immediately, HT-MEK can be used to study new enzymes, as it can be applied to any enzyme with a direct or coupled fluorogenic assay. HT-MEK, and extensions to it currently under development, can assay protein stability as well as function, and important applications include dissecting the functional effects of human alleles that do or may cause disease; providing foundational information to guide protein engineering and design efforts using current approaches; and combining enzymes to efficiently engineer metabolic pathways.

We envision a future wherein large-scale mutational studies are routinely performed with HT-MEK. In this future, analysis, interpretation, and modeling are the rate-limiting steps of advancing protein biochemistry, instead of experimentation. To drive future advances in these models, conformational ensembles will provide ground-truth atomic positions and motions and describe how these are altered in mutants of differing functions. Ensembles can be assembled for many enzymes from the vast data available in the PDB [72], and ensemble information can now be readily attained for new enzyme complexes and variants through advances in room temperature data collection [65].

We suspect that a ‘critical assessment of quantitative protein function’ will be needed. Although blind protein function prediction challenges exist, these contests typically use *in vivo* coarse phenotypic data (e.g. whether or not particular mutations are deleterious) as targets, reflecting the absence of and need for large-scale *in vitro* quantitative measurements of specific functional parameters [118,119]. A new effort would involve solicitation of large-scale quantitative functional data sets ahead of publication and creation of objective metrics for success that scale across different parameters and sizes of data sets. We look forward to contributing to this effort by basic scientists, engineers, and theoreticians.

Finally, with deep functional data in hand, it will also be possible to extend predictive models to the systems level, connecting the basic enzyme properties responsible for selective advantages observed in DMS experiments and in natural and laboratory evolution. Here, comparisons of effects predicted from metabolic models for particular kinetic perturbations with experimental measurements of fitness and of metabolite levels will provide a path to develop robust models to understand cellular metabolism and to engineer new metabolic pathways.

Conflict of interest statement

Nothing declared.

Acknowledgements

The authors dedicate this article to the memory of Dan Tawfik who recently and tragically passed away. Dan's creativity and enthusiasm was an inspiration to us and many others. The authors thank Craig Markin, Siyuan Du, and members of the Herschlag and Fordyce laboratories for helpful discussions and review. This work was supported with funding from the National Institutes of Health (NIH) R01 grant (GM064798), the Gordon and Betty Moore Foundation grant, and the Ono Pharma Foundation Breakthrough Initiative Prize awarded to D.H. and P.M.F and the National Science Foundation (NSF) grant (MCB-1714723) awarded to D.H. D.A.M. acknowledges support from the Stanford Medical Scientist Training Program and the Stanford Interdisciplinary Graduate Fellowship (Anonymous Donor) affiliated with Stanford ChEM-H. P.M.F. is a Chan Zuckerberg Biohub investigator.

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