Structure-Based Experimental Datasets for Benchmarking of Protein Simulation Force Fields

Chapin E. Cavender[1], David A. Case[2], Julian C.-H. Chen[3], Lillian T. Chong[4], Daniel A. Keedy[5], Kresten Lindorff-Larsen[6], David L. Mobley[7], O. H. Samuli Ollila[8], Chris Oostenbrink[9], Paul Robustelli[10], Vincent A. Voelz[11], Michael E. Wall[12], David C. Wych[12,13], Michael K. Gilson[1]*

1. Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, 9500 Gilman Drive, La Jolla, CA 92093
2. Dept. of Chemistry & Chemical Biology. Rutgers University,Piscataway, NJ 08854 USA
3. Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545; Department of Chemistry and Biochemistry, The University of Toledo, Toledo, OH 43606
4. University of Pittsburgh, 219 Parkman Avenue, Pittsburgh, PA 15260
5. Structural Biology Initiative, CUNY Advanced Science Research Center, New York, NY 10031; Department of Chemistry and Biochemistry, City College of New York, New York, NY 10031; and PhD Programs in Biochemistry, Biology, and Chemistry, CUNY Graduate Center, New York, NY 10016
6. Linderstrøm-Lang Centre for Protein Science, Department of Biology, University of Copenhagen, DK-2200 Copenhagen N, Denmark
7. Pharmaceutical Sciences Department, University of California Irvine, Irvine, CA, USA
8. University of Helsinki, Institute of Biotechnology, Helsinki, Finland
9. Institute for Molecular Modeling and Simulation, University of Natural Resources and Life Sciences, Muthgasse 11, 1190 Vienna, Austria
10. Dartmouth College, Department of Chemistry, Hanover, NH, 03755, USA
11. Department of Chemistry, Temple University, Philadelphia, PA, USA
12. Computer, Computational, and Statistical Sciences Division, Los Alamos National Laboratory, Los Alamos, NM, 87545
13. The Center for Nonlinear Studies, Los Alamos National Laboratory, Los Alamos, NM, 87545

Abstract

This review article provides an overview of structurally oriented, experimental datasets that can be used to benchmark protein force fields, focusing on data generated by nuclear magnetic resonance (NMR) spectroscopy and room temperature (RT) protein crystallography. We discuss why these observables are useful for assessing force field accuracy, how they can be calculated from simulation trajectories, and statistical issues that arise when comparing simulations with

experiment. The target audience for this article is computational researchers and trainees who develop, benchmark, or use protein force fields for molecular simulations.

1 Introduction

It is a truth universally acknowledged that a research group in possession of a good force field must be in want of a benchmark. While the earliest computer simulations of biomolecules explored sub-nanosecond phenomena[1–3], recent advances in computing hardware[4] and simulation algorithms[5] have enabled atomistic simulations to study processes that occur on biological timescales. Molecular simulation can now make quantitative predictions about protein conformational changes[6,7], ligand binding[8–10], protein folding[11,12], and assembly of multi-protein complexes[13] that can generate hypotheses to guide wet lab experiments. Hypotheses from molecular simulations have found utility in improving our understanding of protein functions, elucidating molecular mechanisms of human disease, and making it easier and faster to design small molecule therapies that target proteins.

Importance-weighted sampling methods[14], including molecular dynamics and Monte Carlo simulations, make predictions about molecular phenomena by sampling conformations from a protein's Boltzmann-weighted ensemble, where the Boltzmann weight is based on a potential function. As long as the simulation samples a sufficiently large number of conformations, the accuracy of the resulting predictions is limited by the accuracy of the energy model. While knowledge-based heuristic approaches[15] and approaches based on deep learning[16] have been incredibly successful at predicting the structures of proteins in low energy conformations[17], the study of phenomena such as conformational changes and ligand binding requires accurate sampling of conformations away from energy minima. On the other hand, highly accurate but computationally expensive quantum chemical methods are often too slow for phenomena on biologically relevant timescales. Therefore, the simulation community has a continued interest in a class of parameterized, physics-based energy models—called force fields—that use simple approximations of interatomic interactions and can be evaluated quickly to yield conformational energies and atomic forces.

Force field development is a time-consuming and iterative process because experimental datasets for training and validating force field parameters for proteins are sparse, and because it requires optimization in a high-dimensional parameter space. As the histories of modern force fields used to simulate proteins have been reviewed extensively elsewhere, we present only a very general picture of these histories here[18–22]. Protein force fields developed in the 1980s and 1990s typically derived force field parameters from quantum chemical calculations or from neat liquid bulk properties of small molecule analogs of protein fragments[23–25]. These parameters were validated by monitoring the root mean square deviation of atomic coordinates from a structural model of a protein derived from a crystal diffraction experiment over the course of a simulation trajectory. The increased availability of structural data on proteins from x-ray diffraction and nuclear magnetic resonance experiments enabled more detailed evaluations of the conformational ensembles produced by protein force fields, and deficiencies identified in

these assessments led to bespoke protein-specific corrections to the parameters used to model protein backbone and sidechain torsions[26–31].

While modern protein force fields have been fruitfully applied, they are still approximations to the true quantum mechanical energies of proteins' conformations. Additionally, research groups that develop force fields pursue different strategies for deriving parameters that prioritize often-conflicting goals. Individual protein force fields are thus expected to model some features of protein conformational ensembles better and others less well. Assessments of protein force fields that evaluate these diverse features are vital to the iterative cycle of force field development. In this review article, we describe experimental datasets that interrogate a range of structural and dynamical features of proteins and that can be used to benchmark the accuracy of protein force fields. In particular, we focus on datasets consisting of observables that provide detailed information about protein conformational ensembles under conditions similar to those of interest for protein simulations. Second, we consider only datasets consisting of peptides and non-membrane proteins, without ligands or cofactors.

The target audience for this review article is computational researchers and trainees who develop, benchmark, or use protein force fields for molecular simulations. We thus assume familiarity with molecular dynamics techniques, force field terms, and the basics of protein structure. We do not assume familiarity with experimental techniques or interpretation of results from structural biology experiments.

The remainder of the review is organized into three sections. Section 2 describes experiments using nuclear magnetic resonance (NMR) spectroscopy, and Section 3 describes experiments using room temperature (RT) crystallography. In each of these two sections, we first review types of observables provided by the experiments, why they are useful for interrogating protein conformational dynamics, and how they can be calculated from simulation trajectories. Then, we describe specific datasets containing measurements of these observables for peptide or protein systems. Finally, Section 4 touches on statistical issues that arise when comparing simulations with experiment.

2 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy measures the responses of nuclear magnetic moments in a strong external magnetic field to perturbations by weak external magnetic fields oscillating at the resonant frequency of the nuclei. The observed responses are sensitive to the local magnetic fields at the nuclei, so NMR spectroscopy provides information about the local chemical environments of atoms and hence about the conformational distributions and dynamics of the molecules they belong to.

NMR is applicable to nuclei with an odd number of nucleons and hence nonzero nuclear spin—such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P—because these possess a magnetic dipole moment. In the presence of a strong external magnetic field, this dipole precesses around the external field, much as a spinning top precesses around a downward gravitational field. The angular frequency

of the precession, which is called the Larmor frequency, is proportional to the strength of the magnetic field at the nucleus with a proportionality constant characteristic of the nuclear isotope. If a second, weak magnetic field that oscillates near the Larmor frequency is applied, the axis of precession will rotate away from the direction of the strong external field. An NMR spectrometer detects this transverse magnetization by measuring the electrical current it induces in a coil of wire. Although the value of the Larmor frequency is determined chiefly by the strong external magnetic field, the local electronic structure, which is influenced by through-bond and through-space interactions, near each nucleus modulates the field felt by the nucleus and hence its Larmor frequency, so NMR probes the local environment of the nucleus. Additionally, magnetization can be transferred to nearby nuclei via through-space interactions, so NMR can report on the relative dispositions in space of pairs of nuclei. These two effects give NMR spectroscopy its sensitivity to the structure and dynamics of proteins.

NMR observables have several useful features that have led to their adoption as targets for both training and validation of protein force fields[32]. First, NMR experiments are typically performed in laboratory conditions that are approximated closely by the desired setup for most simulation applications, namely dilute aqueous solution. Note, however, that NMR studies are often done at low pH, and it is essential that simulations meant for comparison against NMR data assign pH-appropriate protonation states of titratable residues. Second, in contrast with typical x-ray or neutron crystallography experiments, NMR spectroscopy can provide useful information about disordered proteins, i.e., proteins that do not fold into a well-defined structure that can crystallize. Furthermore, whereas other methods applicable to disordered proteins, such as small angle x-ray scattering, provide only low-resolution structural information, NMR observables can report on specific structural features that are closely related to specific force field terms, e.g. the torsional energy terms for a particular dihedral angle. Finally, because NMR observables are averages over an ensemble that includes deviations from native structures, comparisons to NMR spectroscopy can reveal native state biases in protein force fields that are more difficult to diagnose by comparison to crystal diffraction experiments.

Here, Section 2.1 discusses NMR observables that can be used to assess the accuracy of molecular simulations and hence of the force fields used in simulations. The observables considered are chemical shifts, scalar couplings (also known as J-couplings), residual dipolar couplings, the nuclear Overhauser effect (NOE), spin relaxation, paramagnetic relaxation enhancement, and salt bridges. Section 2.2 then presents specific experimental NMR datasets that are well suited for evaluating simulations.

2.1 NMR Observables

2.1.1 Chemical shifts

2.1.1.1 General principles

The chemical shift of a nucleus is the difference of its Larmor frequency from that of the same isotope in a reference compound. The chemical shift of a nuclear spin probes the degree to which it “feels” the externally imposed magnetic field. This is determined by its electronic environment, which in turn is controlled by the details of the local molecular structure. In a protein, one can consider the chemical shift of a nucleus as having a baseline offset resulting from its local covalent connectivity and bond hybridization, and an additional shift determined by both its local geometry (bond lengths, bond angles, and dihedral angles) and through-space interactions resulting from electric fields, hydrogen bonds, and the proximity of chemical groups that contain substantial magnetic anisotropy such as aromatic rings[33–35]. The sensitivity of a given nucleus to each of these influences depends on its chemical identity (atomic number), covalent structure (bond hybridization) and chemical environment.

A number of empirical algorithms have been developed to predict the chemical shifts of protein backbone atoms for a given set of three-dimensional coordinates. These algorithms are implemented in software packages that include Sparta+, Shiftx2/Shiftx+, Camshift, PPM, and UCB-Shift[36–43]. These empirical chemical prediction algorithms are trained on databases of proteins for which both high resolution x-ray structures and solution backbone NMR chemical shift assignments are available. They take protein coordinates as inputs, and output a chemical shift prediction ($\boldsymbol{\delta}_{\text{Prediction}}$) for each backbone nucleus (C$\boldsymbol{\alpha}$, C$\boldsymbol{\beta}$, C’, N, H_N, H$\boldsymbol{\alpha}$) in a protein structure. The development of chemical shift prediction algorithms has revealed the dependence of the chemical shift of each backbone nucleus on a number several conformational features of proteins. The structural features of proteins that have been found to have the largest influence on the backbone nuclei of a given residue *i* are the $\phi/\psi/\chi$ dihedral angles of that residue; the $\phi/\psi/\chi$ angles of neighboring residues (i-2,i-1,i+1,i+2); the distances and orientations of nearby aromatic rings and other chemical groups with substantial magnetic anisotropy; the presence and geometry of hydrogen bonds; the proximity of polar and charged nuclei; and the solvent exposure of the residue. Accordingly, chemical shift predictions can be estimated as a combination of additive effects:

$$\boldsymbol{\delta}_{\text{Prediction}}=\boldsymbol{\delta}_{\text{Random Coil}}+\boldsymbol{\delta}_{\text{Backbone}}+\boldsymbol{\delta}_{\text{Sidechan}}+\boldsymbol{\delta}_{\text{Ring Currents}}+\boldsymbol{\delta}_{\text{Hydrogen Bonds}}+\boldsymbol{\delta}_{\text{Electric Fields}}\boldsymbol{\delta}_{\text{Solvent}}$$

where $\boldsymbol{\delta}_{\text{Random Coil}}$ is a baseline offset that is determined for each nucleus in each amino acid[44–47], $\boldsymbol{\delta}_{\text{Backbone}}$ reflects the ϕ/ψ angles for the residue and its neighboring residues, $\boldsymbol{\delta}_{\text{Sidechain}}$ reflects sidechain χ angles, $\boldsymbol{\delta}_{\text{Ring Currents}}$ results from magnetic anisotropy effects from nearby aromatic rings, $\boldsymbol{\delta}_{\text{Hydrogen Bonds}}$ results from hydrogen bonding interactions, $\boldsymbol{\delta}_{\text{Electric Fields}}$ is the contribution of nearby charged and polar nuclei, and $\boldsymbol{\delta}_{\text{Solvent}}$ is the effect of exposure to solvent.

The main determinant of backbone chemical shifts are local dihedral angles, and different nuclei are sensitive to different dihedral angles to different extents. For example, for a given residue number *i*, C$\boldsymbol{\alpha}$ and C$\boldsymbol{\beta}$ shifts are most sensitive to the ϕ and ψ angles of that residue (ϕ_i/ψ_i), C’ shifts are most sensitive to the ψ angles of residue *i* and the following (*i*+1) residue (ψ_i/ψ_{i+1}), and N shifts are more sensitive to the χ_1 angle of that residue and the ψ angle of the preceding (*i* -1) residue ($\chi_{1i,}\psi_{i-1}$). Proton shifts are more sensitive to non-bonded interactions than carbon and nitrogen atoms. H_N and H$\boldsymbol{\alpha}$ shifts are both very sensitive the presence of aromatic ring currents,

H_N shifts are particularly sensitive to hydrogen bond geometries, and Hα shifts are more sensitive to the presence of electric fields.

The field of empirical protein backbone chemical shift prediction is relatively mature, with a high degree of consensus in the predictions of algorithms published in the last 20 years. The SHIFTS, SHIFTX, and PROSHIFT algorithms, published from 2001 to 2003, represented significant advances in the accuracy of empirical protein backbone chemical shift predictions from protein structures, with SHIFTX generally producing the most accurate predictions on protein structures not contained in its training databases and requiring only a few seconds to predict all backbone shifts in a protein. Subsequently, the program SPARTA, published in 2007, provided a small improvement in prediction accuracy, with somewhat slower calculation times. The program Camshift, published in 2009, produced comparable accuracy to SHIFTX and SPARTA, but utilizes interatomic distance-based equations that can be evaluated in milliseconds and are differentiable with respect to atomic coordinates, enabling the computationally efficient incorporation of chemical shifts as structural restraints in MD simulations. Taken together, SPARTA, SHIFTX, and Camshift represented important milestones in the field, as their predictions were accurate enough to enable the calculation of accurate protein structures using only NMR chemical shifts as restraints, when combined with molecular mechanics force fields or knowledge-based potential energy functions[48–52]. These predictors were also found to be sensitive to the conformational fluctuations of proteins observed in MD simulations, and were utilized to generate and validate protein conformational ensembles that accurately model the dynamics of proteins[53–56], and to guide the optimization of protein force field torsion terms[57,58].

A more recent generation of empirical shift predictors, including SPARTA+[37], SHIFTX2/SHIFTX+[36], PPM_One[39], UCB-Shift[40], and Graph NMR[41], were developed using machine learning techniques. These predictors provide improved accuracy and have very similar accuracy to one another. They typically produce very similar results in practical applications such as the validation of MD ensembles, the fitting of force field corrections, the calculation of protein structures and structural ensembles, and the reweighting of MD trajectories. Despite the accuracy of these methods, their errors are still an order of magnitude larger than experimental uncertainties in chemical shifts, so experimental uncertainties can generally be neglected when comparing calculation to experiment.

Prior to the publication of the most recent generation of NMR chemical shift predictors, SPARTA+, and SHIFTX+ had been widely used to assess the accuracy of simulations and in the generation of protein conformational ensembles from chemical data, so a number of published force field benchmarks relied on these predictors. It is notable that empirical prediction algorithms trained on databases of protein structures, which can produce predictions for all backbone atoms in proteins in milliseconds to seconds, have consistently provided more accurate predictions than those obtained by using quantum mechanical calculations to compute chemical shifts, although these can take orders of magnitude more computational time[59–64]. Although programs have been developed to calculate NMR chemical shifts of protein side-chain atoms, these predictors have generally been less accurate than backbone chemical

shift predictors, and less applicable for the applications described above[36,39,65,66]. Their lower accuracy presumably stems from a smaller database of training data, a smaller spread in the experimental chemical shifts for each atom type, and the high conformational variability of sidechain positions in protein structures.

2.1.1.2 Evaluation of simulations by direct comparison of computed and measured chemical shifts

Deviations between chemical shifts predicted from MD trajectories and experimental shifts are sensitive indicators of errors in MD ensembles relating to the structural features discussed above. Accordingly, NMR chemical shift predictions are routinely used to assess the accuracy of MD simulations, and torsion terms in protein force fields have been parameterized by optimizing the agreement between experimental and simulated chemical shifts[57,58].
Backbone chemical shift predictions, computed from MD ensembles, with large deviations from experiment, i.e., >1-2x the average predictor accuracy for a given backbone atom type (e.g. C**α**) as assessed on a predictor's training databases, indicate substantial inaccuracies in the average structural features of the ensemble. Large deviations between predictions and experiments can occur when residues sample an incorrect dihedral distribution, and the largest deviations generally reflect incorrect secondary structures and/or side-chain rotamers. Large chemical shift deviations can also be observed, particularly for proton shifts, when aromatic side-chains are positioned incorrectly in MD ensembles, so that aromatic ring current effects do not match experiment. Smaller deviations in predicted shifts can result from over-populated or under-populated hydrogen bond interactions in MD ensembles, or inaccurate distributions of charged groups around a given atom. In the case of disordered proteins, backbone chemical shifts are very sensitive to the presence of partially populated secondary structure elements, and deviations from experiment can indicate that MD ensembles over- or underestimate residual helical, ppII, or **β**-sheet propensities.

When comparing calculated and experimental backbone chemical shifts, one should keep the following considerations in mind.

1. Chemical shift prediction algorithms remain subject to random errors, so sporadic errors are to be expected throughout the protein. A string of residues with several atoms having large deviations between predicted and experimental shifts probably is sampling spurious conformations.

2. The chemical shifts of the backbone nuclei (atom types C**α**, C**β**, C', N, H_N, H**α**) of each residue type have large baseline offsets that are determined by chemical identity and their covalent structure. This baseline offset is often referred to as a "random coil" chemical shift, because it approximates the chemical shift expected for the backbone atom type of a given residue if it freely sampled all accessible conformational space[44–47]. The random coil shifts of a given backbone atom type (e.g. C**β**) can vary more strongly

across residue types (e.g., Val, Ser) than does the chemical shift of the same backbone atom type (e.g. Cβ) across residues of a given type (e.g. Val), due to environmental variations. For example, the random coil shifts of Cβ nuclei can vary by as much as 50 ppm among amino acid types, while the standard deviation of measured Cβ shifts within individual residue types is typically only 3 to 5 ppm[67,68]. As a result, it can be highly misleading to visualize correlations and report correlation coefficients between experimental chemical shifts and shifts predicted from MD simulations, as most of the magnitude in deviation between different residues can be explained by differences in their baseline $\boldsymbol{\delta}_{\text{Ranom Coil}}$ values. Indeed, extremely high correlation coefficients (close to 1.0) can be obtained by comparing experimental chemical shifts to database random coil chemical shift values, without utilizing any structural information. A more informative visualization of the accuracy of chemical shift predictions from an MD simulation is provided by comparing the deviations between predicted and experimental shifts for each residue type. Alternatively, one can compare predicted and experimental "secondary" chemical shifts, which subtract the random coil chemical shifts values from both experimental and predicted shifts, to indicate regions of systematic disagreement with experimental values.

3. Empirical chemical shift predictions depend on many structural features, so one cannot be sure what a given prediction error means for a single atom. For example, a deviation of 1.5 ppm for a N atom may result from a sidechain populating an incorrect rotamer or an aromatic group being incorrectly positioned.

4. Chemical shift prediction error is sequence- and conformation-specific and so should not be used to compare the accuracy of simulations of two different proteins or of two different regions of one protein. For example, chemical shift prediction errors for beta-sheet proteins are substantially higher than for alpha-helical proteins[41], so a simulation of a beta-sheet protein may yield worse agreement with experimental chemical shifts than an equally accurate simulation of an alpha-helical protein. In contrast, when one protein is simulated with multiple force fields, the accuracy of the chemical shift predictions is a clear indication of the relative accuracy of the simulations and hence of the force fields. Scalar couplings (Section 2.1.2) do not have this limitation.

5. The average prediction accuracy on the database of x-ray structures used to train a given predictor provides a reasonable baseline to identify problematic simulations. For example, the standard deviation of Ca shift predictions made by Sparta+ is 0.94 ppm for its training database, so a contiguous stretch of residues with Ca prediction errors greater than 1.5 ppm probably does not reflect the true solution ensemble of this region, whereas a simulation where the Ca shift predictions are less than 1.00 ppm is relatively reliable.

6. In principle, chemical shift predictions based on accurate, thermalized, conformational ensembles should lead to more accurate chemical shift predictions than predictions made on static protein structures. However, the most accurate chemical shift prediction

algorithms are trained against static, high-resolution x-ray structures. As a result, some amount of conformational averaging is "baked in" to these prediction algorithms; that is, chemical shifts predicted from static conformations already implicitly account for the solution-phase averaging over conformations. This fact may help account for the fact that chemical shifts predicted based on static high-resolution x-ray structures are often (though not always[53–56]) more accurate than predictions from MD ensembles. However, the greater accuracy obtained with static structures does not necessarily reflect inaccuracies of the MD ensembles. Indeed, MD simulations that yield excellent agreement with other NMR observables, such as NMR scalar couplings or residual dipolar couplings (see below) may produce less accurate chemical shift predictions than static high-resolution x-ray structures.[53,55,58]

7. Comparisons between simulated and experimental chemical shifts are particularly informative regarding the accuracy of simulations of intrinsically disordered proteins (IDPs) and peptides. The lack of stable tertiary structure in such systems means that through-space interactions are largely washed out so that chemical shift predictions are dominated by backbone dihedral angles, which are the most accurately parametrized and least noisy relationships in backbone chemical shift predictors[69]. As a result, MD ensembles that are known to give accurate backbone dihedral distributions, based on other types of NMR data such as scalar couplings (Section 2.1.2) and residual dipolar couplings (Section 2.1.3) frequently achieve chemical shift predictions with RMSDs substantially lower than the average prediction errors obtained on training databases of x-ray structures of folded proteins. For example, Ca chemical shift predictions obtained from simulations of IDPs and peptides that agree with orthogonal NMR data or secondary structure populations estimated from circular dichroism frequently have RMSDs from experiment less than 0.5 ppm, whereas average Ca prediction errors for folded proteins average ~1 ppm. Indeed, simulations of IDPs with state-of-the art force fields regularly achieve prediction RMSDs a factor of 2 lower than the average predictor errors observed on databases of folded proteins, with a substantial dynamic range that correlates well with the agreement of orthogonal experimental data[56,58,69,70]. Therefore, when several different force fields yield predicted chemical shifts for disordered proteins with RMSDs lower than typically observed for folded proteins, differences across the force fields being tested should not be dismissed as being equivalently within prediction error but instead should be regarded as meaningful and informative.

8. There is not a one-to-one mapping between conformational distributions and chemical shifts, and many different conformational ensembles can produce identical agreement with experiment[71–73]. For example, the same CA chemical shift prediction can be obtained from many different phi/psi dihedral distributions; and ensembles with extremely different phi/psi dihedral distributions for a given residue can produce identical chemical shift predictions for a CA atom if it is exposed to an aromatic ring current in one ensemble and not the other. Nonetheless, if many chemical shift predictions are wrong, then there is very likely something wrong with the predicted conformational distribution.

9. Different programs for predicting chemical shifts tend to yield very similar results, so the choice of method is not expected to have much effect on conclusions. However, it is recommended to use the same method consistently across the trajectories being compared.

2.1.1.3 Evaluation of force fields via structural properties derived from chemical shifts

Measured chemical shifts can be used to derive structural information, such as the helicity of a peptide in solution. Simulations can then be evaluated based on their agreement with these experimentally derived structural data. This approach avoids the complications of predicting chemical shifts from simulations (Section 2.1.1.2). Instead, it relies on the availability of reliable methods to map from chemical shifts to structural properties. Two examples of structural models that can be used in this way, helical propensities and stabilities of salt bridges and salt bridge analogs, are now considered.

Accurate protein force fields should be able to model the preferences for proteins to adopt particular secondary structures. Helical propensity in a particular sequence context is often modeled using the expected fraction of time that a particular non-terminal residue adopts an alpha-helical backbone conformation, and the fractional helicity of a residue can be measured because alpha helical residues form backbone hydrogen bonds that alter the chemical shift of the carbonyl carbon in ^{13}C-labeled proteins[74]. Assuming a two-state helix-coil transition, the helical fraction can be calculated using

$$f_{helix} = \frac{\delta_{obs} - \delta_{coil}}{\delta_{helix} - \delta_{coil}}$$

where δ_{obs}, δ_{coil}, and δ_{helix} are the ^{13}C chemical shifts observed in the experiment, in the reference helical state, and in the reference coil state. Meanwhile, a simulation can be analyzed to provide the fraction of time each residue is in a helical conformation, based on its backbone dihedral angles, and the results can be compared with the results inferred from chemical shifts[75]. Alternatively, researchers can fit simulated conformations to a helix-coil transition model such as the Lifson-Roig model[76] and then compare model parameters to those fit to experimental data[75].

Another important characteristic of protein force fields is the ability to accurately model the formation of salt bridges—pairs of amino acids whose oppositely charged side-chains are within hydrogen bonding distance[77]. When a salt bridge is formed, the presence of the anionic sidechain alters the chemical shift of nitrogen in the cationic side chain, and this perturbation can be measured in ^{15}N-labeled proteins or in small molecule analogs of these side chains. Similar to the helical fraction, the fraction of salt bridge formation can be calculated from the chemical shifts observed in the experiment, along with reference shifts measured in the presence and absence of the salt bridge. Simulations can then be analyzed to provide the fraction of time the salt bridge is present based on geometric criteria for hydrogen bonding between the charged side chains.

2.1.2 Scalar couplings

2.1.2.1 General principles

NMR scalar couplings, also known as indirect couplings or J-couplings (units = Hz), are through-bond, electron-mediated, spin-spin couplings[78,79]. The magnetic field of a nuclear spin modifies the Hamiltonian of nearby electrons. Because the spins of bonded electrons are paired, this results in an indirect coupling of nuclear spin-states across bonds. These couplings can propagate through multiple bonds, and three-bond couplings are of particular interest because they provide a readout of the dihedral angle of the central bond. Accordingly, three-bond ^{3}J-coupling constants have been utilized extensively in the conformational analysis of small molecules[79] and peptides[80] since the initial discovery of the Karplus relationship[78], also called the Karplus equation, which relates the magnitude of the ^{3}J-coupling constant between two nuclear spins to the intervening dihedral angle ϕ:

$$^3J(\phi) = A\cos^2(\phi) + B\cos(\phi) + C$$

Here the Karplus coefficients—A, B, and C—depend on the identities of the coupled nuclei (e.g., ^{13}C, ^{1}H) and their local chemical environments, including bond hybridizations, bond lengths, bond angles, and the electronegativities of nearby substituents[81]. Karplus equation coefficients used in conformational analyses are generally empirically determined, based on the measurement of ^{3}J-coupling constants in molecules of known structure and then transferred to analyze the dihedral angles between nuclei in similar chemical environments[78,79,81–83]. For a given pair of coupled nuclei, the experimentally observed ^{3}J coupling is the probability-weighted average of their instantaneous coupling.

In proteins and peptides, the ^{3}J-couplings between backbone amide protons and alpha protons ($^3J_{HN\text{-}Ha}$) report on the φ dihedral angle of the peptide backbone and thus distinguish between alpha and beta secondary structure. These couplings were adopted as structural restraints in early NMR protein structure calculations[84]. The values of the Karplus coefficients for these ^{3}J coupling constants have been the subject of frequent reexamination and scrutiny[83,85–90], including studies that examine the consequences of harmonic motion and conformational dynamics[83,86,87,90,91]. Although $^3J_{HN\text{-}Ha}$ are the most frequently measured and reported ^{3}J-couplings for the protein backbone, five additional coupling constants also report on the phi dihedral angle[92]: $^3J_{HN\text{-}C'}$, $^3J_{HN\text{-}Cb}$, $^3J_{C'(i\text{-}1)\text{-}Ha}$, $^3J_{C'(i\text{-}1)\text{-}C'}$, and $^3J_{C'(i\text{-}1)\text{-}Cb}$. It has also been shown that 1-bond (^{1}J) scalar couplings, such as $^1J_{Ca\text{-}Ha}$ and $^1J_{Ca\text{-}Cb}$, are sensitive to the psi angle of protein backbones[93–95]. The chi$_1$ angles of protein sidechains can be analyzed via the following ^{3}J-couplings: $^3J_{Ha\text{-}Hb}$, $^3J_{N\text{-}Hb'}$, $^3J_{C'\text{-}Hb}$, $^3J_{Ha\text{-}Cg}$, $^3J_{N'\text{-}Cg}$, and $^3J_{C'\text{-}Cg}$[96,97]. Additionally, $^{3H}J_{N\text{-}C'}$ hydrogen bond scalar couplings (scalar couplings between protein backbone nitrogen and carbonyl atoms in different residues that are mediated through hydrogen bonds) provide quantitative information about hydrogen bond geometries[98]. Once Karplus coefficients are known, no specialized software is required to compute scalar couplings for a protein structure or MD trajectory. One simply needs to calculate the angles of interest and use them to predict their scalar couplings with the Karplus relationship and the appropriate Karplus coefficients.

2.1.2.2 Evaluation of force fields using scalar couplings

^{3}J-couplings can be computed from MD simulations as \frac{1}{N}^{3}J(φ_i), where i indexes the N simulation snapshots and ^{3}J(φ) is given by the Karplus Relationship (above), and the results can be compared with the corresponding measured ^{3}J-couplings. One may therefore use ^{3}J-coupling data to test[30,99–101] and parameterize[27,30,58,75,102,103] simulation force fields. In such calculations, one must choose between "static" and "dynamics-corrected" Karplus coefficients. Static coefficients are obtained from empirical fits of ensemble-averaged solution data to high-resolution x-ray structures and therefore do not explicitly account for the complexities of conformational distributions during parametrization. Dynamics-corrected Karplus coefficients are obtained from empirical fits that seek to account for the distributions of dihedral angles of the molecule in solution, using a variety of approaches including single and multiple well harmonic motion models as well as fitting coefficients to rotamer population distributions obtained from orthogonal data[83,86,87,90,91,97].

A recent study[58] examined the accuracy of ^{3}J-couplings computed with long MD simulations using several sets of Karplus coefficients and seven different force fields, for a large set of NMR data spanning folded and disordered proteins. The static $^3J_{HN\text{-}Ha}$ Karplus coefficients from Vogeli et al[83] (A=7.97, B=-1.26, C=0.63) and the static $^3J_{C'(i\text{-}1)\text{-}C'}$ Karplus coefficients from Li et al[89] (A=1.61, B=-0.93, C=0.66) produced the lowest RMSD values from experiment on average across force fields and protein systems. The dynamics-corrected Karplus coefficients from Lee et al[90] gave similar trends in accuracy across force fields, but larger average deviations from the experimental couplings. In principle, Karplus coefficients could be derived not empirically but from quantum calculations for specific conformations, thus avoiding the question of conformational averaging. However, Karplus coefficients derived in this way have generally given worse agreement with experiment than those derived empirically[83,86,87,90,91,97].

When interpreting the NMR scalar couplings calculated from MD simulations, it is essential to consider the large uncertainty in the Karplus coefficients. This is frequently done using a χ^2 value[58,103]:

$$\chi^2 = \frac{1}{N}\sum_i \frac{\left(J_{exp}(i) - J_{calc}(i)\right)^2}{\sigma_i^2}$$

where $J_{exp}(i)$ and $J_{calc}(i)$ are the *i*th experimental and computed J-couplings, respectively, and $sigma_i$ is the uncertainty in the predictions made by the Karplus equation, i.e. the RMSD between predicted and measured scalar couplings for the fit of the Karplus parameters. A χ^2 value less than 1.0 indicates agreement within the estimated uncertainty. It is also worth recalling the words of Martin Karplus[79]: "the person who attempts to estimate dihedral angles to an accuracy of one or two degrees does so at …[their] own peril".

2.1.3 Residual dipolar couplings

2.1.3.1 General principles

The direct interaction of the magnetic dipoles associated with two nuclear spins leads to an experimentally measurable coupling (units = Hz), known as a dipolar coupling. The magnitude of the dipolar coupling between the nuclear spins of atoms A and B for an instantaneous configuration and orientation of the molecule is given by

$$D_{AB} = D_{AB}^{\max}\left(3\cos^2\theta_{AB} - 1\right)$$
$$= D_{AB}^{\max}\mathbf{r}_{AB}^{T}\mathbf{A}\mathbf{r}_{AB}$$

where D_{AB}^{max} depends on the identities of the nuclei and the distance between them, and θ_{AB} is the angle between the magnetic field imposed by the NMR instrument and the vector connecting the two atoms. The second line re-expresses the quantity in parentheses in terms of $\mathbf{r}_{AB}$, the unit vector joining the two nuclei, defined in the internal coordinates of the molecule, and the alignment tensor **A**, which relates the internal coordinate system to the the lab-frame magnetic field. In a solution of freely tumbling molecules, θ takes on all possible values with equal probability, and the observed dipolar couplings—which are averages over molecules and time—are zero.

However, dipolar couplings can report on the structure and conformational dynamics of proteins and other macromolecules if the molecules can be even weakly aligned relative to the instrument's magnetic field, because then D_{AB} no longer averages to zero. Such alignment may be achieved by linking the protein to prosthetic groups that interact with the field or by placing the protein in an aqueous liquid crystal formed by, for example, bicelles[104,105] or anisotropic compression of acrylamide gels[106]. The dipolar coupling measured in a weakly aligned sample is called a residual dipolar coupling (RDC). RDCs are often measured between atoms that are directly bonded—in proteins, often an amide proton and nitrogen resulting in a $^1D_{NH}$ RDC— but it is also possible to measure RDCs between nuclei that are not directly bonded. Although the alignment procedure could in principle perturb the protein's conformational ensemble, studies looking for this effect have not reported significant perturbations. One may compute the alignment vector from a single protein conformation with available software[107], or from an MD trajectory of a folded protein by applying singular value decomposition to the unit vectors of the bonds of interest following alignment of the MD frames.

2.1.3.2 Evaluation of MD simulations using residual dipolar couplings

In order to obtain a structural interpretation of a protein's RDCs to benchmark a molecular simulation of the protein, one must estimate the alignment tensor, **A**. For MD simulations of folded proteins, it is often reasonable to assume that the internal motions of the protein and the alignment are mostly decoupled or that any coupling does not contribute substantially to the

RDCs. In this case, one may keep the concept of an alignment tensor, noting that this should be fitted over the full ensemble rather than using a single structure. The procedure and equations used in this fitting are the same as for rigid proteins and typically rely on SVD. With a set of experimental RDCs and a simulation one can fit the five independent parameters of the alignment tensor. For unfolded proteins, flexible peptides, and intrinsically disordered proteins, it is not possible to fit an average alignment from the data because the alignment varies across conformations. Therefore most analyses use a physical model to predict the alignment tensor for each conformation and use this to calculate per-frame RDCs that can then be averaged. These physical models can be tested against data for folded proteins, where the alignment can be determined from experiment. Such tests suggest that the predicted alignments are accurate enough to be useful, in particular when alignment is dominated by steric interactions between the macromolecule and the alignment medium. Among the different methods for predicting alignment tensors, the PALES software is probably the most commonly used[107]. While it is possible to predict alignment for the full chain, sometimes local alignment over short stretches of ca. 15 residues is used instead.

2.1.4 Nuclear Overhauser effect spectroscopy

2.1.4.1 General principles

The nuclear Overhauser effect (NOE) is the change in intensity of the resonance peak of one nucleus that occurs when the resonance of a nearby nucleus is saturated by radio frequency irradiation. The NOE is a through-space, rather than through-bond, effect, and dies off as r^{-6}, where r is the internuclear distance. It is typically detectable only when an ensemble average of the distance is less than ~6 Angstroms. The strength of an NOE between two atoms is often interpreted as placing an upper bound on the mean interatomic distance; e.g. strong (<2.5 Å), medium (<3.0 Å), weak <4.5 Å[108]. NOE's are measured in a nuclear Overhauser effect spectroscopy (NOESY) experiment, a so-called 2-dimensional study where one dimension is the irradiation frequency and the other is the readout frequency. NOESY experiments usually focus on hydrogen nuclei (protons), because using carbons or nitrogens would require isotopic labeling.

2.1.4.2 Evaluation of MD simulations using NOESY

NOE data can be used to check the accuracy of a protein simulation by computing the mean interatomic distances corresponding to the available NOEs and checking how well they comply with the distance bounds determined from the NOE strengths. Due to subtleties related to the different timescales of overall molecular rotation and motions within the molecular frame of reference, the time-averaged distance between two atoms in a molecular dynamics simulation is usually computed as $\langle r^{-3}\rangle^{-1/3}$, rather than $\langle r^{-6}\rangle^{-⅙}$. Alternatively, one may use the simulation to calculate the NOESY correlation functions themselves, rather than derived properties, but this approach is more complex[109]. In some cases, the experimental study may not distinguish individual hydrogen atoms, either because they are genuinely indistinguishable (*e.g.* the protons in a methyl group), or because one cannot assign the stereospecific hydrogen atoms (*e.g.* the

β-hydrogens in an amino acid side chain). These cases may either be omitted from the evaluation, or addressed by “center average” or other approaches[110,111].

2.1.5 Spin relaxation

2.1.5.1 General principles

Spin relaxation is the decay of a nucleus’s spin magnetization back to its equilibrium distribution following a radiofrequency pulse at the nucleus’s resonant frequency, that both tips the spin away from its precession around the axis of the external field and also makes the spins precess coherently. Spin relaxation is typically characterized by two exponential decay processes. Spin-lattice relaxation, or T_1 relaxation, is the decay of the component of the nuclear spin magnetization parallel to the external magnetic field, conventionally taken to be the z axis. This is caused by interaction of the aligned spins with the environment. This longitudinal component of the magnetization, $M_z(t)$, decays according to

$$M_z(t) = M_{z,eq} - (M_{z,eq} - M_z(0)) \exp(-\frac{t}{T_1})$$

where $M_{z,eq}$ is the longitudinal component of the magnetization at thermal equilibrium and T_1 is the decay constant for the spin-lattice relaxation. Spin-spin relaxation or T_2 relaxation is the decay of the net magnetization transverse to the external magnetic field due to dwindling coherence of the phases of the spins of individual nuclei. The transverse component of the magnetization, $M_{xy}(t)$, decays according to

$$M_{xy}(t) = M_{xy}(0) \exp(-\frac{t}{T_2}))$$

where T_2 is the decay constant for the spin-spin relaxation. For most systems, spin-spin relaxation is faster than spin-lattice relaxation; i.e., $T_1 > T_2$. Spin relaxation times, i.e. T_1 and T_2, can be detected for NMR-active isotopes, in proteins typically ^{15}N in labeled backbone amides or ^{13}C and ^{2}H labeled side chains. Although protein force fields are usually benchmarked against backbone spin relaxation times, it is also possible to to use side chain data[112].

Spin relaxation times are determined by both dynamics and the conformational ensemble sampled over time. However, they depend in a complex manner on the magnetic field strength, and their connection to molecular dynamics is non linear, so intuitive interpretation is often not straightforward. Standard spin relaxation time analyses typically employ the simplifying Lipari-Szabo approach[113–117], where the timescales of overall protein rotation and internal motions are assumed to be widely separated. One then quantifies the orientational freedom of a bond vector, such as that of a ^{13}C-H bond, in the protein frame of reference in terms of a generalized order parameter. The order parameter ranges between 0 and 1[118], where 0 implies free isotropic rotation of the bond vector, and 1 implies a complete absence of freedom within the molecular frame of reference.

2.1.5.2 Evaluation of MD simulations using spin relaxation

Spin relaxation times can be calculated directly from MD simulations using the Redfield equations[119–121], instead of using the approximations of the Lipari-Szabo model[73,120]. However, the results often deviate from experiment due to an incorrect rate constant for overall protein rotational diffusion that results from errors in the viscosity of water in the simulations, and/or from insufficiently sampled simulations[120,122]. In such cases, the protein internal motions can be compared to order parameters and fast time scales extracted from experiments using the Lipari-Szabo model[113–117,123,124], or the effect of overall motions can be manually corrected using available mathematical tools[112,119,120,125,126]. On the other hand, sufficiently long simulations[127] with water models having the correct viscosity can reproduce experimental spin relaxation times without any further corrections[73]. This is useful for proteins with disordered regions for which the rotational diffusion tensor cannot be defined and the number of relevant rotational timescales is not known a priori. Thus, spin relaxation times can contribute to the evaluation of MD simulations, and can be uniquely informative for proteins with long disordered linkers[73].

2.1.6 Paramagnetic relaxation enhancement

2.1.6.1 General principles

Paramagnetic relaxation enhancement (PRE) is an increase in the NMR relaxation rate of a nuclear spin due to its dipolar interactions with unpaired electrons at a paramagnetic site; i.e., an atom with an unpaired electron[128]. Like an NOE, this effect dies off as r^{-6}. However, because the magnetic moment of an unpaired electron is much larger than that of a nucleus, PRE ranges over much longer distances, to as far as ~25 Angstroms or more. Some metal atoms in metalloproteins are paramagnetic and generate measurable PREs. For other proteins, paramagnetic sites can be artificially introduced by using chemical reactions to attach extrinsic labels, called spin-labels. This is usually done by engineering proteins to have just a single, reactive cysteine residue which can then be reacted with a nitroxide-containing compound such as MTSL or a chelating agent, such as an EDTA derivative, carrying a paramagnetic metal. Although it is possible to measure the PRE for longitudinal (R_1) NMR relaxation rates, most applications focus on transverse relaxation rates (R_2), and these will be the focus of this section. PRE depends both on distribution of distances and the timescales of motion of the protein, along with the location and dynamics of the spin-label. Its strength, range, and strong dependence on distance make it particularly suitable to detect and quantify transient and low-probability interactions.

In a typical PRE experiment, one measures the transverse relaxation rates of various groups in both the spin-labeled (paramagnetic) protein and in the same protein without the spin label (the diamagnetic protein). It is possible to measure PREs of different nuclei and chemical groups, but they are most commonly measured at backbone amides. When a nitroxide spin-label is used, the diamagnetic protein can be generated simply by reducing the nitroxide with ascorbic acid. The PRE for nuclei across the protein is obtained from the difference between the spin relaxations of these two measurements. Alternatively, because the PRE leads to line-broadening, one may estimate the PRE from the ratio of the intensities (peak heights) in

e.g. heteronuclear single quantum correlation spectra of the paramagnetic and diamagnetic samples. While this has commonly been done and may lead to useful insights, the analysis of such data comes with additional uncertainty and assumptions so that one should, if possible, measure the PRE via relaxation rates.

2.1.6.2 Evaluation of MD simulations using PRE

The PRE has been used most extensively to benchmark MD simulations of disordered proteins with weak and/or transient interactions, but can also be used to probe transient interactions between folded proteins and between proteins and nucleic acids, and could thus be used to benchmark simulations of such systems. Often experiments involve measurements on proteins labeled—one at a time—at multiple sites to get a global view of the structural dynamics of a protein. In each experiment one effectively probes the distance between the spin-label site and all backbone amide protons. Thus, one is faced with the challenge of assessing a simulation of a protein based on all these measurements.

When calculating PREs from MD simulations and comparing to spin-label experiments, one must decide on whether to and how to represent the spin-label. One approach is to model each variant of the protein with its covalently linked spin label. This requires generating force field parameters for the covalently modified protein and often also requires mutating the sequence to exclude/include cysteine residues, to match the experimental protein constructs. One also must repeat the simulation for each spin-label site of interest. An easier alternative is to simulate the unlabeled wild-type protein and calculate distances between protein atoms as a proxy for the distances between the spin-label and amide protons. For example, one may use an atom in the sidechain of the reference (wild-type) sequence as a proxy for the location of the unpaired electron. A compromise between these two extremes is to perform simulations of the wild-type protein and then model the spin-label onto this simulation using a rotamer library developed to describe the structural preferences of the label. Such rotamer libraries are available for the commonly used MTSL spin-label[129]. For applications to large MD simulations, placing the spin-labels and sampling the rotamers may be achieved by tools such as Rotamer-ConvolveMD in the MDAnalysis package[130,131] and DEER-PREdict[132], with the latter also implementing calculations of the PREs from the simulations. Which of these approaches to choose depends in part on the desired accuracy and whether there is experimental evidence that the spin-label itself introduces a change in the conformational ensemble.

There can also be complexities and decisions in determining how to account for dynamics when computing the PREs. The relationship between structure, dynamics and the PRE is rigorously described by the Solomon and Bloembergen equation[133]. This captures key conformational and dynamical effects in the spectral density function $J(\omega_I)$, which is essentially the probability that a given nucleus is rotating at frequency ω_I. However, computing the spectral density from a simulation can be nontrivial, so sometimes more simplified expressions are used[134].

2.2 NMR datasets

Many NMR datasets for proteins and peptides are available in the literature and in the Biological Magnetic Resonance Bank (BMRB)[67,68]. We focus here on datasets that appear particularly useful for benchmarking force field parameters because they largely meet the following criteria: 1) the data are available in a machine readable format; 2) estimates of experimental uncertainty are included; 3) a diversity of structural motifs, including different secondary and tertiary structure elements and disordered regions, are present; 4) the proteins are small enough that relatively short simulations suffice to provide well-converged estimates of the NMR observables.

2.2.1 Beauchamp short peptides and ubiquitin

Beauchamp et al.[101] curated from the literature and BioMagResBank 524 NMR chemical shifts and scalar J-couplings for 19 dipeptides (Ace-X-Nme, where X are non-proline amino acids), 11 tripeptides, tetra-alanine, and the protein ubiquitin. The short peptides in this dataset provide an opportunity to assess the backbone preferences of amino acid residues in the absence of a defined secondary structure. It is important for a force field to capture these preferences in order to accurately predict the conformational distributions of flexible loops in folded proteins and of unfolded and intrinsically disordered proteins.

2.2.2 Designed beta-hairpins and Trp-cage miniproteins

Many groups have used NMR to characterize the solution structure, stability, and dynamics of designed beta-hairpin sequences[135–155] and miniproteins[156–162]. Of note is a series of studies of Trp-cage miniprotein sequences[163–168]. For Trp-cage, these data include NOE restraints for four solution structures (PDB entries 1L2Y[163], 2JOF[165], 2M7D[167], 6D37[168]), and folding rates measured by NMR resonance line-broadening due to folded/unfolded-state exchange[150,169] . Temperature-dependent chemical shift deviations (CSDs) have been measured for dozens of related sequences, offering a high-quality benchmark set that reports on how mutations perturb folding. Molecular simulations are now more routinely able to access the microsecond timescales required to make accurate comparisons with these observables.

2.2.3 Mao folded proteins

This is a collection of 41 folded proteins for which both x-ray structures and NMR data, comprising backbone chemical shifts and NOESY intensities, have been measured by the Northeast Structural Genomic Consortium[170]. These data have been used to assess the accuracy of NMR structures, X-ray structures, and Rosetta refinements[170] and to compare the accuracy of MD simulations run with different force fields[58,171].

2.2.4 Robustelli a99SB-disp benchmark dataset of folded and disordered proteins

Robustelli and co-workers assembled a dataset that probes the ability of a protein force field to simultaneously describe the properties of folded proteins, weakly structured peptides, fast-folding proteins, and disordered proteins with a range of residual secondary structure propensities[58,134,171]. This a99SB-disp benchmark set [https://github.com/paulrobustelli/Force-Fields] spans 21 proteins and peptides with over 9,000 experimental data points.

The a99SB-disp dataset includes four folded proteins (ubiquitin, GB3, hen egg white lysozyme (HEWL) and bovine pancreatic trypsin inhibitor) with extensive NMR data, including backbone and sidechain J-couplings, RDCs, and backbone and sidechain spin relaxation order parameters. The protein calmodulin, which contains two folded domains connected by a flexible linker, is also included to assess the ability of a force field to simultaneously describe the flexibility of the linker region, the stability of folded domains, and the propensity of the folded domains to associate. The NMR data for calmodulin comprise chemical shifts, RDCs. The benchmark also includes the bZip domain of the GCN4 transcription factor, a partially disordered dimer with an ordered, helical, coiled-coil, dimerization domain, for which NMR chemical shifts and backbone amide spin-relaxation parameters are available.

The dataset also includes nine proteins that are disordered under physiological conditions and for which extensive sets of NMR (and SAXS) data are available. These test the ability of a force field to accurately describe the dimensions and secondary structure propensities of intrinsically disordered proteins. The proteins range in size from 40 to 140 amino acids, which was important, as a number of force fields that produced reasonable dimensions for proteins containing <70 amino acids produced conformations that were substantially over-collapsed for longer sequences. The available NMR data for these disordered proteins include chemical shifts, RDCS, backbone J-couplings, and PREs. Scalar couplings of the disordered Ala5 peptide were also included.

The a99SP-disp dataset is enriched by a number of non-NMR data, including radii of gyration obtained by various experimental methods, and data on the temperature-dependent stability of fast-folding proteins, and was subsequently expanded[171] to include the free energies of association of 14 protein-protein complexes, the osmotic coefficients of 18 organic and inorganic salts, the position of the first peak of the radial distribution function of seven ion-water and ion-ion pairs, comparisons of Ramachandran distributions of blocked amino-acids in water and Ramachandran distributions obtained from x-ray coil libraries, Lifson-Roig helix extension parameters estimated from NMR for the 20 amino acids from Ace-(AAXAA)3-Nme peptides, the relative folding free energies of mutants of 22 mutants of Trp-cage, the folding enthalpies of 10 fast-folding proteins, the Kirkwood-Buff integrals of ethanol water-mixtures, and the melting curves of the Trpzip1 and GB1 β-hairpin forming peptides.

2.2.5 Spin relaxation datasets

Spin relaxation data for a large number of proteins are reported in literature; see for example Jarymowycz and Stone[172], and some of these data are available in the BMRB. Most of these data have not so far been analyzed in conjunction with molecular simulations, but there are at least two promising examples. First, the membrane-bound, bacterial, TonB proteins possess a long, disordered region that links their C-terminal domain to their transmembrane N-terminus. Spin relaxation times have been measured for the C-terminal domain alone with varying linker lengths (HpTonB194-285, HpTonB179-285, HpTonB36-285)[173], and these data can be used to evaluate the conformational ensembles predicted by MD simulations. In one such study[73], simulations with the Amber ff03ws force field reproduced the experimental spin relaxation times of TonB as well as Engrailed 2, while CHARMM36m and Amber ff99-ILDN gave less accurate results, apparently because they yielded overly collapsed conformational ensembles. Interestingly, the three force fields gave similar accuracy for chemical shifts. Second, spin relaxation data at multiple magnetic fields are available for the partially disordered 143–259 region of the Engrailed 2 transcription factor[174]. This region is highly conserved and is involved in the binding of transcriptional regulators. Simulations with the Amber ff03ws force field yielded good agreement with the experimental data, except for serine and aspartate residues[73].

2.2.6 Salt bridge stabilities via NMR

In some cases, NMR data can be used to measure secondary properties which can then be compared with simulations. For example, the thermodynamic stability of a salt bridge can be assessed by monitoring NMR chemical shifts as a function of pH. One such study examined three potential salt bridges in the context of a folded protein, the B1 domain of protein G (GB1)[175]. These salt bridges involve lysine-carboxylate ionic interactions and were identified from crystal structures. To examine whether these salt bridges are present in solution, NMR experiments were performed to monitor both lysine nitrogen and protein chemical shifts, and the hydrogen-deuterium isotope effects on the ammonium group, while titrating the carboxylates to protonate them. Based on the NMR data, two of the salt-bridges are not formed in solution while a third was only weakly formed, providing an important test of the ability of force fields to model the strengths of weak salt bridges. Interestingly, most force fields tested overestimated the stability of the salt bridges[175], a result also reported by a previous study[176] looking at the association constants of oppositely charged amino acids in water, where the experimental data were obtained by potentiometric titration rather than NMR. Here, the results were significantly improved by atomic charge derivation strategies that implicitly incorporate solvent polarization[177] and by the use of the more expensive, polarizable CHARMM Drude-2013[178] and AMOEBA force fields[179].

3 Room temperature protein crystallography

The earliest protein crystal structures were determined by x-ray diffraction for specimens at or near room temperature[180]. Later, methods of working with protein crystals at low temperature

were developed, and these were widely adopted for their practical advantages[181,182]. More recently, however, there has been growing interest in room temperature protein crystallography because it gives insight into molecular motions that are quenched at cryogenic temperatures[181–184]. Thus, although most crystallographic data available in the Protein Data Bank (PDB) were measured at cryogenic temperatures, the PDB contains a growing number of data measured at room temperature.

Moreover, room temperature (RT) crystallography is better suited than cryocrystallography for benchmarking molecular simulations because, at cryogenic temperatures, there is little motion to simulate, and it is also not clear that the conformational variability observed corresponds to a well-defined thermodynamic ensemble at any particular temperature. The data provided by RT crystallography are valuable because they bear both on structure and on conformational fluctuation. Neutron diffraction crystallography, which can also be carried out at room temperature, goes beyond x-ray crystallography in its ability to resolve hydrogen atoms in protein structures. However, there are not many neutron diffraction structures because this method can only be done at a few appropriate neutron sources.

Water typically occupies about 50% of a protein crystal's volume[185], so crystallized proteins are usually quite well-solvated. Nonetheless, the conformational distribution of a crystallized protein is likely to differ from that of the same protein in solution, due to protein-protein contacts, perturbations of water structure, and the possible presence of cosolutes added to facilitate formation of suitable crystals. Therefore, when one uses crystallographic data to benchmark molecular simulations, one should simulate the crystal, rather than the protein in solution. This requirement adds complexity and makes for larger and hence slower simulations, compared with the conventional simulations that can be used with NMR benchmark data, since the latter pertain to proteins and peptides in solution. Different proteins, and even different crystal structures of the same protein, have different levels and characters of both conformational variation and experimental error. Therefore, much as for NMR benchmarking, force fields should be compared against a single crystallographic dataset, rather than attempting to compare force fields based on benchmarks against different structures. Methods of simulating protein crystals have been discussed in recent reviews [186,187].

This section reviews crystallographic observables that are relatively well developed for use in benchmarking protein simulations, touches on the additional topic of diffuse scattering, and lists crystallographic datasets that are well-suited for benchmarking.

3.1 Crystallographic observables

3.1.1 Bragg diffraction data

3.1.1.1 General principles

X-ray scattering from a perfect crystal would be focused into sharp spots on the detector; these are generally called the Bragg positions. Real crystals also exhibit "diffuse" scattering at all

orientations. Intensities at the Bragg positions are sensitive to the time- and space-average of the electron density, and in this section we will limit our discussion to Bragg intensities. Diffuse scattering, discussed in a subsequent section, reports on correlated motions and crystalline disorder[188]. The Bragg intensities are related to the mean unit-cell electron density through the complex structure factors, or the Fourier transform of the unit-cell electron density distribution. In particular, the Bragg intensities are proportional to the squared amplitudes of the structure factors. In the typical case of an absence of anomalous signal from which experimental phases can be obtained, the phases must be obtained from an atomic model to compute the electron density. The electron density therefore is not typically an "experimental observable" in the usual sense. Still, for a well-refined model, with good resolution and an R-factor below 15% or so, it is common to refer to the “observed electron density”.

3.1.1.2 Evaluation of MD simulations using Bragg diffraction data

The simplest way to benchmark a simulation of a protein crystal is to compare the average structure from a simulation with a model structure available in the Protein Data Bank (PDB[189–191]). This intuitive and quick comparison may be useful when there are large differences between a proposed new force field and results from some current, widely-used force field. This focus on a single structure may also help to identify specific problems in the proposed force field, and thus can yield feedback to improve the parameters. To assess protein backbone agreement, RMSD of Cα atoms or of all backbone heavy atoms can be used. To assess protein side-chain agreement, RMSD of side-chain atoms, dihedral-angle agreement within an angular threshold, or match between defined rotamers[192] can be used. Note that a rigorous measure of RMSD must account for symmetries; for example, a 180° rotation of the χ_2 angle of a phenylalanine in the model should not alter the computed RMSD. Also, because the Debye-Waller factors (B-factors, Section 3.1.2) report on the mean squared uncertainty of an atom’s position, which may include contributions from sources other than intramolecular motion (e.g., lattice vibrations), constant offsets often are ignored in B-factor profile comparisons. The possibility of alternate conformations (Section 3.1.3) should also be factored in.

A second approach is to compute the mean electron density from the simulation using mdv2map in AmberTools with CCP4[193,194] or xtraj in LUNUS with CCBTX[195,196], use crystallographic software to refine an atomic model into this density, and then use structural metrics (see prior paragraph) to compare this model with the experimental structure model. Such a comparison is, at least in principle, a sound method to assess agreement in the presence of disorder.

A third approach is to compute the mean electron density from the simulation[193–196] and compare it with the crystallographic electron density. This avoids the intermediary of a structural model inferred from the crystallographic density. Note that the observed electron density is itself a time and space average over many unit cells and hence reflects disorder in the experimental system. Because the density is often sharply peaked around atomic positions, standard methods of comparing density, such as the Pearson correlation, can be more sensitive to small, local differences than is desired for assessing the relative accuracy between force fields, although these issues may be addressed by approaches such as local model-based map alignments[197].

Existing crystallographic software, such as EDSTATS in CCP4[194] and phenix.real_space_correlation in PHENIX[198], can be used to compare electron density maps.

Other methods of comparing simulations to crystallographic data are possible. For example, one could directly compare computed and measured Bragg intensities or electron densities. This properly takes care of disorder, but can be more difficult to interpret, as modest deviations (by an RMSD less than 0.5 Å) of the protein away from the "correct" structure can greatly reduce the real-space correlations between computed and experimental densities[199]. In such cases, other measures of accuracy than correlation may be more useful. One may also focus on the accuracy of protein-protein contacts at interfaces. This highlights non-covalent and solvent-mediated interactions that are important to get correct. Most crystal contact analysis methods (such as the PISA software[200]) use a single average structure as input, so it is more difficult to account directly for disorder. As with the comparison of average protein structures discussed above, this sort of comparison may be most useful when there are relatively large differences between the results from different proposed force fields.

3.1.2 Debye-Waller factors (B-factors)

3.1.2.1 General principles

The Debye-Waller factor of an atom, also known as its B-factor or temperature factor, is closely related to the atom's fluctuations about its modeled position. B-factors are, in principle at least, related to the mean square displacement of the atom, $\langle u^2 \rangle$, by the equation

$$B = \frac{8}{3}\pi^2 \langle u^2 \rangle$$

However, B-factors can also have contributions from various sources of error, and are sometimes viewed as "slop factors" that absorb other errors in the model to improve the fit to the diffraction data. They may mask the identity of atoms of similar Z number, i.e. K vs. Ca, and may also mask incorrect modeling of side chains, alternate conformations, and disordered regions. Debates continue between crystallographers about when a region that exhibits signs of disorder should be left unmodeled, modeled with zero occupancy, or modeled with B-factors allowed to refine to high values; as a result, their meaning can vary between different crystal structures. However, when derived from well-validated structural models[201], that are modeled correctly and carefully interpreted, they provide a meaningful measure of the mobility of atoms within a protein structure. For structures with resolutions better than ~1.5 Å, it is often appropriate to refine anisotropic B-factors, such that each atom is assigned a tensor of six parameters that define a three-dimensional Gaussian distribution of atomic fluctuations.

3.1.2.2 Evaluation of MD simulations using Debye-Waller factors

Given a simulation of a protein crystal, it is straightforward to compute the mean square displacement of each atom from its mean position and thus obtain computed B-factors; a

modest amount of additional complexity is involved in computing anisotropic B-factors. Alternatively, one may, again, refine a structural model into the electron density computed from the simulation, and compare the resulting simulated B-factors with the experimental B-factors.

3.1.3 Alternate conformations

3.1.3.1 General principles

In contrast to B-factors, which in principle describe harmonic motion within a local energy well (vide supra), alternate conformations explicitly describe anharmonic motion between discrete conformational states in separate local energy wells. For example, alternate locations —also known as alternative conformations, alt confs, alternate locations, or altlocs — can be used to model amino-acid side chains that switch between different rotamer[202] conformations. As they are a powerful mechanism for modeling protein conformational heterogeneity, particularly at high resolution, alternate conformations are a promising avenue for comparison to and improvement of simulations. In a protein structure file, an alternate conformation is given as an additional set of coordinates for a group of atoms (often a residue or series of residues) and marked with a single-character identifier (A, B, C, etc.) that is unique within the file. Each alternate conformation is also assigned a partial occupancy (i.e. probability) from 0 to 1 that is determined by the crystallographic refinement. For covalently bound atoms, like those of proteins, the occupancies of all alternate conformations for a given atom typically sum to 1. By contrast, for molecules that are not covalently bound to the protein, like ligands and water molecules, the occupancies often sum to less than 1. A crystal structure model that contains alternate conformations is termed a multiconformer model.

Alternate conformations are often left unmodeled in crystal structure models even when they are evident in the electron density maps[203]. Such missing alternate conformations can be modeled in an automated and unbiased manner with tools such as the qFit software[204]. Crystalline MD simulations can be used to find alternate conformations that are missed by such automated methods[186]. Alternate conformations are significantly more prevalent at RT than at cryogenic temperatures (cryo)[205]. Indeed, some alternate conformations that are observed only at RT and not at cryo are critical to biological function[183] (see “Cyclophilin A” below) and can modulate ligand binding in important ways[206]. This highlights the importance of RT crystallography data for revealing biologically relevant conformational heterogeneity in protein structures.

While different parts of a biomolecule may have alternate conformations, these conformations are not always independent, e.g. a rearrangement of one part of a protein to an alternate conformation might restrict which conformations its neighbors might have, etc. The RCSB PDB format for crystal structure models does not provide a mechanism for specifying which alternate conformations are physically compatible with one another, other than the A, B, C, etc. identifiers themselves. This bookkeeping issue creates ambiguity in situations where, for example, residue X with conformations A and B is near (in the tertiary structure) to residue Y with conformations A, B, and C. In such a case, it is unclear which conformations of residue X are energetically

compatible with conformation C of residue Y. The corresponding alternate conformations might be coupled, uncoupled, or partially coupled.

3.1.3.2 Evaluation of MD simulations using alternate conformations

The fractional occupancies of alternate conformations inferred from RT crystallography can be readily compared with the corresponding result from a simulation. Note that small changes in the computed free energy difference between two conformations will lead to large changes in occupancy if the free energy difference is near zero, but essentially no change if the free difference is far from zero. This is because, given two conformers A and B, the probability of being in state A is

$$p_A = \left(1 + e^{-\Delta G_{AB}/RT}\right)^{-1}$$

where ΔG_{AB} is the free energy change on going from A to B; and $p_B = 1 - p_A$, assuming only two accessible conformations. Conformational probabilities can be extracted from simulations based on the occupancy of rotameric energy wells, e.g. the rotamer name strings[202] that are output by software such as the model validation suite MolProbity[201] or the related tool phenix.rotalyze from PHENIX[198]. Such occupancies can also be coupled to temperature[206]. In addition, the coordinates and occupancies of alternate conformations can be combined with B-factors to calculate so-called crystallographic order parameters, which can be used to compare to experimental NMR order parameters[207]; a similar framework could be useful for comparing RT crystallographic models to simulations. In these ways, among others, crystallographic alternate conformations can be rich targets for force field optimization.

3.1.4 Diffuse scattering (non-Bragg reflections)

Diffuse or continuous scattering refers to the cloudy, streaked, speckled, halo-shaped, or otherwise patterned weak scattering that lies between the Bragg peaks. In contrast with the Bragg scattering, which is associated with correlations in the mean electron density, the diffuse scattering is associated with spatial correlations in the deviation of the density from the mean. In principle, this means that, much as the Bragg peaks can be used to model the mean structure of the crystals constituents, the diffuse scattering can be used to model their time-averaged fluctuations. There have been several protein crystal MD simulations of diffuse scattering, and it is straightforward to compute diffuse intensities from simulation snapshots[199,208–215].Although a limited study did show that the force field can influence the simulated diffuse scattering[213], it is not yet clear what sort of variation one should expect. Future crystal simulations should help clarify whether analysis of diffuse scattering can be a useful component of benchmarking of force fields.

3.1.5 Hydrogen coordinates from neutron diffraction

Protein x-ray crystallography generally resolves the coordinates only of atoms with atomic number greater than one; hydrogens are visible only in well-ordered regions of ultra-high resolution x-ray structures. This is because x-rays are scattered by electrons and a hydrogen atom, with only one electron, scatters only weakly. In contrast, neutrons are scattered by atomic nuclei and neutron diffraction protein crystallography can provide experimental information on

hydrogen atom positions and protonation states. These data are valuable because hydrogens make up nearly half the atoms in proteins and can play critical functional and structural roles. Thus, neutron diffraction studies are uniquely suited to discern hydrogen bonding patterns, the orientations of solvents and side chain groups, and the protonation states of critical catalytic amino acids such as the histidine in the catalytic triad of a serine protease. By the same token, they provide distinctive information to benchmark protein force fields.

Although first demonstrated in the mid-1960s, macromolecular neutron crystallography has become more practical with the commissioning of new higher flux neutron sources worldwide, as well as improved techniques for crystal growth and molecular biology techniques for producing large amounts of purified proteins. Neutron diffraction is generally weak, and requires large crystals and long data collection times. On the other hand, in contrast to X-rays, there is little to no radiation damage involved, so it is straightforward to collect room temperature data.

3.2 Protein crystallography datasets

Here, we highlight protein crystallography datasets that are well-suited to benchmark force field accuracy by running simulations of protein crystals and comparing the results to experiment. We prioritize the following features, which should be taken as desiderata rather than strict criteria: 1) high resolution (better than 1.2 Å), so that observable data have low uncertainty, 2) relatively unambiguous assignment of protonation states, 3) absence of non-covalent ligands or co-factors, 4) diversity of secondary and tertiary structural motifs, 5) availability of crystal data for the same protein system in multiple symmetry groups to explore the possible impact of different crystallographic contacts, and 6) availability of crystal data for the same protein system at multiple temperatures to identify biases toward structural artifacts that appear only at low temperatures.

3.2.1 Scorpion toxin

Scorpion toxin (PDB ID 1aho) is a 64-residue globular protein with a room-temperature x-ray data set at 0.96 Å resolution. It has only a small amount of regular secondary structure (one 9-residue helix and two short beta strands) but is stabilized by four disulfide bonds. It was the subject of an early MD simulation[216] that compared simulations of the crystal with four force fields, all of which would now be considered obsolete. The notable finding at the time was how diverse the simulation results were, even for simple metrics like average backbone structure and computed B-factors. This study supported the idea that crystal simulations could be used for testing certain aspects of protein simulations, both in terms of the fairly strong interactions that determine the conformation of an individual chain, and also in terms of the weaker (often solvent-mediated) interactions that influence the stability of the crystal lattice. The small size of the unit cell was a more important consideration back in 2010 than it would be today. Although the resolution is below 1 Å, the deposited model has a fairly high R-factor of 16.3%. The small amount of secondary structure makes it a challenging problem, but the paucity of experimental

analysis of alternate conformations (only four residues are assigned alternate conformers in the deposited structure) may make it less useful than other protein systems discussed below.

3.2.2 Hen egg-white lysozyme

Hen egg-white lysozyme (HEWL) was one of the first proteins to have its three-dimensional structure determined by x-ray crystallography, and it is widely used as an experimental model system in part because of its ease of crystallization. This protein has 129 residues, eight helices and two beta-sheets, and four disulfide bonds. Given these constraints, simulations with different force fields may not yield markedly different results, but these data should nonetheless be useful, as discussed below.

There are three RT crystal structures of the triclinic form (P1 space group) of HEWL (2lzt, 2.0 Å resolution; 6o2h, 1.2 Å; 4lzt, 0.95 Å), as well as a cryo version (2vb1, 0.65 Å). Nitrate ions predominate in neutralizing the protein, and a large number of solvent waters are visible in the experimental electron density. The crystal density of the solvent has been determined to high precision[217] and is very close to that estimated by the MD simulations discussed below; this suggests that we know the total number of water molecules in the unit cell to within an uncertainty of just a few water molecules. Two MD simulations of the triclinic HEWL crystal provide guidance for future studies.

First, Janowski et al.[218] simulated a supercell with 12 protein chains and made extensive comparisons to the reflection intensities from 4lzt. Perhaps most insightful was a comparison of two newly-refined atomic models, one refined against the experimental data and a second refined against the average electron density from a three-microsecond MD simulation. The backbone of the simulated structure was about 0.4 Å away from the experimental structure, which is well outside the expected experimental error, but B-factors were in good agreement between the two refinements, aside from a small region near the N-terminus. Comparisons between four protein force fields showed small but significant differences in how faithful the simulations were to the experimental data. Second, Meisburger et al. simulated triclinic HEWL[214] with a primary focus on interpreting diffuse x-ray intensities. Since collective motions of many protein chains are important for diffuse scattering, simulations were carried out using 1, 27, 125, and 343 unit cells, but they used only a single protein force field, so this study did not address the suitability of diffuse scattering for force field benchmarking.

There are also RT crystal structures for two other crystal forms, orthorhombic (8dyz) and tetragonal (8dz7), of HEWL, and these may further probe the ability of force fields to capture crystal-packing interactions[215].

3.2.3 Crambin

The small hydrophobic protein crambin, isolated from the seeds of the Abyssinian cabbage (*Crambe abyssinica*), was found early on to form exceptionally well-ordered crystals[219] and has been used for the development of experimental phasing techniques. Of note here is a study combining RT x-ray and neutron diffraction to 1.1 Å resolution (3U7T) and allowing for the

modeling of anisotropic displacement parameters on select (exchanged) deuterium atoms[220]. Crambin is particularly amenable to simulations because it has only 46 residues, contains both alpha helical and beta strand secondary structure, and is held together by disulfide bridges.

3.2.4 Cyclophilin A

Human cyclophilin A (CypA) is a proline *cis*/*trans* isomerase that is attractive for training force fields due to its well-studied conformational dynamics and high-resolution RT crystallographic data. Several residues (Ser99, Phe113, Met61, Arg55) in the active site form a network that exhibits correlated conformational dynamics on a similar timescale (ms) as catalytic turnover—even in apo CypA—as measured by NMR relaxation experiments[221]. These dynamics are critical for catalysis, as determined by RT crystallography that revealed the alternate conformations involved in this dynamic active-site network detail, and subsequent mutagenesis to rationally perturb them[183]. The alternate conformations in the CypA active site are primarily related to one another by transitions between side-chain rotamers, but they also involve a backbone motion called the backrub[222] that was also used previously for other systems to model conformations that better fit NMR relaxation data[223]. More recently, multi-temperature crystallography across a series of eight temperatures for CypA added nuance to our understanding of its dynamic active-site network, revealing evidence for more complex, hierarchical coupling in which dynamics for some active-site residues are dependent on the conformation of another key active-site residue (Phe113)[224]. In addition to these data, x-ray crystallographic diffuse scattering[225] and x-ray solution scattering[226] have also been measured for CypA. Thus, CypA has rich dynamics that are well-studied by RT crystallography and other experiments and are ripe for comparison to MD simulations.

An attractive crystallography dataset for validating simulations of CypA is the high-resolution (1.20 Å) RT structure (PDB ID 4yuo)[224], whose structure model contains two (A, B) or three (A, B, C) alternate conformations for different residues in the active-site network. This complicates the creation of a single-conformer starting model for a given simulation; we recommend beginning with either state A or state B, which have distinct rotamers for Phe113, which is thought to be the linchpin of the network[224]. One challenge to using CypA to validate simulations is that its catalysis and matching dynamics have been reported to be on the ms timescale[221]. However, multi-temperature crystallography suggests dynamics on multiple timescales within the active-site network, including faster motions at nanosecond timescales[224]. Mutagenesis and solution scattering also point to a separate, independent loop region with faster dynamics[226]. Thus, even simulations at shorter than ms timescales are likely to uncover relevant dynamic features in CypA that can be useful for validation and force field optimization/development. Moreover, CypA is moderately sized, with 165 amino acid residues, which will facilitate reasonably long simulations, where enhanced sampling technologies may also be of use.

In addition to the 1.20 Å RT crystal structure, a similarly high-resolution (1.25 Å) cryo structure of CypA (PDB ID 3k0m)[183] in the same crystal lattice is also available. Such RT-cryo pairs could in principle be used to optimize force fields to match the RT data better than the cryo data. This strategy would not only select for force field parameters that result in a better match to the more

realistic RT data, but could also be instructive regarding the pitfalls of benchmarking against potentially idiosyncratic[184] and at times artifactual cryo data[206].

3.2.5 Ubiquitin variants

Computational protein design and directed evolution have been combined to engineer ubiquitin variants with enhanced affinity to a desired binding partner (USP7) by stabilizing a specific conformation of the β1β2 loop[227]. In a subsequent study, RT x-ray crystallography was used to examine the structural basis for the increasing affinity of these variants over the course of the design/selection process[228]. The resulting high-resolution RT crystal structures revealed that the earlier “core” mutant of ubiquitin (with 6 mutations) exhibits multiple discrete alternate conformations of the β1β2 loop, whereas the later “affinity-matured” mutant (with 3 additional mutations) adopts a singular conformation of this loop. Also, elsewhere in the protein, both variants exhibit a distinct mode of backbone flexibility: alternate conformations for a peptide flip (residues 52-53).

High-resolution RT crystallography data and models are available for both ubiquitin mutant proteins, at a resolution of 1.12 Å for the “core” mutant (PDB ID 5tof), and 1.08 Å for “affinity-matured” mutant (PDB ID 5tog). Simulations based on these datasets should ideally capture the differences in β1β2 loop conformational heterogeneity observed in the crystallographic electron density maps for the two variants (more flexible for the “core” mutant, and more rigid for the “affinity-matured” mutant) as well as the peptide flip shared by both variants. For this system, a number of metrics could be used to quantify the match between simulations and experimental data: root-mean-square fluctuations (RMSF) of backbone atoms in the loop for simulations vs. for multi-conformer crystal structures, local real-space fit to the electron density map in the β1β2 loop region, recapitulation of fractional occupancies for the different loop conformations (perhaps after clustering the simulation snapshots), etc.

Overall, the high-resolution RT crystallography datasets available for these ubiquitin variants provide a powerful opportunity to optimize force fields—not only in general, but also targeting two specific goals: (1) accurate simulations of protein backbone conformational heterogeneity, and (2) accurate predictions of the effects of amino acid substitutions on conformational heterogeneity.

3.2.6 Protein tyrosine phosphatase 1B

Human protein tyrosine phosphatase 1B (PTP1B; also known as PTPN1) exhibits significant structural dynamics across a range of time scales and length scales, as revealed by numerous structural biophysics experiments. It therefore holds promise as a useful system for training force fields to simulate dynamic proteins more robustly.

Recently, multi-temperature X-ray crystallography of apo PTP1B across a spectrum of temperatures from cryo to RT[229] provided insights into correlated conformational heterogeneity in this protein. The resulting series of crystal structures featured alternative conformations, each

modeled with partial occupancy, for the active-site WPD loop (open vs. closed) as well as distal allosteric regions. As temperature increased, the WPD loop shifted crystallographic occupancy from the closed to the open state. Simultaneously, the distal α7 helix, a key component of PTP1B's allosteric network, shifted occupancy from the ordered state to a disordered state, as evidenced by diminished electron density for this helix at high temperature; an adjacent solvent channel in the crystal lattice was able to accommodate this temporarily disordered protein region. Several residues in between these regions exhibited smaller-scale conformational shifts between alternate conformations that appeared to lubricate the larger-scale motions of the WPD loop and α7 helix, mimicking the shifts seen previously for an allosteric inhibitor that displaces α7[230].

Complementing multi-temperature X-ray crystallography, other studies have used NMR spectroscopy to characterize timescales of motion for various regions of PTP1B. NMR relaxation experiments showed that the active-site WPD loop closes on a timescale corresponding to the rate of catalysis (ms)[231]. Beyond the active site, NMR relaxation experiments, mutagenesis, and molecular dynamics simulations restrained by NMR chemical shifts showed that faster, relatively uncoupled dynamics are key to allosteric regulation via α7[232]. Thus, PTP1B exhibits motions that may be amenable to various different types of simulations, from short, traditional simulations to long, enhanced-sampling simulations.

Both of the major states of the protein are modeled in the 1.74 Å RT (278 K) crystal structure of apo PTP1B (PDB ID 6b8x): the closed state (alternate conformation A) and the open state (B). Most regions of the structure are modeled with either no alternate conformations, or both A and B conformations. By contrast, because it is disordered in the open state of the protein, the α7 helix is modeled as only the A conformation with partial occupancy, with no coordinates for the B conformation. Note that crystallography was performed with residues 1-321 of PTP1B, but only residues 1-298 are visible in the electron density, even in the closed state with α7 ordered; the remaining residues are always disordered in a solvent channel in the crystal lattice. Although 6b8x is not as high-resolution as some other very high-resolution room-temperature crystal structures discussed in this manuscript, the wealth of types and extents of conformational heterogeneity it features make it a promising candidate for force field evaluation. Simulations of PTP1B based on 6b8x should be initiated from either the open state or the closed state of the protein and should be assessed on the basis of their ability to recapitulate the allosteric coupling observed in various experiments: as the WPD loop opens, the allosteric network should shift toward the corresponding open-like state, and α7 should become disordered. In addition to unbiased simulations, one could perform biased or guided simulations to enforce a shift in one region (WPD or α7), then examine whether the other region allosterically responds as expected.

3.2.7 Endoglucanase

Particularly high quality data are available for endoglucanase (EG) from *Phanerochaete chrysosporium*, making this a particularly appealing target for molecular dynamics studies. The EG datasets combine two relatively rare aspects. The EG crystal structure was obtained using both x-ray and neutron diffraction data (PDB 3X2P)[233] at RT allowing direct comparison of

simulations with experimental data.(Note, however, that this structure includes a non-standard amino acid and an oligosaccharide ligand.) Furthermore, the neutron diffraction data makes it possible to use experimentally-derived protonation states for protein residues[234–236], removing a potential cause of modeling errors and uncertainties, while also allowing direct determination of the orientation of ordered waters and other factors. Water structure in this case is also much more clearly defined, and amenable to careful curation[237,238].

In one particular prior modeling study[238], the EG-cellopentaose complex from 3X2P was re-refined from 1.5 Å neutron and 1.0 Å X-ray diffraction data, with careful attention to placing H/D atoms based on neutron scattering data and H-bond interactions with the local environment. This particular study focused on whether and to what extent MD simulations of crystals recapitulated experimental observables, and with a particular focus on water placement and occupancy. The study computed electron density maps from crystalline simulations of a 2x2x2 periodic supercell under several different solvent conditions, with several different restraints of the protein heavy atoms and ligand atoms. Clear indications of force field limitations were identified. For example, although recovery of experimentally observed crystallographic waters was over 90% when the protein was restrained modestly to crystallographic positions, this fraction dropped to 50% without restraints. The drop traced to small, local protein motions that disrupted entire water networks[238] consistent with prior work[239].

While this study focused primarily on recovery of crystallographic water molecules, it suggests a route forward in terms of benchmarking force fields on this and similar systems[238]. The carefully curated re-refined structures provide a valuable starting point and should be used as input for simulations. Force fields could be benchmarked by repeating the simulation approach used here, while comparing results at different restraint strengths. Presumably, as force fields are improved, this will result in better recovery of crystallographic water structures at lower protein restraint strengths or with no restraints. Additional benchmarking studies could more closely focus on how well various force fields preserve the structure of the protein.

3.2.8 Staphylococcal nuclease

Staphylococcal nuclease (SNase) has provided a particularly valuable dataset for diffuse scattering studies[240], in part because until fairly recently, this was the only complete, high-quality three-dimensional diffuse scattering dataset available for a protein crystal[212]. Crystalline MD simulations greater than one microseconds in length allowed calculation of diffuse scattering intensity for direct comparison with experiment. More recent work[199] extended these simulations to cover a 2x2x2 supercell, with roughly 5 μs of data, and obtained improved agreement with experimental scattering data, perhaps because the simulations better represented the crystalline environment. Still, agreement with experiment was far from perfect, giving room for further improvement. Different force fields gave different diffuse scattering patterns and different levels of agreement with experiment[213], further supporting this idea.

4 Statistical analysis of benchmark simulations

When comparing sets of simulations against experimental data, it is essential to determine whether the differences are statistically significant[241]. Statistical uncertainties in simulated data are perhaps most reliable if obtained by running multiple independent simulations, but can also be obtained by analysis of a single simulation[242]. The raw experimental data also have uncertainties, and additional uncertainty may result from model assumptions, such as the values of the Karplus parameters for ^{3}J-values or the representation of NOEsy intensities as upper bounds to interatomic distances. When multiple proteins are simulated and several properties are considered, it may not be straightforward to determine if two sets of simulations are significantly different or even which simulation shows the overall better agreement with the available experimental data[243–245]. The differences in a particular property between different force fields will be affected by both the variability due to the choice of protein and the variability between independent replicate simulations. Therefore a statistical approach that takes mixed effects explicitly into account seems most appropriate.

References

(1) Case, D. A.; Karplus, M. Dynamics of Ligand Binding to Heme Proteins. *J. Mol. Biol.* **1979**, *132* (3), 343–368. https://doi.org/10.1016/0022-2836(79)90265-1.

(2) McCammon, J. A.; Gelin, B. R.; Karplus, M. Dynamics of Folded Proteins. *Nature* **1977**, *267* (5612), 585–590. https://doi.org/10.1038/267585a0.

(3) Levitt, M.; Sharon, R. Accurate Simulation of Protein Dynamics in Solution. *Proc. Natl. Acad. Sci.* **1988**, *85* (20), 7557–7561. https://doi.org/10.1073/pnas.85.20.7557.

(4) Stone, J. E.; Phillips, J. C.; Freddolino, P. L.; Hardy, D. J.; Trabuco, L. G.; Schulten, K. Accelerating Molecular Modeling Applications with Graphics Processors. *J. Comput. Chem.* **2007**, *28* (16), 2618–2640. https://doi.org/10.1002/jcc.20829.

(5) Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald: An N·log(N) Method for Ewald Sums in Large Systems. *J. Chem. Phys.* **1993**, *98* (12), 10089–10092. https://doi.org/10.1063/1.464397.

(6) Grant, B. J.; Gorfe, A. A.; McCammon, J. A. Large Conformational Changes in Proteins: Signaling and Other Functions. *Curr. Opin. Struct. Biol.* **2010**, *20* (2), 142–147. https://doi.org/10.1016/j.sbi.2009.12.004.

(7) Anandakrishnan, R.; Drozdetski, A.; Walker, R. C.; Onufriev, A. V. Speed of Conformational Change: Comparing Explicit and Implicit Solvent Molecular Dynamics Simulations. *Biophys. J.* **2015**, *108* (5), 1153–1164. https://doi.org/10.1016/j.bpj.2014.12.047.

(8) Gilson, M. K.; Zhou, H.-X. Calculation of Protein-Ligand Binding Affinities. *Annu. Rev. Biophys. Biomol. Struct.* **2007**, *36* (1), 21–42. https://doi.org/10.1146/annurev.biophys.36.040306.132550.

(9) Wang, K.; Chodera, J. D.; Yang, Y.; Shirts, M. R. Identifying Ligand Binding Sites and Poses Using GPU-Accelerated Hamiltonian Replica Exchange Molecular Dynamics. *J. Comput. Aided Mol. Des.* **2013**, *27* (12), 989–1007. https://doi.org/10.1007/s10822-013-9689-8.

(10) Reif, M. M.; Oostenbrink, C. Net Charge Changes in the Calculation of Relative

Ligand-Binding Free Energies via Classical Atomistic Molecular Dynamics Simulation. *J. Comput. Chem.* **2014**, *35* (3), 227–243. https://doi.org/10.1002/jcc.23490.
(11) Lindorff-Larsen, K.; Piana, S.; Dror, R. O.; Shaw, D. E. How Fast-Folding Proteins Fold. *Science* **2011**, *334* (6055), 517–520. https://doi.org/10.1126/science.1208351.
(12) Shaw, D. E.; Maragakis, P.; Lindorff-Larsen, K.; Piana, S.; Dror, R. O.; Eastwood, M. P.; Bank, J. A.; Jumper, J. M.; Salmon, J. K.; Shan, Y.; Wriggers, W. Atomic-Level Characterization of the Structural Dynamics of Proteins. *Science* **2010**, *330* (6002), 341–346. https://doi.org/10.1126/science.1187409.
(13) S. Saglam, A.; T. Chong, L. Protein–Protein Binding Pathways and Calculations of Rate Constants Using Fully-Continuous, Explicit-Solvent Simulations. *Chem. Sci.* **2019**, *10* (8), 2360–2372. https://doi.org/10.1039/C8SC04811H.
(14) Frenkel, D.; Smit, B. *Understanding Molecular Simulation: From Algorithms to Applications*; Elsevier, 2001.
(15) Rohl, C. A.; Strauss, C. E. M.; Misura, K. M. S.; Baker, D. Protein Structure Prediction Using Rosetta. In *Methods in Enzymology*; Numerical Computer Methods, Part D; Academic Press, 2004; Vol. 383, pp 66–93. https://doi.org/10.1016/S0076-6879(04)83004-0.
(16) Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S. A. A.; Ballard, A. J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A. W.; Kavukcuoglu, K.; Kohli, P.; Hassabis, D. Highly Accurate Protein Structure Prediction with AlphaFold. *Nature* **2021**, *596* (7873), 583–589. https://doi.org/10.1038/s41586-021-03819-2.
(17) Kryshtafovych, A.; Schwede, T.; Topf, M.; Fidelis, K.; Moult, J. Critical Assessment of Methods of Protein Structure Prediction (CASP)—Round XIV. *Proteins Struct. Funct. Bioinforma.* **2021**, *89* (12), 1607–1617. https://doi.org/10.1002/prot.26237.
(18) Lifson, S. Recent Developments in the Consistent Force Field Calculations. In *Dynamic Aspects of Conformation Changes in Biological Macromolecules*; Sadron, C., Ed.; Springer Netherlands: Dordrecht, 1973; pp 421–429. https://doi.org/10.1007/978-94-010-2579-9_28.
(19) Ponder, J. W.; Case, D. A. Force Fields for Protein Simulations. In *Advances in Protein Chemistry*; Protein Simulations; Academic Press, 2003; Vol. 66, pp 27–85. https://doi.org/10.1016/S0065-3233(03)66002-X.
(20) Guvench, O.; MacKerell, A. D. Comparison of Protein Force Fields for Molecular Dynamics Simulations. In *Molecular Modeling of Proteins*; Kukol, A., Ed.; Humana Press: Totowa, NJ, 2008; pp 63–88. https://doi.org/10.1007/978-1-59745-177-2_4.
(21) Zhu, X.; Lopes, P. E. M.; MacKerell Jr, A. D. Recent Developments and Applications of the CHARMM Force Fields. *WIREs Comput. Mol. Sci.* **2012**, *2* (1), 167–185. https://doi.org/10.1002/wcms.74.
(22) Dauber-Osguthorpe, P.; Hagler, A. T. Biomolecular Force Fields: Where Have We Been, Where Are We Now, Where Do We Need to Go and How Do We Get There? *J. Comput. Aided Mol. Des.* **2019**, *33* (2), 133–203. https://doi.org/10.1007/s10822-018-0111-4.
(23) Jorgensen, W. L.; Tirado-Rives, J. The OPLS [Optimized Potentials for Liquid Simulations] Potential Functions for Proteins, Energy Minimizations for Crystals of Cyclic Peptides and Crambin. *J. Am. Chem. Soc.* **1988**, *110* (6), 1657–1666. https://doi.org/10.1021/ja00214a001.
(24) Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W.; Kollman, P. A. A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules. *J. Am. Chem. Soc.* **1995**, *117* (19), 5179–5197. https://doi.org/10.1021/ja00124a002.

(25) MacKerell, A. D.; Bashford, D.; Bellott, M.; Dunbrack, R. L.; Evanseck, J. D.; Field, M. J.; Fischer, S.; Gao, J.; Guo, H.; Ha, S.; Joseph-McCarthy, D.; Kuchnir, L.; Kuczera, K.; Lau, F. T. K.; Mattos, C.; Michnick, S.; Ngo, T.; Nguyen, D. T.; Prodhom, B.; Reiher, W. E.; Roux, B.; Schlenkrich, M.; Smith, J. C.; Stote, R.; Straub, J.; Watanabe, M.; Wiórkiewicz-Kuczera, J.; Yin, D.; Karplus, M. All-Atom Empirical Potential for Molecular Modeling and Dynamics Studies of Proteins. *J. Phys. Chem. B* **1998**, *102* (18), 3586–3616. https://doi.org/10.1021/jp973084f.

(26) Lindorff-Larsen, K.; Piana, S.; Palmo, K.; Maragakis, P.; Klepeis, J. L.; Dror, R. O.; Shaw, D. E. Improved Side-Chain Torsion Potentials for the Amber Ff99SB Protein Force Field. *Proteins Struct. Funct. Bioinforma.* **2010**, *78* (8), 1950–1958. https://doi.org/10.1002/prot.22711.

(27) Best, R. B.; Zhu, X.; Shim, J.; Lopes, P. E. M.; Mittal, J.; Feig, M.; MacKerell, A. D. Jr. Optimization of the Additive CHARMM All-Atom Protein Force Field Targeting Improved Sampling of the Backbone ϕ, ψ and Side-Chain X1 and X2 Dihedral Angles. *J. Chem. Theory Comput.* **2012**, *8* (9), 3257–3273. https://doi.org/10.1021/ct300400x.

(28) Mackerell Jr., A. D.; Feig, M.; Brooks III, C. L. Extending the Treatment of Backbone Energetics in Protein Force Fields: Limitations of Gas-Phase Quantum Mechanics in Reproducing Protein Conformational Distributions in Molecular Dynamics Simulations. *J. Comput. Chem.* **2004**, *25* (11), 1400–1415. https://doi.org/10.1002/jcc.20065.

(29) Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C. Comparison of Multiple Amber Force Fields and Development of Improved Protein Backbone Parameters. *Proteins Struct. Funct. Bioinforma.* **2006**, *65* (3), 712–725. https://doi.org/10.1002/prot.21123.

(30) Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. Ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from Ff99SB. *J. Chem. Theory Comput.* **2015**, *11* (8), 3696–3713. https://doi.org/10.1021/acs.jctc.5b00255.

(31) Tian, C.; Kasavajhala, K.; Belfon, K. A. A.; Raguette, L.; Huang, H.; Migues, A. N.; Bickel, J.; Wang, Y.; Pincay, J.; Wu, Q.; Simmerling, C. Ff19SB: Amino-Acid-Specific Protein Backbone Parameters Trained against Quantum Mechanics Energy Surfaces in Solution. *J. Chem. Theory Comput.* **2020**, *16* (1), 528–552. https://doi.org/10.1021/acs.jctc.9b00591.

(32) Allison, J. R. Assessing and Refining Molecular Dynamics Simulations of Proteins with Nuclear Magnetic Resonance Data. *Biophys. Rev.* **2012**, *4* (3), 189–203. https://doi.org/10.1007/s12551-012-0087-6.

(33) Wishart, D. S.; Case, D. A. [1] - Use of Chemical Shifts in Macromolecular Structure Determination. In *Methods in Enzymology*; James, T. L., Dötsch, V., Schmitz, U., Eds.; Nuclear Magnetic Resonance of Biological Macromolecules Part A; Academic Press, 2002; Vol. 338, pp 3–34. https://doi.org/10.1016/S0076-6879(02)38214-4.

(34) Neal, S.; Nip, A. M.; Zhang, H.; Wishart, D. S. Rapid and Accurate Calculation of Protein 1H, 13C and 15N Chemical Shifts. *J. Biomol. NMR* **2003**, *26* (3), 215–240. https://doi.org/10.1023/A:1023812930288.

(35) Wishart, D. S.; Sykes, B. D. [12] Chemical Shifts as a Tool for Structure Determination. In *Methods in Enzymology*; Nuclear Magnetic Resonance, Part C; Academic Press, 1994; Vol. 239, pp 363–392. https://doi.org/10.1016/S0076-6879(94)39014-2.

(36) Han, B.; Liu, Y.; Ginzinger, S. W.; Wishart, D. S. SHIFTX2: Significantly Improved Protein Chemical Shift Prediction. *J. Biomol. NMR* **2011**, *50* (1), 43–57. https://doi.org/10.1007/s10858-011-9478-4.

(37) Shen, Y.; Bax, A. SPARTA+: A Modest Improvement in Empirical NMR Chemical Shift Prediction by Means of an Artificial Neural Network. *J. Biomol. NMR* **2010**, *48* (1), 13–22. https://doi.org/10.1007/s10858-010-9433-9.

(38) Li, D.-W.; Brüschweiler, R. PPM: A Side-Chain and Backbone Chemical Shift Predictor for the Assessment of Protein Conformational Ensembles. *J. Biomol. NMR* **2012**, *54* (3), 257–265. https://doi.org/10.1007/s10858-012-9668-8.
(39) Li, D.; Brüschweiler, R. PPM_One: A Static Protein Structure Based Chemical Shift Predictor. *J. Biomol. NMR* **2015**, *62* (3), 403–409. https://doi.org/10.1007/s10858-015-9958-z.
(40) Li, J.; C. Bennett, K.; Liu, Y.; V. Martin, M.; Head-Gordon, T. Accurate Prediction of Chemical Shifts for Aqueous Protein Structure on "Real World" Data. *Chem. Sci.* **2020**, *11* (12), 3180–3191. https://doi.org/10.1039/C9SC06561J.
(41) Yang, Z.; Chakraborty, M.; D. White, A. Predicting Chemical Shifts with Graph Neural Networks. *Chem. Sci.* **2021**, *12* (32), 10802–10809. https://doi.org/10.1039/D1SC01895G.
(42) Xu, X.-P.; Case, D. A. Automated Prediction of 15N, 13Cα, 13Cβ and 13C′ Chemical Shifts in Proteins Using a Density Functional Database. *J. Biomol. NMR* **2001**, *21* (4), 321–333. https://doi.org/10.1023/A:1013324104681.
(43) Meiler, J. PROSHIFT: Protein Chemical Shift Prediction Using Artificial Neural Networks. *J. Biomol. NMR* **2003**, *26* (1), 25–37. https://doi.org/10.1023/A:1023060720156.
(44) Tamiola, K.; Acar, B.; Mulder, F. A. A. Sequence-Specific Random Coil Chemical Shifts of Intrinsically Disordered Proteins. *J. Am. Chem. Soc.* **2010**, *132* (51), 18000–18003. https://doi.org/10.1021/ja105656t.
(45) De Simone, A.; Cavalli, A.; Hsu, S.-T. D.; Vranken, W.; Vendruscolo, M. Accurate Random Coil Chemical Shifts from an Analysis of Loop Regions in Native States of Proteins. *J. Am. Chem. Soc.* **2009**, *131* (45), 16332–16333. https://doi.org/10.1021/ja904937a.
(46) Kjaergaard, M.; Poulsen, F. M. Sequence Correction of Random Coil Chemical Shifts: Correlation between Neighbor Correction Factors and Changes in the Ramachandran Distribution. *J. Biomol. NMR* **2011**, *50* (2), 157–165. https://doi.org/10.1007/s10858-011-9508-2.
(47) Schwarzinger, S.; Kroon, G. J. A.; Foss, T. R.; Chung, J.; Wright, P. E.; Dyson, H. J. Sequence-Dependent Correction of Random Coil NMR Chemical Shifts. *J. Am. Chem. Soc.* **2001**, *123* (13), 2970–2978. https://doi.org/10.1021/ja003760i.
(48) Cavalli, A.; Salvatella, X.; Dobson, C. M.; Vendruscolo, M. Protein Structure Determination from NMR Chemical Shifts. *Proc. Natl. Acad. Sci.* **2007**, *104* (23), 9615–9620. https://doi.org/10.1073/pnas.0610313104.
(49) Wishart, D. S.; Arndt, D.; Berjanskii, M.; Tang, P.; Zhou, J.; Lin, G. CS23D: A Web Server for Rapid Protein Structure Generation Using NMR Chemical Shifts and Sequence Data. *Nucleic Acids Res.* **2008**, *36* (suppl_2), W496–W502. https://doi.org/10.1093/nar/gkn305.
(50) Shen, Y.; Lange, O.; Delaglio, F.; Rossi, P.; Aramini, J. M.; Liu, G.; Eletsky, A.; Wu, Y.; Singarapu, K. K.; Lemak, A.; Ignatchenko, A.; Arrowsmith, C. H.; Szyperski, T.; Montelione, G. T.; Baker, D.; Bax, A. Consistent Blind Protein Structure Generation from NMR Chemical Shift Data. *Proc. Natl. Acad. Sci.* **2008**, *105* (12), 4685–4690. https://doi.org/10.1073/pnas.0800256105.
(51) Robustelli, P.; Kohlhoff, K.; Cavalli, A.; Vendruscolo, M. Using NMR Chemical Shifts as Structural Restraints in Molecular Dynamics Simulations of Proteins. *Structure* **2010**, *18* (8), 923–933. https://doi.org/10.1016/j.str.2010.04.016.
(52) Robustelli, P.; Cavalli, A.; Dobson, C. M.; Vendruscolo, M.; Salvatella, X. Folding of Small Proteins by Monte Carlo Simulations with Chemical Shift Restraints without the Use of Molecular Fragment Replacement or Structural Homology. *J. Phys. Chem. B* **2009**, *113* (22), 7890–7896. https://doi.org/10.1021/jp900780b.
(53) Li, D.-W.; Brüschweiler, R. Certification of Molecular Dynamics Trajectories with NMR Chemical Shifts. *J. Phys. Chem. Lett.* **2010**, *1* (1), 246–248. https://doi.org/10.1021/jz9001345.

(54) Markwick, P. R. L.; Cervantes, C. F.; Abel, B. L.; Komives, E. A.; Blackledge, M.; McCammon, J. A. Enhanced Conformational Space Sampling Improves the Prediction of Chemical Shifts in Proteins. *J. Am. Chem. Soc.* **2010**, *132* (4), 1220–1221. https://doi.org/10.1021/ja9093692.
(55) Robustelli, P.; Stafford, K. A.; Palmer, A. G. I. Interpreting Protein Structural Dynamics from NMR Chemical Shifts. *J. Am. Chem. Soc.* **2012**, *134* (14), 6365–6374. https://doi.org/10.1021/ja300265w.
(56) Robustelli, P.; Trbovic, N.; Friesner, R. A.; Palmer, A. G. I. Conformational Dynamics of the Partially Disordered Yeast Transcription Factor GCN4. *J. Chem. Theory Comput.* **2013**, *9* (11), 5190–5200. https://doi.org/10.1021/ct400654r.
(57) Li, D.-W.; Brüschweiler, R. NMR-Based Protein Potentials. *Angew. Chem.* **2010**, *122* (38), 6930–6932. https://doi.org/10.1002/ange.201001898.
(58) Robustelli, P.; Piana, S.; Shaw, D. E. Developing a Molecular Dynamics Force Field for Both Folded and Disordered Protein States. *Proc. Natl. Acad. Sci.* **2018**, *115* (21), E4758–E4766. https://doi.org/10.1073/pnas.1800690115.
(59) Case, D. A. Using Quantum Chemistry to Estimate Chemical Shifts in Biomolecules. *Biophys. Chem.* **2020**, *267*, 106476. https://doi.org/10.1016/j.bpc.2020.106476.
(60) Frank, A.; Möller, H. M.; Exner, T. E. Toward the Quantum Chemical Calculation of NMR Chemical Shifts of Proteins. 2. Level of Theory, Basis Set, and Solvents Model Dependence. *J. Chem. Theory Comput.* **2012**, *8* (4), 1480–1492. https://doi.org/10.1021/ct200913r.
(61) Sumowski, C. V.; Hanni, M.; Schweizer, S.; Ochsenfeld, C. Sensitivity of Ab Initio vs Empirical Methods in Computing Structural Effects on NMR Chemical Shifts for the Example of Peptides. *J. Chem. Theory Comput.* **2014**, *10* (1), 122–133. https://doi.org/10.1021/ct400713t.
(62) Chandy, S. K.; Thapa, B.; Raghavachari, K. Accurate and Cost-Effective NMR Chemical Shift Predictions for Proteins Using a Molecules-in-Molecules Fragmentation-Based Method. *Phys. Chem. Chem. Phys.* **2020**, *22* (47), 27781–27799. https://doi.org/10.1039/D0CP05064D.
(63) Fritz, M.; Quinn, C. M.; Wang, M.; Hou, G.; Lu, X.; Koharudin, L. M. I.; Struppe, J.; Case, D. A.; Polenova, T.; Gronenborn, A. M. Determination of Accurate Backbone Chemical Shift Tensors in Microcrystalline Proteins by Integrating MAS NMR and QM/MM. *Phys. Chem. Chem. Phys.* **2018**, *20* (14), 9543–9553. https://doi.org/10.1039/C8CP00647D.
(64) Mulder, F. A. A.; Filatov, M. NMR Chemical Shift Data and Ab Initio Shielding Calculations: Emerging Tools for Protein Structure Determination. *Chem. Soc. Rev.* **2010**, *39* (2), 578–590. https://doi.org/10.1039/B811366C.
(65) Sahakyan, A. B.; Vranken, W. F.; Cavalli, A.; Vendruscolo, M. Structure-Based Prediction of Methyl Chemical Shifts in Proteins. *J. Biomol. NMR* **2011**, *50* (4), 331–346. https://doi.org/10.1007/s10858-011-9524-2.
(66) Sahakyan, A. B.; Vranken, W. F.; Cavalli, A.; Vendruscolo, M. Using Side-Chain Aromatic Proton Chemical Shifts for a Quantitative Analysis of Protein Structures. *Angew. Chem.* **2011**, *123* (41), 9794–9797. https://doi.org/10.1002/ange.201101641.
(67) Romero, P. R.; Kobayashi, N.; Wedell, J. R.; Baskaran, K.; Iwata, T.; Yokochi, M.; Maziuk, D.; Yao, H.; Fujiwara, T.; Kurusu, G.; Ulrich, E. L.; Hoch, J. C.; Markley, J. L. BioMagResBank (BMRB) as a Resource for Structural Biology. In *Structural Bioinformatics: Methods and Protocols*; Gáspári, Z., Ed.; Methods in Molecular Biology; Springer US: New York, NY, 2020; pp 187–218. https://doi.org/10.1007/978-1-0716-0270-6_14.
(68) Ulrich, E. L.; Akutsu, H.; Doreleijers, J. F.; Harano, Y.; Ioannidis, Y. E.; Lin, J.; Livny, M.; Mading, S.; Maziuk, D.; Miller, Z.; Nakatani, E.; Schulte, C. F.; Tolmie, D. E.; Kent Wenger, R.; Yao, H.; Markley, J. L. BioMagResBank. *Nucleic Acids Res.* **2008**, *36*

(suppl_1), D402–D408. https://doi.org/10.1093/nar/gkm957.
(69) Salmon, L.; Nodet, G.; Ozenne, V.; Yin, G.; Jensen, M. R.; Zweckstetter, M.; Blackledge, M. NMR Characterization of Long-Range Order in Intrinsically Disordered Proteins. *J. Am. Chem. Soc.* **2010**, *132* (24), 8407–8418. https://doi.org/10.1021/ja101645g.
(70) Schwalbe, M.; Ozenne, V.; Bibow, S.; Jaremko, M.; Jaremko, L.; Gajda, M.; Jensen, M. R.; Biernat, J.; Becker, S.; Mandelkow, E.; Zweckstetter, M.; Blackledge, M. Predictive Atomic Resolution Descriptions of Intrinsically Disordered HTau40 and α-Synuclein in Solution from NMR and Small Angle Scattering. *Structure* **2014**, *22* (2), 238–249. https://doi.org/10.1016/j.str.2013.10.020.
(71) Wood, G. P. F.; Rothlisberger, U. Secondary Structure Assignment of Amyloid-β Peptide Using Chemical Shifts. *J. Chem. Theory Comput.* **2011**, *7* (5), 1552–1563. https://doi.org/10.1021/ct200156e.
(72) Shrestha, U. R.; Smith, J. C.; Petridis, L. Full Structural Ensembles of Intrinsically Disordered Proteins from Unbiased Molecular Dynamics Simulations. *Commun. Biol.* **2021**, *4* (1), 1–8. https://doi.org/10.1038/s42003-021-01759-1.
(73) Virtanen, S. I.; Kiirikki, A. M.; Mikula, K. M.; Iwaï, H.; Ollila, O. H. S. Heterogeneous Dynamics in Partially Disordered Proteins. *Phys. Chem. Chem. Phys.* **2020**, *22* (37), 21185–21196. https://doi.org/10.1039/D0CP03473H.
(74) Shalongo, W.; Dugad, L.; Stellwagen, E. Distribution of Helicity within the Model Peptide Acetyl(AAQAA)3amide. *J. Am. Chem. Soc.* **1994**, *116* (18), 8288–8293. https://doi.org/10.1021/ja00097a039.
(75) Best, R. B.; Hummer, G. Optimized Molecular Dynamics Force Fields Applied to the Helix−Coil Transition of Polypeptides. *J. Phys. Chem. B* **2009**, *113* (26), 9004–9015. https://doi.org/10.1021/jp901540t.
(76) Lifson, S.; Roig, A. On the Theory of Helix—Coil Transition in Polypeptides. *J. Chem. Phys.* **1961**, *34* (6), 1963–1974. https://doi.org/10.1063/1.1731802.
(77) Donald, J. E.; Kulp, D. W.; DeGrado, W. F. Salt Bridges: Geometrically Specific, Designable Interactions. *Proteins Struct. Funct. Bioinforma.* **2011**, *79* (3), 898–915. https://doi.org/10.1002/prot.22927.
(78) Karplus, M. Contact Electron-Spin Coupling of Nuclear Magnetic Moments. *J. Chem. Phys.* **1959**, *30* (1), 11–15. https://doi.org/10.1063/1.1729860.
(79) Karplus, Martin. Vicinal Proton Coupling in Nuclear Magnetic Resonance. *J. Am. Chem. Soc.* **1963**, *85* (18), 2870–2871. https://doi.org/10.1021/ja00901a059.
(80) Bystrov, V. F. Spin—Spin Coupling and the Conformational States of Peptide Systems. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10* (2), 41–82. https://doi.org/10.1016/0079-6565(76)80001-5.
(81) Haasnoot, C. A. G.; de Leeuw, F. A. A. M.; Altona, C. The Relationship between Proton-Proton NMR Coupling Constants and Substituent Electronegativities—I: An Empirical Generalization of the Karplus Equation. *Tetrahedron* **1980**, *36* (19), 2783–2792. https://doi.org/10.1016/0040-4020(80)80155-4.
(82) Wang, A. C.; Bax, A. Determination of the Backbone Dihedral Angles φ in Human Ubiquitin from Reparametrized Empirical Karplus Equations. *J. Am. Chem. Soc.* **1996**, *118* (10), 2483–2494. https://doi.org/10.1021/ja9535524.
(83) Vögeli, B.; Ying, J.; Grishaev, A.; Bax, A. Limits on Variations in Protein Backbone Dynamics from Precise Measurements of Scalar Couplings. *J. Am. Chem. Soc.* **2007**, *129* (30), 9377–9385. https://doi.org/10.1021/ja070324o.
(84) Pardi, A.; Billeter, M.; Wüthrich, K. Calibration of the Angular Dependence of the Amide Proton-Cα Proton Coupling Constants, 3JHNα, in a Globular Protein: Use of 3JHNα for Identification of Helical Secondary Structure. *J. Mol. Biol.* **1984**, *180* (3), 741–751. https://doi.org/10.1016/0022-2836(84)90035-4.
(85) Altona, C. Vicinal Coupling Constants and Conformation of Biomolecules. In *eMagRes*;

John Wiley & Sons, Ltd, 2007. https://doi.org/10.1002/9780470034590.emrstm0587.
(86) Case, D. A.; Scheurer, C.; Brüschweiler, R. Static and Dynamic Effects on Vicinal Scalar J Couplings in Proteins and Peptides: A MD/DFT Analysis. *J. Am. Chem. Soc.* **2000**, *122* (42), 10390–10397. https://doi.org/10.1021/ja001798p.
(87) Lindorff-Larsen, K.; Best, R. B.; Vendruscolo, M. Interpreting Dynamically-Averaged Scalar Couplings in Proteins. *J. Biomol. NMR* **2005**, *32* (4), 273–280. https://doi.org/10.1007/s10858-005-8873-0.
(88) Wang, B.; He, X.; Merz, K. M. Quantum Mechanical Study of Vicinal J Spin–Spin Coupling Constants for the Protein Backbone. *J. Chem. Theory Comput.* **2013**, *9* (10), 4653–4659. https://doi.org/10.1021/ct400631b.
(89) Li, F.; Lee, J. H.; Grishaev, A.; Ying, J.; Bax, A. High Accuracy of Karplus Equations for Relating Three-Bond J Couplings to Protein Backbone Torsion Angles. *ChemPhysChem* **2015**, *16* (3), 572–578. https://doi.org/10.1002/cphc.201402704.
(90) Lee, J. H.; Li, F.; Grishaev, A.; Bax, A. Quantitative Residue-Specific Protein Backbone Torsion Angle Dynamics from Concerted Measurement of 3J Couplings. *J. Am. Chem. Soc.* **2015**, *137* (4), 1432–1435. https://doi.org/10.1021/ja512593s.
(91) Brueschweiler, R.; Case, D. A. Adding Harmonic Motion to the Karplus Relation for Spin-Spin Coupling. *J. Am. Chem. Soc.* **1994**, *116* (24), 11199–11200. https://doi.org/10.1021/ja00103a062.
(92) Schmidt, J. M.; Blümel, M.; Löhr, F.; Rüterjans, H. Self-Consistent 3J Coupling Analysis for the Joint Calibration of Karplus Coefficients and Evaluation of Torsion Angles. *J. Biomol. NMR* **1999**, *14* (1), 1–12. https://doi.org/10.1023/A:1008345303942.
(93) Vuister, G. W.; Delaglio, F.; Bax, A. The Use of 1JCαHα Coupling Constants as a Probe for Protein Backbone Conformation. *J. Biomol. NMR* **1993**, *3* (1), 67–80. https://doi.org/10.1007/BF00242476.
(94) Cornilescu, G.; Bax, A.; Case, D. A. Large Variations in One-Bond 13Cα−13Cβ J Couplings in Polypeptides Correlate with Backbone Conformation. *J. Am. Chem. Soc.* **2000**, *122* (10), 2168–2171. https://doi.org/10.1021/ja993609c.
(95) Gapsys, V.; Narayanan, R. L.; Xiang, S.; de Groot, B. L.; Zweckstetter, M. Improved Validation of IDP Ensembles by One-Bond Cα–Hα Scalar Couplings. *J. Biomol. NMR* **2015**, *63* (3), 299–307. https://doi.org/10.1007/s10858-015-9990-z.
(96) Pérez, C.; Löhr, F.; Rüterjans, H.; Schmidt, J. M. Self-Consistent Karplus Parametrization of 3J Couplings Depending on the Polypeptide Side-Chain Torsion X1. *J. Am. Chem. Soc.* **2001**, *123* (29), 7081–7093. https://doi.org/10.1021/ja003724j.
(97) Chou, J. J.; Case, D. A.; Bax, A. Insights into the Mobility of Methyl-Bearing Side Chains in Proteins from 3JCC and 3JCN Couplings. *J. Am. Chem. Soc.* **2003**, *125* (29), 8959–8966. https://doi.org/10.1021/ja029972s.
(98) Barfield, M. Structural Dependencies of Interresidue Scalar Coupling H3JNC‘ and Donor 1H Chemical Shifts in the Hydrogen Bonding Regions of Proteins. *J. Am. Chem. Soc.* **2002**, *124* (15), 4158–4168. https://doi.org/10.1021/ja012674v.
(99) Graf, J.; Nguyen, P. H.; Stock, G.; Schwalbe, H. Structure and Dynamics of the Homologous Series of Alanine Peptides: A Joint Molecular Dynamics/NMR Study. *J. Am. Chem. Soc.* **2007**, *129* (5), 1179–1189. https://doi.org/10.1021/ja0660406.
(100) Best, R. B.; Buchete, N.-V.; Hummer, G. Are Current Molecular Dynamics Force Fields Too Helical? *Biophys. J.* **2008**, *95* (1), L07–L09. https://doi.org/10.1529/biophysj.108.132696.
(101) Beauchamp, K. A.; Lin, Y.-S.; Das, R.; Pande, V. S. Are Protein Force Fields Getting Better? A Systematic Benchmark on 524 Diverse NMR Measurements. *J. Chem. Theory Comput.* **2012**, *8* (4), 1409–1414. https://doi.org/10.1021/ct2007814.
(102) Piana, S.; Lindorff-Larsen, K.; Shaw, D. E. How Robust Are Protein Folding Simulations with Respect to Force Field Parameterization? *Biophys. J.* **2011**, *100* (9), L47–L49.

https://doi.org/10.1016/j.bpj.2011.03.051.
(103) Best, R. B.; Zheng, W.; Mittal, J. Balanced Protein–Water Interactions Improve Properties of Disordered Proteins and Non-Specific Protein Association. *J. Chem. Theory Comput.* **2014**, *10* (11), 5113–5124. https://doi.org/10.1021/ct500569b.
(104) Sanders, C. R.; Prestegard, J. H. Magnetically Orientable Phospholipid Bilayers Containing Small Amounts of a Bile Salt Analogue, CHAPSO. *Biophys. J.* **1990**, *58* (2), 447–460.
(105) Prosser, R. S.; Hwang, J. S.; Vold, R. R. Magnetically Aligned Phospholipid Bilayers with Positive Ordering: A New Model Membrane System. *Biophys. J.* **1998**, *74* (5), 2405–2418.
(106) Tycko, R.; Blanco, F. J.; Ishii, Y. Alignment of Biopolymers in Strained Gels: A New Way To Create Detectable Dipole−Dipole Couplings in High-Resolution Biomolecular NMR. *J. Am. Chem. Soc.* **2000**, *122* (38), 9340–9341. https://doi.org/10.1021/ja002133q.
(107) Zweckstetter, M. NMR: Prediction of Molecular Alignment from Structure Using the PALES Software. *Nat. Protoc.* **2008**, *3* (4), 679–690. https://doi.org/10.1038/nprot.2008.36.
(108) Smith, L. J.; Sutcliffe, M. J.; Redfield, C.; Dobson, C. M. Structure of Hen Lysozyme in Solution. *J. Mol. Biol.* **1993**, *229* (4), 930–944. https://doi.org/10.1006/jmbi.1993.1097.
(109) Peter, C.; Daura, X.; van Gunsteren, W. F. Calculation of NMR-Relaxation Parameters for Flexible Molecules from Molecular Dynamics Simulations. *J. Biomol. NMR* **2001**, *20* (4), 297–310. https://doi.org/10.1023/A:1011241030461.
(110) Wüthrich, K.; Billeter, M.; Braun, W. Pseudo-Structures for the 20 Common Amino Acids for Use in Studies of Protein Conformations by Measurements of Intramolecular Proton-Proton Distance Constraints with Nuclear Magnetic Resonance. *J. Mol. Biol.* **1983**, *169* (4), 949–961. https://doi.org/10.1016/S0022-2836(83)80144-2.
(111) Fletcher, C. M.; Jones, D. N. M.; Diamond, R.; Neuhaus, D. Treatment of NOE Constraints Involving Equivalent or Nonstereoassigned Protons in Calculations of Biomacromolecular Structures. *J. Biomol. NMR* **1996**, *8* (3), 292–310. https://doi.org/10.1007/BF00410328.
(112) Hoffmann, F.; Mulder, F. A. A.; Schäfer, L. V. Accurate Methyl Group Dynamics in Protein Simulations with AMBER Force Fields. *J. Phys. Chem. B* **2018**, *122* (19), 5038–5048. https://doi.org/10.1021/acs.jpcb.8b02769.
(113) Best, R. B.; Vendruscolo, M. Determination of Protein Structures Consistent with NMR Order Parameters. *J. Am. Chem. Soc.* **2004**, *126* (26), 8090–8091. https://doi.org/10.1021/ja0396955.
(114) Showalter, S. A.; Brüschweiler, R. Validation of Molecular Dynamics Simulations of Biomolecules Using NMR Spin Relaxation as Benchmarks: Application to the AMBER99SB Force Field. *J. Chem. Theory Comput.* **2007**, *3* (3), 961–975. https://doi.org/10.1021/ct7000045.
(115) Showalter, S. A.; Johnson, E.; Rance, M.; Brüschweiler, R. Toward Quantitative Interpretation of Methyl Side-Chain Dynamics from NMR by Molecular Dynamics Simulations. *J. Am. Chem. Soc.* **2007**, *129* (46), 14146–14147. https://doi.org/10.1021/ja075976r.
(116) Maragakis, P.; Lindorff-Larsen, K.; Eastwood, M. P.; Dror, R. O.; Klepeis, J. L.; Arkin, I. T.; Jensen, M. Ø.; Xu, H.; Trbovic, N.; Friesner, R. A.; Palmer, A. G.; Shaw, D. E. Microsecond Molecular Dynamics Simulation Shows Effect of Slow Loop Dynamics on Backbone Amide Order Parameters of Proteins. *J. Phys. Chem. B* **2008**, *112* (19), 6155–6158. https://doi.org/10.1021/jp077018h.
(117) Trbovic, N.; Kim, B.; Friesner, R. A.; Palmer III, A. G. Structural analysis of protein dynamics by MD simulations and NMR spin-relaxation. *Proteins Struct. Funct. Bioinforma.* **2008**, *71* (2), 684–694. https://doi.org/10.1002/prot.21750.

(118) Lipari, G.; Szabo, A. Model-Free Approach to the Interpretation of Nuclear Magnetic Resonance Relaxation in Macromolecules. 1. Theory and Range of Validity. *J. Am. Chem. Soc.* **1982**, *104* (17), 4546–4559. https://doi.org/10.1021/ja00381a009.
(119) Chen, P.; Hologne, M.; Walker, O.; Hennig, J. Ab Initio Prediction of NMR Spin Relaxation Parameters from Molecular Dynamics Simulations. *J. Chem. Theory Comput.* **2018**, *14* (2), 1009–1019. https://doi.org/10.1021/acs.jctc.7b00750.
(120) Ollila, O. H. S.; Heikkinen, H. A.; Iwaï, H. Rotational Dynamics of Proteins from Spin Relaxation Times and Molecular Dynamics Simulations. *J. Phys. Chem. B* **2018**, *122* (25), 6559–6569. https://doi.org/10.1021/acs.jpcb.8b02250.
(121) Hoffmann, F.; Mulder, F. A. A.; Schäfer, L. V. Predicting NMR Relaxation of Proteins from Molecular Dynamics Simulations with Accurate Methyl Rotation Barriers. *J. Chem. Phys.* **2020**, *152* (8), 084102. https://doi.org/10.1063/1.5135379.
(122) Wong, V.; Case, D. A. Evaluating Rotational Diffusion from Protein MD Simulations. *J. Phys. Chem. B* **2008**, *112* (19), 6013–6024. https://doi.org/10.1021/jp0761564.
(123) Lipari, G.; Szabo, A.; Levy, R. M. Protein Dynamics and NMR Relaxation: Comparison of Simulations with Experiment. *Nature* **1982**, *300* (5888), 197–198. https://doi.org/10.1038/300197a0.
(124) Fenwick, R. B.; Dyson, H. J. Classic Analysis of Biopolymer Dynamics Is Model Free. *Biophys. J.* **2016**, *110* (1), 3–6. https://doi.org/10.1016/j.bpj.2015.11.022.
(125) Prompers, J. J.; Brüschweiler, R. General Framework for Studying the Dynamics of Folded and Nonfolded Proteins by NMR Relaxation Spectroscopy and MD Simulation. *J. Am. Chem. Soc.* **2002**, *124* (16), 4522–4534. https://doi.org/10.1021/ja012750u.
(126) Anderson, J. S.; LeMaster, D. M. Rotational Velocity Rescaling of Molecular Dynamics Trajectories for Direct Prediction of Protein NMR Relaxation. *Biophys. Chem.* **2012**, *168–169*, 28–39. https://doi.org/10.1016/j.bpc.2012.05.005.
(127) Bowman, G. R. Accurately Modeling Nanosecond Protein Dynamics Requires at Least Microseconds of Simulation. *J. Comput. Chem.* **2016**, *37* (6), 558–566. https://doi.org/10.1002/jcc.23973.
(128) Clore, G. M.; Tang, C.; Iwahara, J. Elucidating Transient Macromolecular Interactions Using Paramagnetic Relaxation Enhancement. *Curr. Opin. Struct. Biol.* **2007**, *17* (5), 603–616. https://doi.org/10.1016/j.sbi.2007.08.013.
(129) Polyhach, Y.; Bordignon, E.; Jeschke, G. Rotamer Libraries of Spin Labelled Cysteines for Protein Studies. *Phys. Chem. Chem. Phys.* **2011**, *13* (6), 2356–2366. https://doi.org/10.1039/C0CP01865A.
(130) Michaud-Agrawal, N.; Denning, E. J.; Woolf, T. B.; Beckstein, O. MDAnalysis: A Toolkit for the Analysis of Molecular Dynamics Simulations. *J. Comput. Chem.* **2011**, *32* (10), 2319–2327. https://doi.org/10.1002/jcc.21787.
(131) Gowers, R. J.; Linke, M.; Barnoud, J.; Reddy, T. J. E.; Melo, M. N.; Seyler, S. L.; Domański, J.; Dotson, D. L.; Buchoux, S.; Kenney, I. M.; Beckstein, O. MDAnalysis: A Python Package for the Rapid Analysis of Molecular Dynamics Simulations. *Proc. 15th Python Sci. Conf.* **2016**, 98–105. https://doi.org/10.25080/Majora-629e541a-00e.
(132) Tesei, G.; Martins, J. M.; Kunze, M. B. A.; Wang, Y.; Crehuet, R.; Lindorff-Larsen, K. DEER-PREdict: Software for Efficient Calculation of Spin-Labeling EPR and NMR Data from Conformational Ensembles. *PLOS Comput. Biol.* **2021**, *17* (1), e1008551. https://doi.org/10.1371/journal.pcbi.1008551.
(133) Solomon, I.; Bloembergen, N. Nuclear Magnetic Interactions in the HF Molecule. *J. Chem. Phys.* **1956**, *25* (2), 261–266. https://doi.org/10.1063/1.1742867.
(134) Piana, S.; Donchev, A. G.; Robustelli, P.; Shaw, D. E. Water Dispersion Interactions Strongly Influence Simulated Structural Properties of Disordered Protein States. *J. Phys. Chem. B* **2015**, *119* (16), 5113–5123. https://doi.org/10.1021/jp508971m.
(135) Ramírez-Alvarado, M.; Blanco, F. J.; Serrano, L. Elongation of the BH8 β-Hairpin Peptide:

Electrostatic Interactions in β-Hairpin Formation and Stability. *Protein Sci.* **2001**, *10* (7), 1381–1392. https://doi.org/10.1110/ps.52901.
(136) Blanco, F. J.; Rivas, G.; Serrano, L. A Short Linear Peptide That Folds into a Native Stable β-Hairpin in Aqueous Solution. *Nat. Struct. Biol.* **1994**, *1* (9), 584–590. https://doi.org/10.1038/nsb0994-584.
(137) Ramírez-Alvarado, M.; Blanco, F. J.; Serrano, L. De Novo Design and Structural Analysis of a Model β-Hairpin Peptide System. *Nat. Struct. Biol.* **1996**, *3* (7), 604–612. https://doi.org/10.1038/nsb0796-604.
(138) de Alba, E.; Jiménez, M. A.; Rico, M. Turn Residue Sequence Determines β-Hairpin Conformation in Designed Peptides. *J. Am. Chem. Soc.* **1997**, *119* (1), 175–183. https://doi.org/10.1021/ja962325e.
(139) Cochran, A. G.; Skelton, N. J.; Starovasnik, M. A. Tryptophan Zippers: Stable, Monomeric β-Hairpins. *Proc. Natl. Acad. Sci.* **2001**, *98* (10), 5578–5583. https://doi.org/10.1073/pnas.091100898.
(140) Maynard, A. J.; Sharman, G. J.; Searle, M. S. Origin of β-Hairpin Stability in Solution: Structural and Thermodynamic Analysis of the Folding of a Model Peptide Supports Hydrophobic Stabilization in Water. *J. Am. Chem. Soc.* **1998**, *120* (9), 1996–2007. https://doi.org/10.1021/ja9726769.
(141) Stanger, H. E.; Gellman, S. H. Rules for Antiparallel β-Sheet Design: D-Pro-Gly Is Superior to l-Asn-Gly for β-Hairpin Nucleation1. *J. Am. Chem. Soc.* **1998**, *120* (17), 4236–4237. https://doi.org/10.1021/ja973704q.
(142) Espinosa, J. F.; Syud, F. A.; Gellman, S. H. An Autonomously Folding β-Hairpin Derived from the Human YAP65 WW Domain: Attempts to Define a Minimum Ligand-Binding Motif. *Pept. Sci.* **2005**, *80* (2–3), 303–311. https://doi.org/10.1002/bip.20205.
(143) Andersen, N. H.; Olsen, K. A.; Fesinmeyer, R. M.; Tan, X.; Hudson, F. M.; Eidenschink, L. A.; Farazi, S. R. Minimization and Optimization of Designed β-Hairpin Folds. *J. Am. Chem. Soc.* **2006**, *128* (18), 6101–6110. https://doi.org/10.1021/ja054971w.
(144) Fesinmeyer, R. M.; Hudson, F. M.; Andersen, N. H. Enhanced Hairpin Stability through Loop Design: The Case of the Protein G B1 Domain Hairpin. *J. Am. Chem. Soc.* **2004**, *126* (23), 7238–7243. https://doi.org/10.1021/ja0379520.
(145) Honda, S.; Akiba, T.; Kato, Y. S.; Sawada, Y.; Sekijima, M.; Ishimura, M.; Ooishi, A.; Watanabe, H.; Odahara, T.; Harata, K. Crystal Structure of a Ten-Amino Acid Protein. *J. Am. Chem. Soc.* **2008**, *130* (46), 15327–15331. https://doi.org/10.1021/ja8030533.
(146) Pastor, M. T.; López de la Paz, M.; Lacroix, E.; Serrano, L.; Pérez-Payá, E. Combinatorial Approaches: A New Tool to Search for Highly Structured β-Hairpin Peptides. *Proc. Natl. Acad. Sci.* **2002**, *99* (2), 614–619. https://doi.org/10.1073/pnas.012583999.
(147) Peintner, S.; Erdélyi, M. Pushing the Limits of Characterising a Weak Halogen Bond in Solution. *Chem. – Eur. J.* **2022**, *28* (5), e202103559. https://doi.org/10.1002/chem.202103559.
(148) Russell, S. J.; Blandl, T.; Skelton, N. J.; Cochran, A. G. Stability of Cyclic β-Hairpins: Asymmetric Contributions from Side Chains of a Hydrogen-Bonded Cross-Strand Residue Pair. *J. Am. Chem. Soc.* **2003**, *125* (2), 388–395. https://doi.org/10.1021/ja028075l.
(149) Skelton, N. J.; Blandl, T.; Russell, S. J.; Starovasnik, M. A.; Cochran, A. G. *β*–hairpin Polypeptides by Design and Selection. *J. Spectrosc.* **2003**, *17*, 213–230. https://doi.org/10.1155/2003/148024.
(150) Scian, M.; Shu, I.; Olsen, K. A.; Hassam, K.; Andersen, N. H. Mutational Effects on the Folding Dynamics of a Minimized Hairpin. *Biochemistry* **2013**, *52* (15), 2556–2564. https://doi.org/10.1021/bi400146c.
(151) Anderson, J. M.; Jurban, B.; Huggins, K. N. L.; Shcherbakov, A. A.; Shu, I.; Kier, B.; Andersen, N. H. Nascent Hairpins in Proteins: Identifying Turn Loci and Quantitating Turn

Contributions to Hairpin Stability. *Biochemistry* **2016**, *55* (39), 5537–5553. https://doi.org/10.1021/acs.biochem.6b00732.
(152) Jiménez, M. A. Design of Monomeric Water-Soluble β-Hairpin and β-Sheet Peptides. In *Protein Design: Methods and Applications*; Köhler, V., Ed.; Methods in Molecular Biology; Springer: New York, NY, 2014; pp 15–52. https://doi.org/10.1007/978-1-4939-1486-9_2.
(153) Kier, B. L.; Shu, I.; Eidenschink, L. A.; Andersen, N. H. Stabilizing Capping Motif for β-Hairpins and Sheets. *Proc. Natl. Acad. Sci.* **2010**, *107* (23), 10466–10471. https://doi.org/10.1073/pnas.0913534107.
(154) Morales, P.; Jiménez, M. A. Design and Structural Characterisation of Monomeric Water-Soluble α-Helix and β-Hairpin Peptides: State-of-the-Art. *Arch. Biochem. Biophys.* **2019**, *661*, 149–167. https://doi.org/10.1016/j.abb.2018.11.014.
(155) Richaud, A. D.; Zhao, G.; Hobloss, S.; Roche, S. P. Folding in Place: Design of β-Strap Motifs to Stabilize the Folding of Hairpins with Long Loops. *J. Org. Chem.* **2021**, *86* (19), 13535–13547. https://doi.org/10.1021/acs.joc.1c01442.
(156) Imperiali, B.; Ottesen, J. j. Uniquely Folded Mini-Protein Motifs. *J. Pept. Res.* **1999**, *54* (3), 177–184. https://doi.org/10.1034/j.1399-3011.1999.00121.x.
(157) Struthers, M. D.; Cheng, R. P.; Imperiali, B. Design of a Monomeric 23-Residue Polypeptide with Defined Tertiary Structure. *Science* **1996**, *271* (5247), 342–345. https://doi.org/10.1126/science.271.5247.342.
(158) Struthers, M.; Ottesen, J. J.; Imperiali, B. Design and NMR Analyses of Compact, Independently Folded BBA Motifs. *Fold. Des.* **1998**, *3* (2), 95–103. https://doi.org/10.1016/S1359-0278(98)00015-7.
(159) McKnight, C. J.; Matsudaira, P. T.; Kim, P. S. NMR Structure of the 35-Residue Villin Headpiece Subdomain. *Nat. Struct. Biol.* **1997**, *4* (3), 180–184. https://doi.org/10.1038/nsb0397-180.
(160) Vermeulen, W.; Vanhaesebrouck, P.; Van Troys, M.; Verschueren, M.; Fant, F.; Goethals, M.; Ampe, C.; Martins, J. C.; Borremans, F. A. M. Solution Structures of the C-Terminal Headpiece Subdomains of Human Villin and Advillin, Evaluation of Headpiece F-Actin-Binding Requirements. *Protein Sci.* **2004**, *13* (5), 1276–1287. https://doi.org/10.1110/ps.03518104.
(161) Cornilescu, G.; Hadley, E. B.; Woll, M. G.; Markley, J. L.; Gellman, S. H.; Cornilescu, C. C. Solution Structure of a Small Protein Containing a Fluorinated Side Chain in the Core. *Protein Sci.* **2007**, *16* (1), 14–19. https://doi.org/10.1110/ps.062557707.
(162) Gronwald, W.; Hohm, T.; Hoffmann, D. Evolutionary Pareto-Optimization of Stably Folding Peptides. *BMC Bioinformatics* **2008**, *9* (1), 109. https://doi.org/10.1186/1471-2105-9-109.
(163) Neidigh, J. W.; Fesinmeyer, R. M.; Andersen, N. H. Designing a 20-Residue Protein. *Nat. Struct. Biol.* **2002**, *9* (6), 425–430. https://doi.org/10.1038/nsb798.
(164) Lin, J. C.; Barua, B.; Andersen, N. H. The Helical Alanine Controversy: An (Ala)6 Insertion Dramatically Increases Helicity. *J. Am. Chem. Soc.* **2004**, *126* (42), 13679–13684. https://doi.org/10.1021/ja047265o.
(165) Barua, B.; Lin, J. C.; Williams, V. D.; Kummler, P.; Neidigh, J. W.; Andersen, N. H. The Trp-Cage: Optimizing the Stability of a Globular Miniprotein. *Protein Eng. Des. Sel.* **2008**, *21* (3), 171–185. https://doi.org/10.1093/protein/gzm082.
(166) Scian, M.; Lin, J. C.; Le Trong, I.; Makhatadze, G. I.; Stenkamp, R. E.; Andersen, N. H. Crystal and NMR Structures of a Trp-Cage Mini-Protein Benchmark for Computational Fold Prediction. *Proc. Natl. Acad. Sci.* **2012**, *109* (31), 12521–12525. https://doi.org/10.1073/pnas.1121421109.
(167) Byrne, A.; Kier, B. L.; Williams, D. V.; Scian, M.; Andersen, N. H. Circular Permutation of the Trp-Cage: Fold Rescue upon Addition of a Hydrophobic Staple. *RSC Adv.* **2013**, *3* (43), 19824–19829. https://doi.org/10.1039/C3RA43674H.
(168) Graham, K. A.; Byrne, A.; Son, R.; Andersen, N. H. Reversing the Typical PH Stability

Profile of the Trp-Cage. *Biopolymers* **2019**, *110* (3), e23260. https://doi.org/10.1002/bip.23260.
(169) Byrne, A.; Williams, D. V.; Barua, B.; Hagen, S. J.; Kier, B. L.; Andersen, N. H. Folding Dynamics and Pathways of the Trp-Cage Miniproteins. *Biochemistry* **2014**, *53* (38), 6011–6021. https://doi.org/10.1021/bi501021r.
(170) Mao, B.; Tejero, R.; Baker, D.; Montelione, G. T. Protein NMR Structures Refined with Rosetta Have Higher Accuracy Relative to Corresponding X-Ray Crystal Structures. *J. Am. Chem. Soc.* **2014**, *136* (5), 1893–1906. https://doi.org/10.1021/ja409845w.
(171) Piana, S.; Robustelli, P.; Tan, D.; Chen, S.; Shaw, D. E. Development of a Force Field for the Simulation of Single-Chain Proteins and Protein–Protein Complexes. *J. Chem. Theory Comput.* **2020**, *16* (4), 2494–2507. https://doi.org/10.1021/acs.jctc.9b00251.
(172) Jarymowycz, V. A.; Stone, M. J. Fast Time Scale Dynamics of Protein Backbones: NMR Relaxation Methods, Applications, and Functional Consequences. *Chem. Rev.* **2006**, *106* (5), 1624–1671. https://doi.org/10.1021/cr040421p.
(173) Ciragan, A.; Backlund, S. M.; Mikula, K. M.; Beyer, H. M.; Samuli Ollila, O. H.; Iwaï, H. NMR Structure and Dynamics of TonB Investigated by Scar-Less Segmental Isotopic Labeling Using a Salt-Inducible Split Intein. *Front. Chem.* **2020**, *8*.
(174) Khan, S. N.; Charlier, C.; Augustyniak, R.; Salvi, N.; Déjean, V.; Bodenhausen, G.; Lequin, O.; Pelupessy, P.; Ferrage, F. Distribution of Pico- and Nanosecond Motions in Disordered Proteins from Nuclear Spin Relaxation. *Biophys. J.* **2015**, *109* (5), 988–999. https://doi.org/10.1016/j.bpj.2015.06.069.
(175) Ahmed, M. C.; Papaleo, E.; Lindorff-Larsen, K. How Well Do Force Fields Capture the Strength of Salt Bridges in Proteins? **2018**. https://doi.org/10.1101/272799.
(176) Debiec, K. T.; Gronenborn, A. M.; Chong, L. T. Evaluating the Strength of Salt Bridges: A Comparison of Current Biomolecular Force Fields. *J. Phys. Chem. B* **2014**, *118* (24), 6561–6569. https://doi.org/10.1021/jp500958r.
(177) Debiec, K. T.; Cerutti, D. S.; Baker, L. R.; Gronenborn, A. M.; Case, D. A.; Chong, L. T. Further along the Road Less Traveled: AMBER Ff15ipq, an Original Protein Force Field Built on a Self-Consistent Physical Model. *J. Chem. Theory Comput.* **2016**, *12* (8), 3926–3947. https://doi.org/10.1021/acs.jctc.6b00567.
(178) Lopes, P. E. M.; Huang, J.; Shim, J.; Luo, Y.; Li, H.; Roux, B.; MacKerell, A. D. Jr. Polarizable Force Field for Peptides and Proteins Based on the Classical Drude Oscillator. *J. Chem. Theory Comput.* **2013**, *9* (12), 5430–5449. https://doi.org/10.1021/ct400781b.
(179) Shi, Y.; Xia, Z.; Zhang, J.; Best, R.; Wu, C.; Ponder, J. W.; Ren, P. Polarizable Atomic Multipole-Based AMOEBA Force Field for Proteins. *J. Chem. Theory Comput.* **2013**, *9* (9), 4046–4063. https://doi.org/10.1021/ct4003702.
(180) Muirhead, H.; Perutz, M. F. Structure Of Hæemoglobin: A Three-Dimensional Fourier Synthesis of Reduced Human Haemoglobin at 5.5 Å Resolution. *Nature* **1963**, *199* (4894), 633–638. https://doi.org/10.1038/199633a0.
(181) Fischer, M. Macromolecular Room Temperature Crystallography. *Q. Rev. Biophys.* **2021**, *54*, e1. https://doi.org/10.1017/S0033583520000128.
(182) Thorne, R. E. Determining Biomolecular Structures near Room Temperature Using X-Ray Crystallography: Concepts, Methods and Future Optimization. *Acta Crystallogr. Sect. Struct. Biol.* **2023**, *79* (1), 78–94. https://doi.org/10.1107/S2059798322011652.
(183) Fraser, J. S.; Clarkson, M. W.; Degnan, S. C.; Erion, R.; Kern, D.; Alber, T. Hidden Alternative Structures of Proline Isomerase Essential for Catalysis. *Nature* **2009**, *462* (7273), 669–673. https://doi.org/10.1038/nature08615.
(184) Keedy, D. A.; van den Bedem, H.; Sivak, D. A.; Petsko, G. A.; Ringe, D.; Wilson, M. A.; Fraser, J. S. Crystal Cryocooling Distorts Conformational Heterogeneity in a Model Michaelis Complex of DHFR. *Structure* **2014**, *22* (6), 899–910.

https://doi.org/10.1016/j.str.2014.04.016.
(185) Altan, I.; Fusco, D.; Afonine, P. V.; Charbonneau, P. Learning about Biomolecular Solvation from Water in Protein Crystals. *J. Phys. Chem. B* **2018**, *122* (9), 2475–2486. https://doi.org/10.1021/acs.jpcb.7b09898.
(186) Wych, D. C.; Aoto, P. C.; Vu, L.; Wolff, A. M.; Mobley, D. L.; Fraser, J. S.; Taylor, S. S.; Wall, M. E. Molecular-Dynamics Simulation Methods for Macromolecular Crystallography. *Acta Crystallogr. Sect. Struct. Biol.* **2023**, *79* (1), 50–65. https://doi.org/10.1107/S2059798322011871.
(187) Cerutti, D. S.; Case, D. A. Molecular Dynamics Simulations of Macromolecular Crystals. *WIREs Comput. Mol. Sci.* **2019**, *9* (4), e1402. https://doi.org/10.1002/wcms.1402.
(188) Meisburger, S. P.; Thomas, W. C.; Watkins, M. B.; Ando, N. X-Ray Scattering Studies of Protein Structural Dynamics. *Chem. Rev.* **2017**, *117* (12), 7615–7672. https://doi.org/10.1021/acs.chemrev.6b00790.
(189) Bernstein, F. C.; Koetzle, T. F.; Williams, T. F.; G. J. B. Meyer, J.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. The Protein Data Bank: A Computer-Based Archival File for Macromolecular Structures. *J Mol Biol* **1977**, *112*, 535–542.
(190) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucl Acids Res* **2000**, *28*, 235–242.
(191) Rose, P. W.; Prlić, A.; Bi, C.; Bluhm, W. F.; Christie, C. H.; Dutta, S.; Green, R. K.; Goodsell, D. S.; Westbrook, J. D.; Woo, J.; Young, J.; Zardecki, C.; Berman, H. M.; Bourne, P. E.; Burley, S. K. The RCSB Protein Data Bank: Views of Structural Biology for Basic and Applied Research and Education. *Nucleic Acids Res.* **2015**, *43* (Database issue), D345–D356. https://doi.org/10.1093/nar/gku1214.
(192) Dunbrack, R. L.; Karplus, M. Backbone-Dependent Rotamer Library for Proteins Application to Side-Chain Prediction. *J. Mol. Biol.* **1993**, *230* (2), 543–574. https://doi.org/10.1006/jmbi.1993.1170.
(193) Case, D. A.; Aktulga, H. M.; Belfrom, K.; Ben-Shalom, I. Y.; Berryman, J. T.; Brozell, S. R.; Cerutti, D. S.; Cheatham, III, T. E.; Cisneros, G. A.; Cruzeiro, V. W. D.; Darden, T. A.; Duke, R. E.; Giambasu, G.; Gilson, M. K.; Gohlke, H.; Goetz, A. W.; Harris, R.; Izadi, S.; Izmailov, K.; Kasavajhala, K.; Kaymak, M. C.; King, E.; Kovalenko, A.; Kurtzman, T.; Lee, T. S.; LeGrand, S.; Li, P.; Lin, C.; Liu, J.; Luchko, T.; Luo, R.; Machado, M.; Man, V.; Manathunga, M.; Merz, K. M.; Miao, Y.; Mikhailovskii, O.; Monard, G.; Nguyen, H.; O'Hearn, K. A.; Onufriev, A.; Pan, F.; Pantano, S.; Qi, R.; Rahnamoun, A.; Roe, D. R.; Roitberg, A.; Sagui, C.; Schott-Verdugo, S.; Shajan, A.; Shen, J.; Simmerling, C. L.; Skrynnikov, N. R.; Smith, J.; Swails, J.; Walker, R. C.; Wang, J.; Wang, J.; Wei, H.; Wolf, R. M.; Wu, X.; Xiong, Y.; Xue, Y.; York, D. M.; Zhao, S.; Kollman, P. A. *Amber 2022*; University of California, San Fransisco, 2022.
(194) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. Overview of the CCP4 Suite and Current Developments. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67* (4), 235–242. https://doi.org/10.1107/S0907444910045749.
(195) Wall, M. E. Methods and Software for Diffuse X-Ray Scattering from Protein Crystals. In *Micro and Nano Technologies in Bioanalysis: Methods and Protocols*; Foote, R. S., Lee, J. W., Eds.; Methods in Molecular Biology™; Humana Press: Totowa, NJ, 2009; pp 269–279. https://doi.org/10.1007/978-1-59745-483-4_17.
(196) Grosse-Kunstleve, R. W.; Sauter, N. K.; Moriarty, N. W.; Adams, P. D. The Computational Crystallography Toolbox: Crystallographic Algorithms in a Reusable Software Framework. *J. Appl. Crystallogr.* **2002**, *35* (1), 126–136. https://doi.org/10.1107/S0021889801017824.
(197) Pearce, N. M.; Krojer, T.; Bradley, A. R.; Collins, P.; Nowak, R. P.; Talon, R.; Marsden, B. D.; Kelm, S.; Shi, J.; Deane, C. M.; von Delft, F. A Multi-Crystal Method for Extracting

Obscured Crystallographic States from Conventionally Uninterpretable Electron Density. *Nat. Commun.* **2017**, *8* (1), 15123. https://doi.org/10.1038/ncomms15123.
(198) Liebschner, D.; Afonine, P. V.; Baker, M. L.; Bunkóczi, G.; Chen, V. B.; Croll, T. I.; Hintze, B.; Hung, L.-W.; Jain, S.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R. D.; Poon, B. K.; Prisant, M. G.; Read, R. J.; Richardson, J. S.; Richardson, D. C.; Sammito, M. D.; Sobolev, O. V.; Stockwell, D. H.; Terwilliger, T. C.; Urzhumtsev, A. G.; Videau, L. L.; Williams, C. J.; Adams, P. D. Macromolecular Structure Determination Using X-Rays, Neutrons and Electrons: Recent Developments in Phenix. *Acta Crystallogr. Sect. Struct. Biol.* **2019**, *75* (10), 861–877. https://doi.org/10.1107/S2059798319011471.
(199) Wall, M. E. Internal Protein Motions in Molecular-Dynamics Simulations of Bragg and Diffuse X-Ray Scattering. *IUCrJ* **2018**, *5* (2), 172–181. https://doi.org/10.1107/S2052252518000519.
(200) Krissinel, E.; Henrick, K. Inference of Macromolecular Assemblies from Crystalline State. *J. Mol. Biol.* **2007**, *372* (3), 774–797. https://doi.org/10.1016/j.jmb.2007.05.022.
(201) Williams, C. J.; Headd, J. J.; Moriarty, N. W.; Prisant, M. G.; Videau, L. L.; Deis, L. N.; Verma, V.; Keedy, D. A.; Hintze, B. J.; Chen, V. B.; Jain, S.; Lewis, S. M.; Arendall III, W. B.; Snoeyink, J.; Adams, P. D.; Lovell, S. C.; Richardson, J. S.; Richardson, D. C. MolProbity: More and Better Reference Data for Improved All-Atom Structure Validation. *Protein Sci.* **2018**, *27* (1), 293–315. https://doi.org/10.1002/pro.3330.
(202) Lovell, S. C.; Word, J. M.; Richardson, J. S.; Richardson, D. C. The penultimate rotamer library. *Proteins Struct. Funct. Bioinforma.* **2000**, *40* (3), 389–408. https://doi.org/10.1002/1097-0134(20000815)40:3<389::AID-PROT50>3.0.CO;2-2.
(203) Lang, P. T.; Ng, H.-L.; Fraser, J. S.; Corn, J. E.; Echols, N.; Sales, M.; Holton, J. M.; Alber, T. Automated Electron-Density Sampling Reveals Widespread Conformational Polymorphism in Proteins. *Protein Sci.* **2010**, *19* (7), 1420–1431. https://doi.org/10.1002/pro.423.
(204) Riley, B. T.; Wankowicz, S. A.; de Oliveira, S. H. P.; van Zundert, G. C. P.; Hogan, D. W.; Fraser, J. S.; Keedy, D. A.; van den Bedem, H. QFit 3: Protein and Ligand Multiconformer Modeling for X-Ray Crystallographic and Single-Particle Cryo-EM Density Maps. *Protein Sci.* **2021**, *30* (1), 270–285. https://doi.org/10.1002/pro.4001.
(205) Fraser, J. S.; Bedem, H. van den; Samelson, A. J.; Lang, P. T.; Holton, J. M.; Echols, N.; Alber, T. Accessing Protein Conformational Ensembles Using Room-Temperature X-Ray Crystallography. *Proc. Natl. Acad. Sci.* **2011**, *108* (39), 16247–16252. https://doi.org/10.1073/pnas.1111325108.
(206) C. Bradford, S. Y.; Khoury, L. E.; Ge, Y.; Osato, M.; L. Mobley, D.; Fischer, M. Temperature Artifacts in Protein Structures Bias Ligand-Binding Predictions. *Chem. Sci.* **2021**, *12* (34), 11275–11293. https://doi.org/10.1039/D1SC02751D.
(207) Fenwick, R. B.; van den Bedem, H.; Fraser, J. S.; Wright, P. E. Integrated Description of Protein Dynamics from Room-Temperature X-Ray Crystallography and NMR. *Proc. Natl. Acad. Sci.* **2014**, *111* (4), E445–E454. https://doi.org/10.1073/pnas.1323440111.
(208) Faure, P.; Micu, A.; Pérahia, D.; Doucet, J.; Smith, J. C.; Benoit, J. P. Correlated Intramolecular Motions and Diffuse x–Ray Scattering in Lysozyme. *Nat. Struct. Biol.* **1994**, *1* (2), 124–128. https://doi.org/10.1038/nsb0294-124.
(209) Clarage, J. B.; Romo, T.; Andrews, B. K.; Pettitt, B. M.; Phillips, G. N. A Sampling Problem in Molecular Dynamics Simulations of Macromolecules. *Proc. Natl. Acad. Sci.* **1995**, *92* (8), 3288–3292. https://doi.org/10.1073/pnas.92.8.3288.
(210) Héry, S.; Genest, D.; Smith, J. C. X-Ray Diffuse Scattering and Rigid-Body Motion in Crystalline Lysozyme Probed by Molecular Dynamics Simulation. *J. Mol. Biol.* **1998**, *279* (1), 303–319. https://doi.org/10.1006/jmbi.1998.1754.
(211) Meinhold, L.; Smith, J. C. Fluctuations and Correlations in Crystalline Protein Dynamics: A Simulation Analysis of Staphylococcal Nuclease. *Biophys. J.* **2005**, *88* (4), 2554–2563.

https://doi.org/10.1529/biophysj.104.056101.
(212) Wall, M. E.; Van Benschoten, A. H.; Sauter, N. K.; Adams, P. D.; Fraser, J. S.; Terwilliger, T. C. Conformational Dynamics of a Crystalline Protein from Microsecond-Scale Molecular Dynamics Simulations and Diffuse X-Ray Scattering. *Proc. Natl. Acad. Sci.* **2014**, *111* (50), 17887–17892. https://doi.org/10.1073/pnas.1416744111.
(213) Wych, D. C.; Fraser, J. S.; Mobley, D. L.; Wall, M. E. Liquid-like and Rigid-Body Motions in Molecular-Dynamics Simulations of a Crystalline Protein. *Struct. Dyn.* **2019**, *6* (6), 064704. https://doi.org/10.1063/1.5132692.
(214) Meisburger, S. P.; Case, D. A.; Ando, N. Diffuse X-Ray Scattering from Correlated Motions in a Protein Crystal. *Nat. Commun.* **2020**, *11* (1), 1271. https://doi.org/10.1038/s41467-020-14933-6.
(215) Meisburger, S. P.; Case, D. A.; Ando, N. Robust Total X-Ray Scattering Workflow to Study Correlated Motion of Proteins in Crystals. bioRxiv January 2, 2023, p 2022.08.22.504832. https://doi.org/10.1101/2022.08.22.504832.
(216) Cerutti, D. S.; Freddolino, P. L.; Duke, R. E. Jr.; Case, D. A. Simulations of a Protein Crystal with a High Resolution X-Ray Structure: Evaluation of Force Fields and Water Models. *J. Phys. Chem. B* **2010**, *114* (40), 12811–12824. https://doi.org/10.1021/jp105813j.
(217) Moreau, D. Ice Formation and Solvent Nanoconfinement in Protein Crystallography. dissertation or thesis, Cornell University, Ithaca, NY, 2020. https://doi.org/10.7298/2yxj-4e25.
(218) Janowski, P. A.; Liu, C.; Deckman, J.; Case, D. A. Molecular Dynamics Simulation of Triclinic Lysozyme in a Crystal Lattice. *Protein Sci.* **2016**, *25* (1), 87–102. https://doi.org/10.1002/pro.2713.
(219) Teeter, M. M. Water Structure of a Hydrophobic Protein at Atomic Resolution: Pentagon Rings of Water Molecules in Crystals of Crambin. *Proc. Natl. Acad. Sci.* **1984**, *81* (19), 6014–6018. https://doi.org/10.1073/pnas.81.19.6014.
(220) Chen, J. C.-H.; Fisher, Z.; Kovalevsky, A. Y.; Mustyakimov, M.; Hanson, B. L.; Zhurov, V. V.; Langan, P. Room-Temperature Ultrahigh-Resolution Time-of-Flight Neutron and X-Ray Diffraction Studies of H/D-Exchanged Crambin. *Acta Crystallograph. Sect. F Struct. Biol. Cryst. Commun.* **2012**, *68* (2), 119–123. https://doi.org/10.1107/S1744309111051499.
(221) Eisenmesser, E. Z.; Millet, O.; Labeikovsky, W.; Korzhnev, D. M.; Wolf-Watz, M.; Bosco, D. A.; Skalicky, J. J.; Kay, L. E.; Kern, D. Intrinsic Dynamics of an Enzyme Underlies Catalysis. *Nature* **2005**, *438* (7064), 117–121. https://doi.org/10.1038/nature04105.
(222) Davis, I. W.; Arendall, W. B.; Richardson, D. C.; Richardson, J. S. The Backrub Motion: How Protein Backbone Shrugs When a Sidechain Dances. *Structure* **2006**, *14* (2), 265–274. https://doi.org/10.1016/j.str.2005.10.007.
(223) Friedland, G. D.; Linares, A. J.; Smith, C. A.; Kortemme, T. A Simple Model of Backbone Flexibility Improves Modeling of Side-Chain Conformational Variability. *J. Mol. Biol.* **2008**, *380* (4), 757–774. https://doi.org/10.1016/j.jmb.2008.05.006.
(224) Keedy, D. A.; Kenner, L. R.; Warkentin, M.; Woldeyes, R. A.; Hopkins, J. B.; Thompson, M. C.; Brewster, A. S.; Van Benschoten, A. H.; Baxter, E. L.; Uervirojnangkoorn, M.; McPhillips, S. E.; Song, J.; Alonso-Mori, R.; Holton, J. M.; Weis, W. I.; Brunger, A. T.; Soltis, S. M.; Lemke, H.; Gonzalez, A.; Sauter, N. K.; Cohen, A. E.; van den Bedem, H.; Thorne, R. E.; Fraser, J. S. Mapping the Conformational Landscape of a Dynamic Enzyme by Multitemperature and XFEL Crystallography. *eLife* **2015**, *4*, e07574. https://doi.org/10.7554/eLife.07574.
(225) Van Benschoten, A. H.; Liu, L.; Gonzalez, A.; Brewster, A. S.; Sauter, N. K.; Fraser, J. S.; Wall, M. E. Measuring and Modeling Diffuse Scattering in Protein X-Ray Crystallography. *Proc. Natl. Acad. Sci.* **2016**, *113* (15), 4069–4074. https://doi.org/10.1073/pnas.1524048113.

(226) Thompson, M. C.; Barad, B. A.; Wolff, A. M.; Sun Cho, H.; Schotte, F.; Schwarz, D. M. C.; Anfinrud, P.; Fraser, J. S. Temperature-Jump Solution X-Ray Scattering Reveals Distinct Motions in a Dynamic Enzyme. *Nat. Chem.* **2019**, *11* (11), 1058–1066. https://doi.org/10.1038/s41557-019-0329-3.
(227) Zhang, Y.; Zhou, L.; Rouge, L.; Phillips, A. H.; Lam, C.; Liu, P.; Sandoval, W.; Helgason, E.; Murray, J. M.; Wertz, I. E.; Corn, J. E. Conformational Stabilization of Ubiquitin Yields Potent and Selective Inhibitors of USP7. *Nat. Chem. Biol.* **2013**, *9* (1), 51–58. https://doi.org/10.1038/nchembio.1134.
(228) Biel, J. T.; Thompson, M. C.; Cunningham, C. N.; Corn, J. E.; Fraser, J. S. Flexibility and Design: Conformational Heterogeneity along the Evolutionary Trajectory of a Redesigned Ubiquitin. *Structure* **2017**, *25* (5), 739-749.e3. https://doi.org/10.1016/j.str.2017.03.009.
(229) Keedy, D. A.; Hill, Z. B.; Biel, J. T.; Kang, E.; Rettenmaier, T. J.; Brandão-Neto, J.; Pearce, N. M.; von Delft, F.; Wells, J. A.; Fraser, J. S. An Expanded Allosteric Network in PTP1B by Multitemperature Crystallography, Fragment Screening, and Covalent Tethering. *eLife* **2018**, *7*, e36307. https://doi.org/10.7554/eLife.36307.
(230) Wiesmann, C.; Barr, K. J.; Kung, J.; Zhu, J.; Erlanson, D. A.; Shen, W.; Fahr, B. J.; Zhong, M.; Taylor, L.; Randal, M.; McDowell, R. S.; Hansen, S. K. Allosteric Inhibition of Protein Tyrosine Phosphatase 1B. *Nat. Struct. Mol. Biol.* **2004**, *11* (8), 730–737. https://doi.org/10.1038/nsmb803.
(231) Whittier, S. K.; Hengge, A. C.; Loria, J. P. Conformational Motions Regulate Phosphoryl Transfer in Related Protein Tyrosine Phosphatases. *Science* **2013**, *341* (6148), 899–903. https://doi.org/10.1126/science.1241735.
(232) Choy, M. S.; Li, Y.; Machado, L. E. S. F.; Kunze, M. B. A.; Connors, C. R.; Wei, X.; Lindorff-Larsen, K.; Page, R.; Peti, W. Conformational Rigidity and Protein Dynamics at Distinct Timescales Regulate PTP1B Activity and Allostery. *Mol. Cell* **2017**, *65* (4), 644-658.e5. https://doi.org/10.1016/j.molcel.2017.01.014.
(233) Nakamura, A.; Ishida, T.; Kusaka, K.; Yamada, T.; Fushinobu, S.; Tanaka, I.; Kaneko, S.; Ohta, K.; Tanaka, H.; Inaka, K.; Higuchi, Y.; Niimura, N.; Samejima, M.; Igarashi, K. "Newton's Cradle" Proton Relay with Amide–Imidic Acid Tautomerization in Inverting Cellulase Visualized by Neutron Crystallography. *Sci. Adv.* **2015**, *1* (7), e1500263. https://doi.org/10.1126/sciadv.1500263.
(234) Chen, J. C.-H.; Hanson, B. L.; Fisher, S. Z.; Langan, P.; Kovalevsky, A. Y. Direct Observation of Hydrogen Atom Dynamics and Interactions by Ultrahigh Resolution Neutron Protein Crystallography. *Proc. Natl. Acad. Sci.* **2012**, *109* (38), 15301–15306. https://doi.org/10.1073/pnas.1208341109.
(235) Blakeley, M. P. Neutron Macromolecular Crystallography. *Crystallogr. Rev.* **2009**, *15* (3), 157–218. https://doi.org/10.1080/08893110902965003.
(236) Ashkar, R.; Bilheux, H.; Bordallo, H.; Briber, R.; Callaway, D. J. E.; Cheng, X.; Chu, X.-Q.; Curtis, J. E.; Dadmun, M.; Fenimore, P.; Fushman, D.; Gabel, F.; Gupta, K.; Herberle, F.; Heinrich, F.; Hong, L.; Katsaras, J.; Kelman, Z.; Kharlampieva, E.; Kneller, G. R.; Kovalevsky, A.; Krueger, S.; Langan, P.; Lieberman, R.; Liu, Y.; Losche, M.; Lyman, E.; Mao, Y.; Marino, J.; Mattos, C.; Meilleur, F.; Moody, P.; Nickels, J. D.; O'Dell, W. B.; O'Neill, H.; Perez-Salas, U.; Peters, J.; Petridis, L.; Sokolov, A. P.; Stanley, C.; Wagner, N.; Weinrich, M.; Weiss, K.; Wymore, T.; Zhang, Y.; Smith, J. C. Neutron Scattering in the Biological Sciences: Progress and Prospects. *Acta Crystallogr. Sect. Struct. Biol.* **2018**, *74* (12), 1129–1168. https://doi.org/10.1107/S2059798318017503.
(237) Blakeley, M. P.; Kalb (Gilboa), A. J.; Helliwell, J. R.; Myles, D. A. A. The 15-K Neutron Structure of Saccharide-Free Concanavalin A. *Proc. Natl. Acad. Sci.* **2004**, *101* (47), 16405–16410. https://doi.org/10.1073/pnas.0405109101.
(238) Wall, M. E.; Calabró, G.; Bayly, C. I.; Mobley, D. L.; Warren, G. L. Biomolecular Solvation Structure Revealed by Molecular Dynamics Simulations. *J. Am. Chem. Soc.* **2019**, *141*

(11), 4711–4720. https://doi.org/10.1021/jacs.8b13613.
(239) Lexa, K. W.; Carlson, H. A. Full Protein Flexibility Is Essential for Proper Hot-Spot Mapping. *J. Am. Chem. Soc.* **2011**, *133* (2), 200–202. https://doi.org/10.1021/ja1079332.
(240) Wall, M. E.; Ealick, S. E.; Gruner, S. M. Three-Dimensional Diffuse x-Ray Scattering from Crystals of Staphylococcal Nuclease. *Proc. Natl. Acad. Sci.* **1997**, *94* (12), 6180–6184. https://doi.org/10.1073/pnas.94.12.6180.
(241) van Gunsteren, W. F.; Daura, X.; Hansen, N.; Mark, A. E.; Oostenbrink, C.; Riniker, S.; Smith, L. J. Validation of Molecular Simulation: An Overview of Issues. *Angew. Chem. Int. Ed.* **2018**, *57* (4), 884–902. https://doi.org/10.1002/anie.201702945.
(242) Grossfield, A.; Patrone, P. N.; Roe, D. R.; Schultz, A. J.; Siderius, D.; Zuckerman, D. M. Best Practices for Quantification of Uncertainty and Sampling Quality in Molecular Simulations [Article v1.0]. *Living J. Comput. Mol. Sci.* **2019**, *1* (1), 5067–5067. https://doi.org/10.33011/livecoms.1.1.5067.
(243) Setz, M. Molecular Dynamics Simulations of Biomolecules: From Validation to Application, 2018. http://zidapp.boku.ac.at/abstracts/search_abstract.php?paID=3&paLIST=0&paSID=12393 (accessed 2023-02-28).
(244) Wassenaar, T. A.; Mark, A. E. The Effect of Box Shape on the Dynamic Properties of Proteins Simulated under Periodic Boundary Conditions. *J. Comput. Chem.* **2006**, *27* (3), 316–325. https://doi.org/10.1002/jcc.20341.
(245) Villa, A.; Fan, H.; Wassenaar, T.; Mark, A. E. How Sensitive Are Nanosecond Molecular Dynamics Simulations of Proteins to Changes in the Force Field? *J. Phys. Chem. B* **2007**, *111* (21), 6015–6025. https://doi.org/10.1021/jp068580v.

Acknowledgements

O.H.S.O. acknowledges the Academy of Finland (grants 315596 and 319902) for financial support.
C.E.C. acknowledges funding from the National Institutes of Health (R01GM132386) and the Open Force Field Consortium
J.D.C. acknowledges support from NIH grant P30~CA008748, NIH grant R01~GM132386, and the Sloan Kettering Institute.
L.TC. acknowledges support from the National Science Foundation (CHE-1807301)
M.K.G. acknowledges funding from the National Institutes of Health (R01GM132386).
D.A.K. acknowledges funding from the National Institutes of Health (R35GM133769).
These findings are solely of the authors and do not necessarily represent the views of the NIH, the NSF, or any other funder.
V.A.V. acknowledges funding from the National Institutes of Health (R01GM123296).
P.R. acknowledges support from the National Institutes of Health under award R35GM142750.
D.C.W. and M.E.W. acknowledge support from the Exascale Computing Project (grant No. 17-SC-20-SC), a collaborative effort of the US Department of Energy Office of Science and National Nuclear Security Administration.
D.C.W. acknowledges support from the Center for Nonlinear Studies at Los Alamos National Laboratory (under US Department of Energy contract No. 89233218CNA000001 to Triad National Security)

Disclosures

- D.L.M. serves on the scientific advisory boards of OpenEye Scientific Software and Anagenex, and is an Open Science Fellow with Psivant Sciences.
- J.D.C. is a current member of the Scientific Advisory Board of OpenEye Scientific Software, Redesign Science, Ventus Therapeutics, and Interline Therapeutics, and has equity interests in Redesign Science and Interline Therapeutics. The Chodera laboratory receives or has received funding from multiple sources, including the National Institutes of Health, the National Science Foundation, the Parker Institute for Cancer Immunotherapy, Relay Therapeutics, Entasis Therapeutics, Silicon Therapeutics, EMD Serono (Merck KGaA), AstraZeneca, Vir Biotechnology, Bayer, XtalPi, Interline Therapeutics, the Molecular Sciences Software Institute, the Starr Cancer Consortium, the Open Force Field Consortium, Cycle for Survival, a Louis V. Gerstner Young Investigator Award, and the Sloan Kettering Institute. A complete funding history for the Chodera lab can be found at http://choderalab.org/funding.
- M.R.S. is an Open Science Fellow with Psivant Sciences and consults for Relay Therapeutics.
- M.K.G. has an equity interest in and is a cofounder and scientific advisor of VeraChem LLC and a member of the Scientific Advisory Board of InCerebro Co., Ltd.
- L.T.C. serves on the scientific advisory board of OpenEye Scientific Software and is an Open Science Fellow with Psivant Sciences.
- M.E.W. has an equity interest in and is a consultant for Eli Lilly and Company