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REVIEW ARTICLE

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Toward physics-based precision medicine: Exploiting protein dynamics to design new therapeutics and interpret variants

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Abstract

The goal of precision medicine is to utilize our knowledge of the molecular causes of disease to better diagnose and treat patients. However, there is a substantial mismatch between the small number of food and drug administration (FDA)-approved drugs and annotated coding variants compared to the needs of precision medicine. This review introduces the concept of physics-based precision medicine, a scalable framework that promises to improve our understanding of sequence–function relationships and accelerate drug discovery. We show that accounting for the ensemble of structures a protein adopts in solution with computer simulations overcomes many of the limitations imposed by assuming a single protein structure. We highlight studies of protein dynamics and recent methods for the analysis of structural ensembles. These studies demonstrate that differences in conformational distributions predict functional differences within protein families and between variants. Thanks to new computational tools that are providing unprecedented access to protein structural ensembles, this insight may enable accurate predictions of variant pathogenicity for entire libraries of variants. We further show that explicitly accounting for protein ensembles, with methods like alchemical free energy calculations or docking to Markov state models, can uncover novel lead compounds. To conclude, we demonstrate that cryptic pockets, or cavities absent in experimental structures, provide an avenue to target proteins that are currently considered undruggable. Taken together, our review provides a roadmap for the field of protein science to accelerate precision medicine.

KEYWORDS

conformational ensembles, drug discovery, machine learning, Markov state models, precision medicine, protein dynamics

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1 | INTRODUCTION

The goal of precision medicine is to utilize our knowledge of the molecular causes of disease to better diagnose and treat patients. In the precision medicine framework, diseases are subdivided by their underlying etiologies, and treatment is based on a patient's unique genetic background, unlike the current state of medicine where most patients with a given diagnosis are treated the same way. Precision medicine has the potential to substantially improve response rates to therapies and reduce unwanted side effects. There has been some early success in adopting precision medicine. Notable examples include precision oncology, pharmacogenomics, and the treatment of cystic fibrosis where patients are matched to treatments based on their mutations (Ashley, 2016).

However, significant hurdles remain to fully realizing the promise of precision medicine. Firstly, the number of variants that need to be annotated is daunting. To use just protein coding missense variants as an example, there are almost 500,000 missense variants of unknown clinical significance in the ClinVar repository of genetic variants (Landrum et al., 2018). Additionally, even when patient carry pathogenic variants, there are rarely specific molecular therapies that treat their particular disease process. Precision medicine requires an arsenal of drugs that is much larger than the current pool of FDA-approved therapies.

Computational modeling has the potential to bridge the mismatch between the low numbers of annotated variants and FDA-approved drugs and the needs of precision medicine. Experiments from biochemical assays to animal models can provide insight into how a variant affects function at different scales. However, these experiments are typically low throughput and require substantial time and effort. More high throughput approaches, like deep mutational scanning, can exhaustively determine how mutations in a protein affect its function, but they are difficult and expensive to perform (Fowler & Fields, 2014). Similarly, high throughput screening with purified proteins, cell lines, or complex organoids can uncover new lead compounds (Gilmartin et al., 2014; Macarron et al., 2011; Malik et al., 2011). However, lead compounds revealed in these assays are often difficult to improve without a detailed understanding of how a compound binds its target. For these reasons, there has been great interest in using computational modeling to improve variant interpretation and drug discovery. In principle, computational modeling can be used to predict the functional impact of a very large number of variants or screen large libraries of drug candidates.

To date, the field has focused primarily on solving structures of proteins and using these to understand sequence–function relationships and discover new drugs. Biophysicists have typically placed great weight on all-atom models of a protein structure, which are typically generated through x-ray crystallography or, increasingly, cryogenic electron microscopy (Chim et al., 2021; Martynowycz et al., 2021; Scapin et al., 2018). More recently, with the emergence of highly accurate predictive models of protein structures like AlphaFold, we now have access to reliable structures for nearly all human proteins (Jumper et al., 2021). It has long been suggested that protein structure can inform which variants are likely to be pathogenic (Schmidt et al., 2023). After all, variants that fall in functionally relevant parts of a protein (e.g., an active site) may be more likely to have deleterious consequences. In theory, a single structure could aid in the interpretation of all missense variants that affect a given protein. Additionally, structure-based drug design offers the tantalizing promise of rational drug design. By docking small molecules against experimental or predicted structures, it should be possible to identify novel drugs against targets identified in population genetic studies.

However, single structures have substantial limitations that constrain their utility for variant interpretation and drug discovery. Combining machine learning with protein structure to predict variant pathogenicity has shown substantial promise, but even the best-performing models fail in many cases and usually do not distinguish between activating and inactivating mutations (see Section 2). An illustrative example comes from myosin motors, a class of proteins frequently mutated in human disease (Trivedi et al., 2020). To predict the effects of mutations based on a single structure, one typically uses heuristics, like assuming that mutations at nearby sites have similar effects. However, in many cases, myosin mutations cause opposite phenotypes (i.e., hypertrophic cardiomyopathy vs. dilated cardiomyopathy) but are found at neighboring residues, or even at the same residue (Lehman et al., 2023). Hence, a structure can provide some clues as to how a variant will affect function, but the predictive power of this approach is limited.

Similarly, rational drug design methods largely assume that proteins adopt a single structure, which is greatly limiting in several ways. Firstly, this assumption limits drug design to inhibiting proteins by identifying compounds that bind key functional sites, thereby physically blocking the protein from performing functions like catalysis or binding other proteins. It is all but impossible to imagine designing a drug to enhance a desirable function if proteins are essentially rigid bodies. Moreover, many proteins must be written off as undruggable because their structures lack pockets where an inhibitor has the potential to bind tightly enough to serve as a

valuable drug (Borrel et al., 2015; Cox et al., 2014; Hopkins & Groom, 2002). Finally, current computational drug design methods struggle to quantitatively predict protein–ligand binding affinities, suggesting there is a fatal flaw in the single structure assumption (Jones et al., 2021; Meller, de Oliveira, et al., 2023).

A long-standing hypothesis is that accounting for the entire ensemble of structures a protein adopts in solution would be vastly superior to assuming a single structure encodes all the relevant information (Karplus & McCammon, 2002; Prakash et al., 2015; Stank et al., 2016). For example, sequence variation can produce distinct biochemical phenotypes by modulating the relative probabilities of an ensemble of conformations primed for different functional roles. There is a growing body of evidence for this hypothesis (see Section 2). For example, variants that cause increases in the probability of structures with a favorable alignment of catalytic residues lead to elevated catalytic efficiencies (Lim et al., 2001; Soeung et al., 2020). Similarly, within protein families, differences in the distributions of conformations adopted in simulation can predict functional differences, even when crystal structures and phylogeny cannot (Porter et al., 2020; Sultan et al., 2018). Thus, our ability to predict which patient missense variants are pathogenic is likely to improve when we explicitly consider protein ensembles.

Our ability to discover new drugs will also greatly improve thanks to an ensemble perspective. A protein–ligand affinity is an ensemble measurement, which reflects contributions from each state in the protein structural ensemble and that of the ligand. Thus, by incorporating knowledge of proteins' conformational ensembles, we may be able to finally develop universally accurate methods for predicting protein–ligand affinity. Additionally, we may be able to design specific allosteric modulators of proteins that are currently considered undruggable but may form cryptic pockets in their excited states.

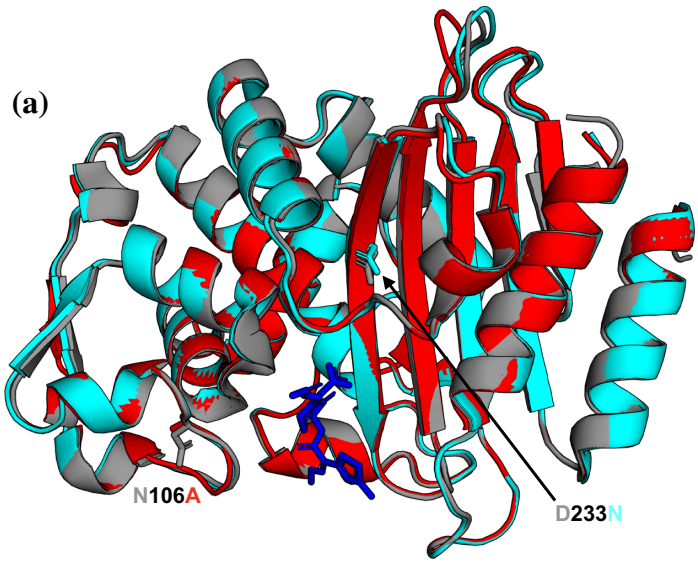
Here, we describe how computer simulations are providing a general and scalable means to take an ensemble perspective toward sequence–function relationships and drug discovery, thereby enabling a physics-based approach to precision medicine. We will highlight exciting new uses of Markov state models (MSMs) and deep learning to predict the effects of missense variants and perform virtual screening (Chodera & Noé, 2014; Corso et al., 2022; Gentile et al., 2022; Husic & Pande, 2018; Meller, Bhakat, et al., 2023; Prinz et al., 2011). In addition to facilitating traditional drug discovery approaches, this perspective also opens novel opportunities. For example, cryptic pockets that are absent in structural snapshots of a protein but form due to protein dynamics are providing new targets for drug discovery. Finally, we discuss some of the remaining hurdles standing between the current state of physics-based precision medicine and direct impact on clinical care.

2 | PREDICTING SEQUENCE–FUNCTION RELATIONSHIPS FROM AN ENSEMBLE PERSPECTIVE

Models that can predict function from sequence are urgently needed to interpret the effects of sequence variation within protein families and in patient cohorts. Within protein families, closely related isoforms often perform different functions because of variation in underlying biochemical parameters (Bloemink & Geeves, 2011; Greenberg et al., 2015; Palzkill, 2018; Patel et al., 2018; Porter et al., 2020). For example, some myosin motors function as cargo transporters while others participate in filaments to drive muscle contraction (Robert-Paganin et al., 2020; Trivedi et al., 2020). Structural biologists have solved structures across several protein families with relevance to human disease, like myosins, kinases, and G-protein-coupled receptors (GPCRs) (Canagarajah et al., 1997; Day et al., 2009; Dominguez et al., 1998; Shiriaeva et al., 2023; Wacker et al., 2017). Comparing structures of these closely related proteins could, in principle, provide clues as to why proteins within the same family vary in their function. By extension, models which integrate a protein's structure with its evolutionary history to predict function could enable exciting avenues in protein design. For example, it may be possible to predict what minimal set of mutations is needed to modify the biochemical properties of naturally occurring proteins (e.g., modifying a myosin's nucleotide binding properties).

Moreover, because of the genomic revolution, there is a growing need for methods that can accurately predict whether a given protein variant affects function. There are now almost 500,000 missense variants of unknown significance in ClinVar and many more likely to be discovered as whole genome sequencing becomes routine in clinical care (Landrum et al., 2018). This has created a significant need for tools to determine whether patient mutations are deleterious or benign to a protein's function. If protein function is encoded in protein structure, then experimental structures or even AlphaFold-predicted structures could assist in interpreting these variants of unknown significance.

However, in many cases, it remains unclear how sequence differences contribute to functional differences when biochemically distinct proteins have nearly indistinguishable experimental structures (Figure 1). For



(b)

CTX-M Variant	k_{cat} (s^{-1})	All-atrom RMSD (Å)
CTX-M-14	47.2 ± 4.3	-
CTX-M-14 N106A	100 ± 4	0.926
CTX-M-14 D233N	19.0 ± 7.7	0.782

FIGURE 1 CTX-M β-lactamase variants exhibit functional divergence despite highly similar structures. (a) Overlay of wildtype CTX-M-14 (gray), N106A mutant (red), and D233N mutant (cyan). Bound cefotaxime substrate is shown in blue. (b) Comparison of catalytic rate (k_{cat}) of ampicillin hydrolysis among variants depicted in (a) (Adamski et al., 2015; Kemp et al., 2021; Lu et al., 2023). Root mean square deviation of each mutant is given with respect to wildtype CTX-M-14.

example, motors from the myosin superfamily vary greatly in the percent of time they spend bound to actin in their mechanochemical cycle (0.05–0.95) (Bloemink & Geeves, 2011). Despite this, crystal structures of two divergent myosin motor domains, the part of the protein that hydrolyzes ATP and binds actin, overlay with less than 1 Å of backbone root mean square deviation (RMSD) even though the sequence identity between these motors is only 35% (Planelles-Herrero et al., 2017; Ropars et al., 2016; Trivedi et al., 2020). Similarly, crystal structures of single point variants often produce very similar structures. The CTX-M β-lactamases mediate antibiotic resistance by hydrolyzing β-lactam antibiotics (Brown et al., 2020). Because many CTX-M clinical variants confer higher catalytic efficiency or protein stability, substantial effort was dedicated to solving their crystal structures (Adamski et al., 2015; Chen et al., 2005; Kemp et al., 2021; Lu et al., 2023). As is seen in Figure 1a, these CTX-M variants have highly similar crystal structures with a backbone RMSD of <1 Å. Perhaps unsurprisingly then, predicted structures from homology modeling or AlphaFold also produce highly similar structures for single point variants (Buel & Walters, 2022; Feyfant et al., 2007; Pak et al., 2023). Even if different variants produce similar ground state structures, it has long been suggested that a mutation's location in a protein's structure can at least inform whether that mutation is deleterious.

Mapping genetic variation to experimental structures has not transformed our ability to predict how sequence variation modulates protein function, though there has been substantial progress toward this goal. It is reasonable to expect that mutations which fall in functionally relevant regions or disrupt the overall structure of the protein (i.e., by introducing steric clash) are more likely to affect protein function and hence cause deleterious downstream consequences. This logic has been pursued by several methods that use a mutation's 3D neighborhood or spatial location to predict its functional consequences or pathogenicity (Adzhubei et al., 2010, 2013; Cheng et al., 2023). Recently published models like PrimateAI and AlphaMissense distinguish benign variants from pathogenic variants with relatively high accuracy across several variant pathogenicity prediction benchmarks (e.g., ROC-AUC 0.797 and 0.809, respectively on the Deciphering Developmental Disorders benchmark) (Cheng et al., 2023; Gao et al., 2023). Notably, these models leverage not only a residue's location in a 3D structure but also that protein's evolutionary history embedded in its multiple sequence alignment. Though they are a highly useful community resource, AlphaMissense predictions are worse for disordered regions, suggesting that explicitly accounting for dynamics may be required to understand how residues in these regions contribute to pathogenicity. Additionally, AlphaMissense classifies many hyperactivating mutations (e.g., those in the human glucose sensor GCK) as ambiguous or benign. Pathogenic variants that diminish protein function will likely require different drug design strategies than variants that enhance protein function. Hence, new

predictive models of sequence–function relationships are still needed. Informatics approaches could mature to fill this need, but mechanistic models would still be valuable for informing the development of new therapeutics.

Considering protein dynamics can not only improve upon the performance of machine learning models using protein structure and evolutionary history but also enable more quantitative predictions of how variants affect a protein's function, especially for enzymes. Previous studies have shown that pathogenic mutations tend to alter the dynamics of regions critical for protein function like active sites or protein interfaces (Butler et al., 2015; Kumar et al., 2015; Ose et al., 2022). Additionally, incorporating dynamical features in machine learning models can improve variant pathogenicity prediction (Ponzoni & Bahar, 2018). Moreover, there are instances where similar mutations in adjacent residues cause opposite phenotypes. For example, a A223T mutation in β-cardiac myosin causes *dilated* cardiomyopathy but is spatially adjacent to another mutation, I263T, that causes *hypertrophic* cardiomyopathy (Spudich et al., 2016; Ujfalusi et al., 2018). This suggests that considering a protein's conformational distribution may be necessary to leverage structural data for improved predictions of variants' functional effects.

Fortunately, new computational tools are now providing unprecedented access to protein structural ensembles. Molecular dynamics (MD) simulations provide an atomic-resolution lens into how proteins behave in solution at room or physiological temperatures (McCammon et al., 1977; Voelz et al., 2010; Zimmerman et al., 2021). Recent advances in custom hardware like Anton and massively parallel distributed computing platforms like folding@home have helped MD simulations reach unprecedented scales (Shim et al., 2022; Voelz et al., 2023). Several tools have been developed to facilitate the comparison of ensembles. Highly parallel simulations can be aggregated with MSMs to create a map of a protein's free energy landscape (Pande et al., 2010; Prinz et al., 2011; Suárez et al., 2021). A sequence invariant extension of MSMs enables quantitative comparisons of protein dynamics across protein families (Sultan & Pande, 2018). Finally, multiple machine learning approaches, like self-supervised autoencoders, time-lagged autoencoders, and support vector machines have been successfully applied to identify differences between ensembles (Fleetwood et al., 2020; Mardt et al., 2018; Wang et al., 2020; Ward et al., 2021). In our experience, comparing ensembles with self-supervised autoencoders called DiffNets is a relatively automated approach for identifying conformational differences that explain functional patterns across GPCRs, myosins, and β-lactamases (Lee et al., 2023; Malik et al., 2021). Overall, these advances promise to not only accelerate our ability to gather the kind of large simulation datasets required for accurate estimation of thermodynamic parameters but also enable rapid analysis of the high-dimensional datasets that are generated.

Within protein families, differences in conformational distributions predict functional differences. When we examined simulations of eight myosin isoforms, we found that simulations sample conformational diversity in the nucleotide-binding P-loop of myosin motors that is rarely observed in crystal structures (Porter et al., 2020). Furthermore, the balance between nucleotide-favorable and nucleotide-unfavorable conformations could predict a myosin's propensity to stay bound to actin (Figure 2). Motors with an intrinsic preference for nucleotide-favorable states (i.e., higher ensemble probability) were more likely to stay bound to actin likely because the dominant actin-bound state is also adenosine diphosphate (ADP)-bound. Further work found correlations between the behavior of a myosin active site loop and the probability with which a myosin motor adopts a biochemical state associated with a slow rate of ATP hydrolysis (Lee et al., 2023). Like myosins, kinases also have highly conserved structures but varying biochemical properties (Sultan et al., 2018). Sultan et al. showed that kinases from the Src family differ in their propensity to adopt catalytically active conformations. Kinases with higher equilibrium probabilities of the catalytically active state tend to display higher specific activities. These examples illustrate that sequence variation can produce distinct biochemical phenotypes by modulating the relative probabilities of an ensemble of conformations primed for different functional roles (Campbell et al., 2016).

Conformational preferences can also help explain how single point mutations contribute to biochemical differences that lead to human disease. It is especially important to consider dynamics when a mutation falls outside an active site or known binding site. For example, in the case of p53, a tumor suppresser protein nicknamed the "guardian of the genome" because of its importance in cancer, the Y220C mutation falls outside of its DNA-binding interface (Boeckler et al., 2008). MSMs constructed from p53 simulations revealed that this mutation allosterically modulated the behavior of the DNA-binding interface (Barros et al., 2021). Specifically, the mutation was associated with stabilizing a structural state where a lysine residue that typically interacts with DNA is sequestered in a salt bridge. Importantly, this structural state had not been captured in existing crystal structures, highlighting the importance of protein dynamics. Likewise, considering dynamics is crucial when mutations at the same or nearby positions have variable effects. For

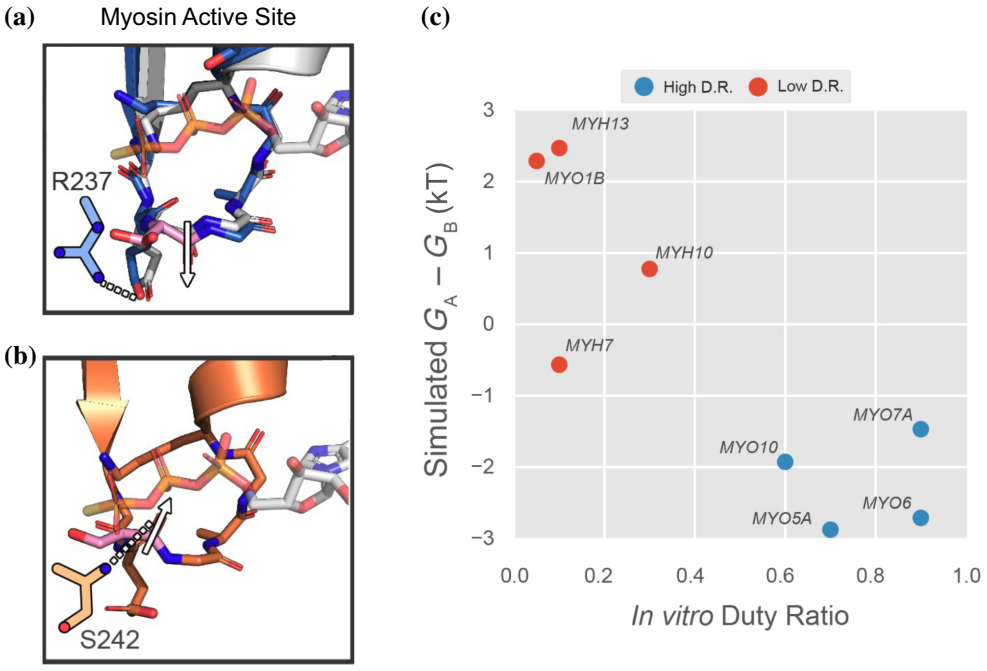


FIGURE 2 The conformational dynamics of isolated myosin motor domains predict myosin biochemical properties. (a) In one of the commonly observed conformations (i.e., macrostates) in the isolated myosin motor domain ensemble, an active site loop known as the P-loop has its central carbonyl oriented downward away from ATP (gray silhouette). This state is similar to ground state experimental structures (gray structure) and is referred to as state A. (b) In another high probability macrostate of the isolated (i.e., ATP-free) motor domain ensemble, this carbonyl points toward the ATP position. This orientation is stabilized by interactions with nearby residues like S242 (MYH7 numbering). (c) The free energy difference between state A and state B is correlated with a myosin motor's in vitro duty ratio. High duty motors primarily occupy the nucleotide-compatible state A while low duty motors have a higher probability of adopting the nucleotide-incompatible state B in their apo ensembles.

example, only certain allosteric mutations in the TEM β-lactamase confer stability to the enzyme and allow for bacteria to evolve antibiotic resistance. Atomically detailed simulations coupled to MSMs showed that a M182T mutation acts as a cap on a helix that stabilizes numerous hydrogen bonds in that helix (Zimmerman et al., 2017). Biochemical intuition suggests that an M182N mutation should have the same effect as asparagine is an even better cap on helices. However, simulations predicted that M182N would not be stabilizing due to competition between alternative hydrogen bonding networks it can form. Importantly, this prediction was proved correct by subsequent experimental measurements of the variants' stability. Another simulation study of TEM β-lactamase demonstrated that the propensity to adopt conformations where an active site loop is pinned down was strongly correlated with the catalytic efficiency of 15 TEM variants (Hart et al., 2016). Thus, conformational preferences can explain how even highly similar mutations can produce different biochemical properties.

Future work is needed to develop computational approaches that can leverage a detailed understanding of protein dynamics to make accurate predictions for entire libraries of clinical variants. Because of the very large number of variants of unknown significance, it is not realistic to generate separate ensembles for each variant. Instead, future methods will need to leverage existing simulations, typically of a wildtype ensemble, to predict how a variant will perturb an ensemble. Plattner et al. (2017) developed a method that avoids running additional simulations for a variant. In this approach, state populations in a wildtype MSM are adjusted based on the difference in potential energy in implicit solvent between a structure with a variant and the corresponding wildtype structure. While this approach showed good agreement with experiment for a model system, it assumes that a variant will occupy the same set of states as the wildtype protein. To avoid this assumption but still leverage a wildtype MSM, one can introduce a mutation to a representative structure from each wildtype MSM state and launch simulations from these structures to generate a new MSM for a variant. In the future, it may be possible to generate statistically weighted ensembles without running simulations, and early methods in this direction are showing promise (del Alamo et al., 2022; Janson et al., 2023; Vani et al., 2023). In the long run, such efforts will be crucial to enabling a detailed understanding of the functional consequences of variants of

unknown significance and the development of novel therapies targeting those variants.

3 | ENABLING ENSEMBLE-BASED DRUG DESIGN

Virtual screening has emerged as a routine tool in drug discovery. While experimentally screening large libraries of compounds in biophysical assays remains a successful paradigm in drug discovery, virtual screening has notable advantages. Because docking software is fast, it enables screening extremely large libraries of compounds (>1 billion) with highly diverse chemistries (Luttens et al., 2022). Improved scoring functions, faster software, and the emergence of "make-on-demand" compound libraries have contributed to notable success stories. Several virtual screening campaigns report hit rates of well above 10% with one campaign uncovering a picomolar binder of the D4 dopamine receptor (Lyu et al., 2019).

However, there are limitations to using single structures in drug discovery. Successful docking campaigns have typically targeted proteins with high-resolution experimental structures and known ligands. It is much less clear what success rates to expect for targets that lack known ligands or experimental structures, but they are likely to be substantially worse. Additionally, to ensure rapid calculations, docking scoring functions make several approximations that result in poor predictions of binding affinities between a compound and its protein target (Trott & Olson, 2010).

Several examples highlight the importance of remembering that proteins exist in a dynamic equilibrium between multiple states, and different receptor conformations may bind different chemistries. For example, one famous class of drug targets, kinases, have different ratios of "DFG-in" and "DFG-out" conformations (Haldane et al., 2016). Docking ligands to *apo* protein conformations that do not reflect the ligand-bound structure may result in poor compound ranking. This may explain why docking success rates are generally higher when using target receptors bound to a known ligand (Rueda et al., 2010). Accounting for receptor heterogeneity remains a substantial challenge in virtual screening.

More recently, progress has been made toward accounting for protein conformational heterogeneity in virtual screening. One promising strategy has been to use multiconformer models of ligand-free electron density maps as input to virtual screening. For example, Fischer et al. (2014) docked to different loop conformations of cytochrome c peroxidase and assigned Boltzmann-weighted energy penalties to each docked pose based on crystallographic occupancies. Loop conformations that had low occupancies received higher penalties to avoid selecting ligands that preferentially stabilized higher energy protein conformations. Impressively, this approach led to the discovery of ligands with new chemotypes and physical properties. On the other hand, other groups have combined ensembles generated using MD simulations with docking to discover new chemical matter. This approach is commonly referred to as "ensemble docking." Lückmann et al. (2019) simulated a GPCR that is an anti-diabetes target and found that removing an agonist from simulations causes closure of a pocket. They reasoned that forcing this pocket open with a ligand would sensitize the receptor to the agonist. Indeed, by docking compounds to four representative structures from MD simulations where that pocket was open, they discovered a novel positive allosteric modulator that sensitized the receptor to stimulation via the agonist. Luckman et al. selected those compounds that had the best scores for any given target receptor conformation (i.e., scores for the same compound across different target conformations were not aggregated). It is likely that the four states that were considered vary in their equilibrium probabilities. Hence, more sophisticated in silico methods that directly estimate the free energy of binding from an ensemble may have an improved ability to predict affinities and rank compounds.

Alchemical binding free energy calculations provide a means to directly estimate protein–ligand affinity. Binding simulations can either be relative to other ligand affinities, in which case they are often called relative binding free energy calculations (RBFE) or free energy perturbation calculations (FEP). Alternatively they can be relative to an unbound receptor, in which case they are called absolute binding free energy simulations. Authoritative reviews and an edited volume on many subtopics in this area exist (Chipot, 2014; Mobley & Gilson, 2017; Pohorille et al., 2010). Briefly, these schemes use the Zwanzig perturbation formula to estimate the free energy of binding. This can either be by enhancing sampling of a conformational change that leads to (un)binding of a ligand in a simulation, or by disappearing or mutating the ligand (Chipot, 2014). In either case, transformations can occur using rapid switching schemes (non-equilibrium switching) or using equilibrium simulations. For both approaches, stratification of the switching process through intermediate states is nearly always beneficial for convergence (Pohorille et al., 2010).

Alchemical free energy methods have a balance of performance and accuracy that is intended to bias toward high accuracy with significant computational resources and require a skilled practitioner to make key decisions. RBFE assume a common binding mode for the scaffold

moiety and are started from a particular ligand-pose receptor conformation. Selecting an appropriate pose is crucial for the accuracy of the calculation (Cournia et al., 2017). Furthermore, the simulations must be manually prepared since transitions between poses are restricted in most formulations. The free energy calculations themselves are performed as replica simulations that are defined in a series along a perturbation parameter (lambda) that morphs the system Hamiltonian from one extreme of the transformation to the other—this provides stratification that enhances convergence. Such calculations must converge several "window" simulations or dissipated work estimates that correspond to the system held at a particular point in the transformation process. Thus, they are resource intensive to perform. Additionally, morphing systems from one charge state to another also poses technical challenges—although there are also proposed solutions to these challenges, changes in charge species tend to be less accurate.

Despite these challenges, free energy calculations have played an increasingly important role in drug discovery (Hu et al., 2023). Thanks to improvements in software and the development of methods to address technical challenges like pose lock-in, relative binding free energy methods have developed a reputation for being chemically accurate (within 1 kcal/mol of experimental affinities) when the ligand binding problem is well suited to their use (Heinzelmann & Gilson, 2021; Sherborne et al., 2016; Wang et al., 2019; Xu, 2023). Indeed, a method called FEP+ exhibited the highest capacity to rank affinity of any of the methods assessed in a recent benchmark (Breznik et al., 2023; Steinbrecher et al., 2015). Recent studies have also demonstrated that free energy calculations can be performed at a large scale. While most applications of alchemical free energy calculations consider tens or hundreds of compounds, the Folding@home community screened over 20,000 ligands in support of the COVID Moonshot, which aims to develop a patent free inhibitor of the SARS-CoV-2 main protease. Impressively, the overall root mean squared error (RMSE) between experimental pIC50s and calculated pIC50s from RBFE ranged from 0.55 to 0.79 kcal/mol across several binder scaffolds (Boby et al., 2023). This is well within the 1 kcal/mol threshold often seen as the level of chemical accuracy needed to truly design novel binders in silco.

However, blind affinity predictions suggest that free energy calculations still have a long way to go and may underestimate protein conformational heterogeneity. A the critical assessment of protein structure prediction (CASP)-like blind prediction competition, the Statistical Assessment of the Modeling of Proteins and Ligands (SAMPL), has emerged as a way for the community to test ideas and to iterate over public datasets (Mobley et al., 2014). SAMPL challenges have historically restricted affinity predictions to host–guest complexes (though the most recent one will include protein–ligand challenges). These complexes—usually macrocycles like crown-ethers with 100 or less heavy atoms—provide a well bounded problem for binding estimation because of their small size and reduced conformational flexibility while still offering the same sorts of physical interactions implicated in noncovalent protein–ligand complexes. That said they result in real challenges to most affinity prediction methodologies, with RMSE above 3 kcal/mol being the norm and only a handful of methods (e.g., groups using the AMOBEA polarizable force field) performing within 2 kcal/mol of experiment (Amezcua et al., 2021). Importantly, some guest complexes, like the "Trimer Trip" host, can adopt multiple ligand-compatible conformations (Kellett et al., 2021). Though most methods failed to make accurate predictions for this more challenging host, one group which determined the relative probabilities of the two host conformations in the absence of ligand was able to accurately predict its affinity for the ligand. Accounting for receptor heterogeneity is therefore one of the more significant gaps present even in this simplified context.

Combining MSMs with methods that predict protein–ligand affinities is an exciting avenue for rigorously accounting for conformational heterogeneity in both the protein and ligand. The main advantage of this approach is that it explicitly considers multiple protein conformations. Though building an MSM is computationally expensive, once built, an MSM produces a compressed representation of the system's ensemble that can be reused for many ligands. One strategy that leverages this compressed representation is performing docking or binding calculations to representative receptor conformations from an MSM. Because the relative weight of each state is known from the *apo* model's equilibrium probabilities, it is possible to calculate an overall affinity between a small molecule and the protein ensemble. In an early formulation of this approach dubbed "Boltzmann docking," an enzyme substrate's binding affinity for different receptors was computed by taking the ensemble average of docking scores across a set of MSM states, weighting each state by its equilibrium probability. Boltzmann docking was better able to predict the catalytic efficiencies of different TEM β-lactamase variants than docking to a single structure, suggesting it could predict substrate affinity (Hart et al., 2016). Boltzmann docking was also used to identify novel allosteric activators and inhibitors of TEM activity by screening over 10,000 compounds against structures from simulation (Hart et al., 2017). Though these results were promising,

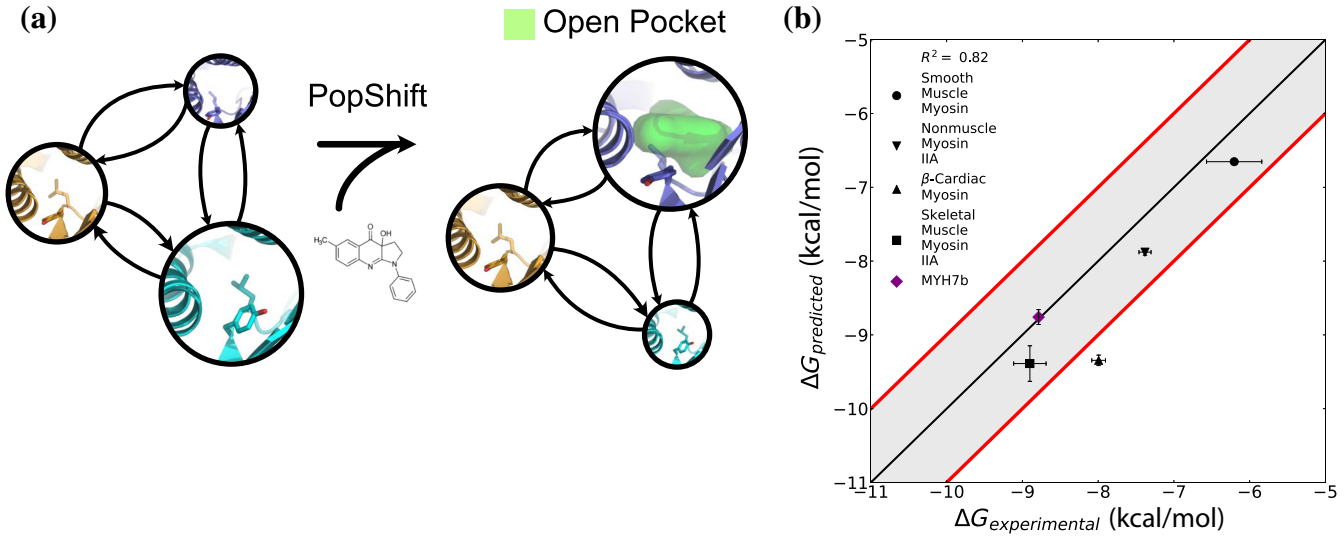


FIGURE 3 PopShift provides a framework for determining ligand–protein binding affinities and allosteric effects using apo Markov state models. (a) Cartoon illustration of a Markov state model demonstrating how a ligand can adjust the probability of conformational states. The network on the left shows a three-state model with different pocket conformations. The size of each circle corresponds to the state's equilibrium probability. When a ligand perturbation is applied to the Markov state model (e.g., a ligand is docked to each of these conformations and an affinity for each pose is estimated), the probabilities are updated with the state containing an open pocket (shown as a green cavity) increasing in probability while a closed state decreases in probability. (b) The PopShift approach accurately predicts affinities between blebbistatin, a myosin inhibitor (chemical structure shown in a), and different myosin motors.

compounds were only docked to a subset of MSM states that had open pockets, suggesting that binding affinities may be overestimated.

Recent theoretical improvements have not only expanded the potential utility of MSMs in drug discovery but also provided a framework for understanding how ligands achieve their biological effects. Inspired by Boltzmann docking, Smith et al. took a binding polynomial approach to derive a more physically accurate way to calculate the average affinity of a ligand to a set of protein structures (Louis et al., 2023). This approach, called "PopShift," outperforms Boltzmann docking, traditional ensemble docking approaches, and other common estimators of binding free energies. It also provides a formula for reweighting individual states based on the affinity between the ligand and protein and the *apo* equilibrium probability. Hence, PopShift can probe which states are the most populated in the bound state and predict a ligand's allosteric effects (Figure 3a). Furthermore, this framework accommodates multiple ligand poses, receptor conformational heterogeneity, and the effect ligand binding has on receptor states. In another study, this updated PopShift approach (previously referred to as MSM-docking) was able to accurately predict one compound's binding affinity for different myosin motors (Figure 3b) (Meller, Lotthammer, et al., 2023). Though only docking has been used with this framework so far, our view is that in future iterations of this method, binding affinity estimates for each state can be done with implicit solvent methods (e.g., generalized Born Poisson–Boltzmann calculations) or alchemical free energy simulations. Thus, the use of MSMs has great potential in improving computer-aided drug design and expanding the arsenal of drugs required for precision medicine.

4 | EXPANDING THE DRUGGABLE PROTEOME WITH CRYPTIC POCKETS AND ALLOSTERY

Experimental structures are an invaluable input to the computational drug design pipeline, but they do have important limitations. Firstly, many proteins may appear undruggable because they lack pockets in their experimental structures. By one estimate, as many as half of all structured domains lack sufficiently large pockets on their surface to accommodate drug-like molecules (Meller, Ward, et al., 2023). Secondly, though many proteins have pockets where they bind their natural substrates (e.g., enzyme active sites), compounds which bind at these sites are obligate inhibitors. When developing drugs for many diseases, especially genetic diseases caused by loss-of-function mutations, it is more desirable to restore protein function. Thirdly, as proteins are highly dynamic in solution, targeting pockets seen in their ground states may not yield drugs when those pockets

are rare or unrepresentative of the protein's ensemble (Cimermancic et al., 2016).

Cryptic pockets and allostery could greatly expand the set of potentially druggable targets (Nussinov & Tsai, 2013). Protein fluctuations often reveal the formation of pockets absent in ligand-free experimental structures, also known as cryptic pockets (Blamey et al., 2005; Horn & Shoichet, 2004; Oleinikovas et al., 2016). For proteins that lack pockets in their native structures, cryptic pockets may render these targets druggable. For other proteins, like enzymes, that have pockets (e.g., active sites), cryptic pockets open new therapeutic opportunities. Many cryptic pockets are distant from active sites, so compounds targeting them can allosterically inhibit *or* activate protein function, unlike compounds targeting active sites which are limited to competitive inhibition (Hart et al., 2017). Furthermore, while most orthosteric sites are highly conserved as proteins from the same family typically bind the same ligands, cryptic pockets are likely less conserved (Ivetac & McCammon, 2010; Wenthur et al., 2014). This opens the possibility of developing more specific allosteric modulators of protein function. However, cryptic pockets remain difficult to discover intentionally with experimental techniques. Most cryptic pockets are discovered serendipitously when a structure of a protein bound to a ligand reveals a previously unobserved pocket (Allingham et al., 2005; Horn & Shoichet, 2004; Sirigu et al., 2016). Furthermore, while MD simulations of proteins can reveal cryptic pockets, they remain computationally expensive.

Fortunately, machine learning models can aid in identifying which proteins are likely to form cryptic pockets. CryptoSite is an outstanding example of a machine learning tool that takes a structure as input and predicts the probability with which each residue forms a cryptic pocket (Cimermancic et al., 2016). CryptoSite was trained with examples of cryptic pockets from the protein data bank (PDB) and achieves good accuracy on a held-out test set (ROC-AUC: 0.83). Notably, CryptoSite uses features generated from a coarse-grained simulation to make its predictions. This means that running CryptoSite on an example can take up to a day, depending on the size of the input structure. On the other hand, a tool from our lab called PocketMiner uses only single structures as input to a graph neural network to make rapid predictions of cryptic pocket formation (Meller, Ward, et al., 2023). Both CryptoSite and PocketMiner achieve similar performance, but PocketMiner returns a prediction in under a second. PocketMiner was trained using labels derived from MD simulations, suggesting that models trained with simulation data can be used to predict other protein features. Moreover, deploying CryptoSite and PocketMiner at the proteome level suggests that there are thousands of human proteins that lack pockets in their ground state structures but are likely to form druggable cryptic pockets. To generate structures containing cryptic pockets that are not observed in the PDB, it may be possible to use AlphaFold to generate an ensemble of structures that samples cryptic pocket opening (Meller, Bhakat, et al., 2023). For many proteins known to form cryptic pockets, AlphaFold successfully generates open, or partially open, conformations when its input multiple sequence alignment is stochastically subsampled. Thus, machine learning tools can greatly accelerate the prioritization of drug targets and the discovery of cryptic pockets.

Furthermore, great progress has been made in discovering cryptic pockets with computer simulations (Comitani & Gervasio, 2018; Raich et al., 2021; Sztain et al., 2021). We have found that a handful of conventional MD simulations of intermediate length started from a ligand-free experimental structure are often sufficient to observe cryptic pocket formation (Meller, Ward, et al., 2023). We found this to be true in 14 out of 15 experimentally validated cryptic pockets, spanning several different classes of motions. However, there are proteins whose cryptic pocket opening occurs over such slow timescales that sampling the opening event with conventional MD simulations remains difficult (Meller, Bhakat, et al., 2023; Meller, Ward, et al., 2023; Oleinikovas et al., 2016). It may also be important to accurately characterize the free energy landscape of cryptic pocket opening by observing large numbers of opening and closing events. Fortunately, even for pockets that are slow to form in conventional MD simulations, there are several promising strategies to encourage sampling of pocket formation. We have shown that in the case of an antimalarial drug target, it is possible to accelerate cryptic pocket discovery by starting simulations from an AlphaFold-generated ensemble (Meller, Bhakat, et al., 2023). For the antimalarial target, AlphaFold generated partially open states that were more likely to transition to fully open conformations. A Markov state model was then used to build a quantitative map of the free energy landscape of cryptic pocket opening. In other cases, it may be more appropriate to use techniques like enhanced sampling methods, such as metadynamics or a Hamiltonian replica exchange method, or an adaptive sampling strategy like FAST to discover cryptic pockets (Capelli et al., 2022; Comitani & Gervasio, 2018; Valsson et al., 2016; Zimmerman & Bowman, 2015). After collecting simulation data, one can determine where cryptic pockets form by applying a pocket detection algorithm, like LIGSITE, fpocket, or P2Rank, to each state in an ensemble or MSM or using the exposons method (Krivák & Hoksza, 2018; Le Guilloux et al., 2009; Porter,

Moeder, et al., 2019; Porter, Zimmerman, et al., 2019; Valsson et al., 2016).

Crucially, multiple lines of experimental evidence corroborate cryptic pockets discovered in simulation and their functional importance. In the case of TEM-1 β-lactamase, simulations have revealed the formation of several cryptic pockets (Bowman et al., 2015; Bowman & Geissler, 2012; Hart et al., 2016). The most commonly occurring cryptic pocket in TEM-1 β-lactamase simulations (partially open as much as 53% of the time) is found at a known cryptic allosteric binding site (Bowman & Geissler, 2012). Moreover, simulations revealed a novel TEM-1 cryptic pocket, known as the omega loop pocket because it forms due to a loop motion (Porter, Moeder, et al., 2019). Thiol labeling experiments showed that this pocket opens faster than the global rate of unfolding, corroborating its existence. Interestingly, it was later shown that opening of this cryptic pocket was important for β-lactamase function. Across different β-lactamases, omega loop cryptic pocket opening was associated with increased benzylpenicillinase activity (Knoverek et al., 2021). As with TEM-1 β-lactamase, simulations of the Ebola virus viral protein 35 (VP35) revealed the formation of a cryptic pocket, even though the VP35 crystal structure lacks large pockets on its surface and might be considered an "undruggable" target (Cruz et al., 2022). With VP35, however, there was no precedence in the literature suggesting that it might harbor a cryptic site. Nonetheless, thiol labeling experiments were used to demonstrate that cryptic pocket opening occurs at rates faster than the global rate of unfolding. Moreover, labeled VP35 (i.e., VP35 where the thiol labeling reagent was covalently attached to a cysteine) with the cryptic pocket forced open, showed decreased binding to double stranded RNA. Together, these experiments suggest targeting cryptic pockets with ligands is likely to have functional consequences.

Additionally, simulation studies of several drug targets have shown that differences in cryptic pocket formation underlie the specificity of compounds known to bind at cryptic pockets. For example, a positive allosteric modulator of a G-protein coupled receptor achieves a high degree of specificity despite binding at an allosteric binding site that is insufficiently large to accommodate its size across all similar receptors, including the sensitive receptor (Abdul-Ridha et al., 2014; Hollingsworth et al., 2019). Hollingsworth et al. (2019) demonstrated that cryptic pocket opening occurs with a much higher probability in the sensitive receptor. Furthermore, they showed that mutational disruption of the cryptic pocket reduces affinity of the selective allosteric modulator. Another compound that binds at a cryptic site is the myosin inhibitor blebbistatin. In all blebbistatin-free myosin structures, including structures of sensitive isoforms, the blebbistatin pocket is closed (Allingham et al., 2005; Meller, Lotthammer, et al., 2023). Thus, blebbistatin's binding site is cryptic, though we have not seen this point regularly acknowledged in the literature. Moreover, blebbistatin has almost 100-fold differences in binding affinity across sensitive isoforms. MD simulations revealed that cryptic pocket opening is unique to simulations of sensitive isoforms and that the probability of pocket opening predicts differences in affinity across sensitive isoforms. Together, these results suggest that differences in cryptic pocket opening can be exploited for the development of specific compounds.

Finally, virtual screening with simulation structures containing cryptic pockets is an increasingly promising strategy for drug discovery. A cryptic pocket opens in simulations of the p53 core domain when this domain carries cancer-causing mutations (Wassman et al., 2013). After assessing this pocket with several pocket detection algorithms, Wassman et al. used molecular docking to identify compounds that might bind at this cryptic pocket. Specifically, they docked against the 15 most-populated clusters from their MD simulations and chose compounds based on their best score against any of the clusters. This approach led to the discovery of a compound that showed dose-dependent activation of p53. In a follow-up study, Durairaj et al. (2022) screened a much larger library of 1.7 million compounds against an open state from simulation and identified compounds that could reactivate p53. The best compounds achieve IC50s of less than 10 μM against mutant p53. Importantly, they also showed that their compounds bind in the target pocket in the p53 core domain using a photo-affinity labeling experiment. Similarly, Hart et al. (2017) discovered an inhibitor and an activator of TEM-1 β-lactamase by docking to simulation structures. To our knowledge, this was the first attempt to dock against an MSM to account for protein conformational heterogeneity. In experiments, the EC50s of compounds prioritized by docking were around 60 μM. Overall, these results suggest that simulation structures containing open cryptic pockets can be used to identify novel drugs (Figure 4).

5 | CONCLUSIONS

Computer simulations of proteins are addressing many of the key barriers to precision medicine. Even when ground state structures are highly similar, differences in conformational ensembles can often explain how genetic variation seen in the clinic contributes to biochemical differences that lead to human disease. Simulations are a

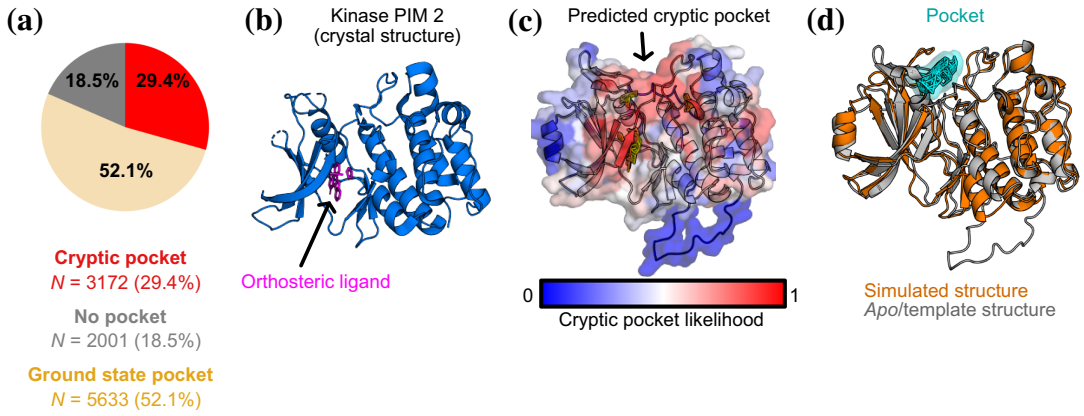


FIGURE 4 Cryptic pockets are common across the proteome and provide a means to drug-challenging targets. (a) Though nearly half of human proteins with a single structured domain lack a pocket in their native, folded state, the majority of these proteins are predicted to have a cryptic pocket. Pie chart shows the number of human proteins containing pockets in their native structures in wheat; proteins that lack pockets in their native structures but are likely to form cryptic pocket(s) in red; and proteins that lack both ground state and cryptic pockets in gray. (b) The crystal structure of the PIM2 kinase contains an orthosteric binding site but does not reveal any allosteric pockets. (c) The PocketMiner graph neural network predicts a cryptic pocket at an allosteric site. (d) Simulations recapitulate the cryptic pocket predicted by PocketMiner. In simulations, a loop peels back to reveal a cryptic pocket (shown in cyan) at the site pinpointed by PocketMiner. The simulated PIM2 structure with a cryptic pocket (orange) is overlaid on the apo PIM2 structure (gray).

promising means to identify differences in conformational ensembles, and new tools are making the acquisition and analysis of simulation data more facile. Simulations are also addressing one of the other key challenges in precision medicine—the need for more drugs tailored to each patient. Ensemble-based drug design provides a scalable framework to discovering new lead compounds and understanding how compounds perturb ensembles. Additionally, simulations are a well-established means to identify novel cryptic pockets that may render challenging protein targets druggable. There are still many theoretical and technical challenges that must be overcome to scale some of these approaches to entire libraries of variants and compounds. Nonetheless, physics-based precision medicine has the promise to improve our understanding of sequence–function relationships and to accelerate drug discovery.

AUTHOR CONTRIBUTIONS

Artur Meller: Conceptualization; investigation; writing – original draft; writing – review and editing; project administration; funding acquisition. **Devin Kelly:** Conceptualization; investigation; writing – review and editing; writing – original draft; visualization. **Louis G. Smith:** Writing – original draft; writing – review and editing; conceptualization; investigation. **Gregory R. Bowman:** Funding acquisition; writing – review and editing; project administration; conceptualization; validation.

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