Representing Structures of the Multiple Conformational States of Proteins

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Highlights

- Improved methods have advanced multi-conformational structural modeling
- Two or more multiple-state conformations often best describe a protein structure
- Single-state representation depicts local model uncertainty on one representative conformer
- Consistent data structures are needed for archiving multiple-state models

Abstract

Biomolecules exhibit dynamic behavior that single-state models of their structures cannot fully capture. We review some recent advances for investigating multiple conformations of biomolecules, including experimental methods, molecular dynamics simulations, and machine learning. We also address the challenges associated with representing single- and multiple-state models in data archives, with a particular focus on NMR structures. Establishing standardized representations and annotations will facilitate effective communication and understanding of these complex models to the broader scientific community.

Abbreviations: AF2 – AlphaFold2 Multimer; BMRB - biological magnetic resonance bank; CEST – chemical exchange by saturation transfer; CPMG – Carr-Purcell-Meiboom-Gill; DEER – double electron-electron resonance; FID - free induction decay NMR data; FRET - Förster resonance energy transfer; LDDT – local-distance difference test; MD – molecular dynamics; ML – machine learning; mmCIF - macromolecular crystallographic information file; MSA – multiple sequence alignment; NMR - nuclear magnetic resonance spectroscopy; PDB - protein data bank; pLDDT - predicted local model confidence score predicted from ML; PRE - paramagnetic relaxation enhancement; RDC – residual dipolar coupling; wwPDB – worldwide PDB.

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Introduction

Biomolecules exhibit dynamic behavior and adopt a distribution of conformations influenced by factors including sequence, temperature, pressure, ligand binding, and solution conditions. Traditionally, structural biology has predominantly focused on single-conformation models. However, it is broadly appreciated that most biomolecules must move to function. One of the earliest experimental demonstrations of protein structure plasticity came from NMR studies of aromatic-ring flips of the small protein bovine pancreatic trypsin inhibitor, where it was observed that conformational fluctuations allow rapid rotations of aromatic rings buried in the hydrophobic core [1]. Recent advances in experimental and computational methods illustrate the importance of multiple-conformation modeling for understanding biomolecule functions. In particular, as the machine learning (ML) methods of AlphaFold2 (AF2) [2], RosettaFold [3], OpenFold [4], ESMFold [5], RaptorX [6], and other advanced techniques have reached the stage where single structure prediction of small proteins is robust and reliable, and a current frontier is multiple-state modeling [7,8]. Establishing consistent ontologies and formats for representing such multiple-state ensemble models is crucial for supporting and advancing this important area of structural biology.

This perspective addresses the significance and handling of multiple-conformation models of biomolecules. We begin with key definitions. Conformers refer to atomic structures capable of interconversion without making or breaking covalent bonds. Conformational ensembles consist of collections of such conformers. Structural models can be categorized as “single-state” or “multiple-state” based on the nature of the experimental data or the theoretical inference. Multiple-state models may constitute a pair of conformers, pairs of conformational ensembles, or ensembles of many states representing the conformational distributions observed for disordered polymer chains. The distinction between states, and determination of the number of states, is determined by the interpretation used in modeling the data. Terms used for such multiple-state ensembles in the literature include alternative conformations, multi-conformer ensemble models, switched folds, metamorphic states, chameleonic states, and conformational excited states. Here we outline the pressing need for standardized representations of multiple-state ensembles and their corresponding data in structural databases and across the structural biology community.

The significance of multiple conformational states

Modeling multiple conformations is fundamental to understanding biomolecule functions, as dynamics determines their ability to carry out these functions. In this perspective, we focus on representing diverse conformational states of proteins, but similar challenges also apply to nucleic acids such as DNA and RNA [9,10]. Conformational dynamics also underlie enzyme function [11,12,] and are especially important for membrane protein activities as receptors and transporters of ions, metabolites, and drugs. Protein-protein interfaces may also exhibit multiple conformational states, as observed for example, in dimers of the influenza A virus non-structural protein NS1 [13] and between domains of MHC class I molecules [14]. Additionally, the significance of intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) is increasingly recognized in biology [15,16].

Biomolecules often undergo conformational changes when interacting with binding partners or substrates, encompassing both induced fit and conformational selection mechanisms. These structural changes can occur at binding sites or be distributed across the biomolecule structure. The significant role of allostery in enzyme function, where an allosteric modulator molecule binds to sites distal to the active site, has been recognized for decades [17]. Recent advances combining experimental data with advanced modeling methods have revealed structural details of allosteric mechanisms [17-20]. Evolutionary coupling (EC) based on sequence covariance analysis has also been utilized to enhance enzyme activities by perturbing allosteric networks with mutations distant from their active sites [21].

Structural heterogeneity is also important in *de novo* protein design, and has been successfully used in to create cyclic chameleon peptides that switch between exposed hydrophobic and hydrophilic surfaces to provide membrane permeability [22], two-state hinge proteins [23], and fold-switching metamorphic proteins [24]. Membrane protein transporters have also been the subject of multiple-state *de novo* design efforts, such as the Zn^{2+}-transporting four-helix bundle transmembrane protein Rocker [25]. Advancements in the controlled design of proteins that switch between alternative conformations are crucial for creating novel protein effectors and catalysts.

Experimental methods for characterizing multiple conformational states

Recent reviews discuss the experimental methods that provide structural information on multiple conformational states of biomolecules [26-28]. Crystallographic studies, using either X-ray or neutron diffraction, may capture different states in different crystal forms. Electron density can often be fit to multiple conformations within a single crystal, and room-temperature (or higher temperature) X-ray crystallography avoids the structural bias from cryogenic cooling and reveals motions crucial for catalysis, ligand binding, and allosteric regulation [12,28-30]. Other experimental data types, such as small-angle X-ray scattering (SAXS) [27] and electron microscopy (cryoEM) [31-33] data, can frequently only be fit to conformational distributions of multi-conformer models. Additionally, Förster resonance energy transfer (FRET), Double Electron-Electron Resonance (DEER) spectroscopy, and chemical cross-linking data have been used to model multiple conformational states since they can characterize interprobe distance distributions in structural ensembles [26]. With all experimental data, multiple-state fitting becomes crucial when a single-state model is inadequate, allowing a better representation of the structural heterogeneity observed in biomolecules.

Nuclear Magnetic Resonance (NMR) spectroscopy is a valuable tool for studying the dynamic behavior of biomolecules. It employs properties such as nuclear relaxation and chemical exchange saturation transfer to determine interconversion rates and populations of conformations [19,34-36]. NMR parameters reflect conformational averaging on parameter-specific timescales: for ^{1}H chemical shift, slow exchange (conformational lifetime >> ~ 1 ms) yields distinct resonances for individual states, fast exchange (conformational lifetime << ~ 1 ms) results in population-weighted average resonance frequencies, and intermediate exchange leads to characteristic resonance lineshapes. The distinction between slow and fast

exchange depends on the difference in the resonance frequencies of the individual states; e.g., for a two-spin system with ^{1}H chemical shift differences between 0.1 and 1 ppm at 800 MHz, intermediate exchange corresponds to rates from ca. 10 to 250,000 s^{-1} [37]. Chemical shift refocusing experiments like Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion and $T1_{rho}$ relaxation experiments, and saturation transfer experiments (e.g., chemical exchange by saturation transfer, CEST), can provide quantitative information about conformational exchange on the intermediate or slow chemical shift timescale [34], and can be used to characterize sparsely-populated states that cannot otherwise be observed in NMR spectra [35]. NOESY and residual dipolar coupling (RDC) data can reveal multiple conformations in fast dynamic equilibrium and provide structural restraints for modeling each state [38-41]. Paramagnetic effects in metal-containing biomolecules provide ensemble-averaged distance restraints and can also determine ensemble-averaged relative orientations of structural domains [42,43]. In many cases, the structures of the conformations in dynamic exchange are modeled by fitting back-calculated NMR parameters (e.g., chemical shifts) to ensemble-averaged NMR data.

Computational methods for modeling multiple conformational states

For decades, molecular dynamics (MD) simulations and normal mode analysis methods have been utilized to study the various conformational states of biomolecules [44]. Integrating experimental data, such as NMR and X-ray crystallographic data, with MD simulations has led to improved conformational heterogeneity evaluation. MD has been combined with X-ray crystallography data to generate multiple-state ensemble models that much better fit X-ray data compared to single-structure models [45,46]. However, conventional MD simulations often fall short in capturing slower motions, particularly allosteric conformational changes. Approaches have also been developed to integrate experimental NMR data with MD simulations, aiming to create more representative conformational ensembles. By incorporating time-averaged distance restraints from NOE data [19,40], MD simulations can better model conformational distributions consistent with experimental data, as in the case of the DNA-binding loops of *E. coli* tryptophan repressor [47]. Bayesian inference and ensemble fitting approaches, which leverage experimental data alongside MD simulations, can also generate improved ensembles [48,49]. Similarly, chemical shift data have been used to guide or interpret computational methods. For example, NMR chemical shift perturbation analysis using programs like CHESCA has been used to characterize allosteric conformational switching upon ligand binding by chemical shift covariance analysis [50]. Alternative conformational state modeling with Ohm, a structural perturbation propagation method, for a set of ~ 20 allosterically-modulated proteins was observed to provide excellent predictions of CHESCA-based chemical shift changes [20]. Accelerated MD methods have also proven effective in modeling multiple conformational states of proteins, which align well with NMR data [19].

Machine learning (ML) approaches show tremendous promise for modeling conformational dynamics. Although AF2 was not trained to model protein dynamics, in some cases, it can provide information about the individual states in dynamic equilibrium. For example, comparison of AF2 and NMR models for CASP14 target T1027, *Gaussia* luciferase, against NMR data suggested that the AF2 prediction corresponds to just one of the multiple conformations in the NMR sample [8]. Subsequently, the

proclivity of AF2 to model one of multiple conformational states was also reported for a collection of ~100 apo / holo protein structure pairs [51]. Recently, methods have emerged to extend AF2 and other machine learning networks to model alternative protein conformations explicitly. These extensions involve leveraging ECs that distinguish multiple conformations [52-54], employing multiple templates with diverse conformations [55,56], using shallow multiple-sequence alignments [56-58], or by perturbing the neural network weights [59] to generate conformational diversity. SPEACH_AF utilizes *in silico* mutations as input to AF2 to model conformational switching in soluble and membrane proteins [60]. AlphaFold2-RAVE utilizes the structural outputs from AF2 for AI-augmented MD, resulting in Boltzmann-ranked ensembles of conformations [61].

Databases of multiple conformational states

The advancements in experimental methods and modeling techniques for determining multiple conformational states of biomolecules necessitate improved methods for representing and archiving information about conformations in dynamic equilibrium. This can be challenging, as definitions of conformational "states" depend on the timescale of the experimental data and/or the modeling methods used. Apart from the well-known Protein Data Bank (PDB) and Nucleic Acid Database (NDB), several other databases (ACMS, CoDNaS, D3PM, and MultiComp) primarily store and annotate data on alternative conformations obtained from diverse crystal structures, as reported in the PDB. These databases, along with other important structural databases that primarily archive single-state models but can also provide data about multiple conformational states, are listed in Table 1 along with their URLs.

Clearly, multiple conformational state information is important in biology, and there is a need for consistent representation of such information in databases. In the following sections, we discuss some challenges in representing collections of molecular models derived from NMR data. While some points are specific to NMR structures and data, most are relevant for representing biomolecule structures obtained via various experimental or predictive modeling techniques.

Representation of biomolecular NMR structures: single-state ensembles

Solution NMR structures are typically represented as ensembles of coordinate sets, where each model in the ensemble is independently generated by fitting experimental data to a single conformer. This is done multiple times under different initialization conditions, generating an ensemble of conformers. The single-state ensemble representation commonly used for NMR structures encodes information about which regions of the protein structure are "well-defined" by the NMR data and which regions are not. Less-well-defined segments of the structure often (but not always) correspond to regions undergoing conformational dynamics. In the simplest case, the coordinates for the ensemble of conformer models are deposited to the PDB, accompanied by restraints, while the chemical shifts, peaks lists, and raw FID data are deposited to the Biological Magnetic Resonance Bank (BMRB).

This single-state ensemble representation can be confusing for scientists using NMR structures. Although each conformer is considered to be a good fit to the data, the coordinate uncertainty in different regions

can only be assessed by analyzing the ensemble as a whole. The individual conformer models in the single-state representation are not meant to describe actual conformers contributing to the Boltzmann distribution of states present in the sample. The single-state ensemble also does not provide a statistically reliable estimate of the atomic coordinate precision based on experimental measurement uncertainties, although Bayesian methods have been proposed for this purpose [48]. Despite its limitations, the prevailing convention for biomolecular structure modeling using NMR data continues to be the single-state ensemble. When using such models, it is crucial to distinguish well-defined regions from the experimental data from less precise regions where atomic positions are highly uncertain. This distinction is critical for the correct application of structure validation methods, which are generally applicable only to well-defined regions [62]. Accordingly, it is important that the single-state ensemble representation is conveyed in a simple way to users of NMR structures.

In X-ray crystallography, single-state models use B-factors to describe the uncertainty of atomic positions. In a similar way, a single-state ensemble for NMR can be represented by a *single representative conformer*, along with information about coordinate uncertainty (Figure 1). The wwPDB uses the medoid conformer as the representative structure, defined as the single conformer in the ensemble most like all the other conformers [62,63]. Tools like Dihedral Angle Order Parameter (DAOP) [63,64], FindCore [65,66], and CYRANGE [67] assess well-defined and not-well-defined residue ranges in NMR ensembles. The cutoffs used by these tools are based on standardized conventions. Presently, the wwPDB has adopted CYRANGE conventions to annotate well-defined residue ranges in the NMR structure validation report. PDBStat [63] also uses these tools and writes information about well-ordered residues as well as atom-specific coordinate variances into a conventional PDB (or mmCIF) coordinate file, allowing graphical rendering of this information onto a single representative conformer (e.g., the medoid conformer) (Figure 1). After aligning the models with respect to the core residues, coordinate uncertainties are converted using the Debye-Waller equation to effective "NMR B-factors" [66], indicating the variability and uncertainty in atomic positions across the ensemble. It is unfortunate that these PDB-file annotations are not more widely used compared to the widespread adoption of the analogous concept of predicted LDDT (pLDDT) scores reported for AlphaFold2 models [2]. These annotations are essential for the informed use of NMR structures.

Representation of biomolecular NMR structures: multiple-state ensembles

Solution NMR can provide valuable insights into multiple conformational states in dynamic equilibrium. Different modeling approaches are employed depending on the timescale of conformational averaging, such as slow or fast chemical shift exchange (Figure 2). Alternative models may be generated for each set of slowly exchanging resonances [22], by deconvoluting the conformational averaging within an ensemble of rapidly exchanging states [39], by fitting to spectral features of intermediate exchange [34,37], by matching chemical shifts of slowly-exchanging states to chemical shifts predicted from known structures [68], or by other methods. These regimes generate different numbers of chemical shift lists, restraint lists, and coordinate sets, which need to be accounted for in creating archive data structures.

The current wwPDB archive does not provide consistent data structures for organizing the experimental data (e.g., FID, chemical shift, and restraint data) supporting multiple conformational states derived from a single sample. Figure 2B illustrates examples of multiple-state model ensembles in the PDB. Some were deposited as pairs of separate PDB files, while in other cases, the multiple-state ensembles were concatenated in a single PDB file. For the separate entries, although comments describing the relationships of these pairs of PDB files are included in their header files, it is not always clear where these multiple conformations were derived from a single sample and a single set of experimental data. Three of the multiple-state ensembles illustrated in Figure 2B were each deposited as a single PBD file. In two other cases (5tm0 and 2lwa), the sets (2 or 3, respectively) of structures were reported as separate chains within a single PDB file. In contrast, the individual states of the multiple-state ensemble reported in the single PDB file 7r95 were not distinguished by any specific designator. In X-ray crystal structures, alternative local conformations are often represented with distinct 'AltLoc IDs', the alternative location indicators, where atoms are assigned unique letters to represent different conformations [28,32,33]. These AltLocs can range from single atoms to sets of connected or non-connected residues, and has the benefit of including relative occupancies as well as the positions for each atom with alternative coordinates. Despite progress in automating the assignment and creation of PDB files with AltLoc annotations, challenges persist regarding interpretation ease and compatibility with other analysis software. In NMR studies, like those shown in Figure 2, multiple-state structures have been refined using multiple complete copies of the entire molecule for each state. This does not easily align with the AltLoc ID usage, which also does not currently support association with the corresponding alternative chemical shift assignments.

To address these inconsistencies and improve data organization, it is crucial to establish standardized conventions for archiving multiple-state ensembles in the PDB and other structural databases. Additionally, it is essential to ensure that the underlying experimental data, including raw FID data, are archived along with the model coordinates [69]. This will allow for future regeneration of models as methods improve. As illustrated in Figure 2C, for cases of multiple coordinate sets derived from NMR data on fast-exchanging systems, there are (i) a set of raw data, (ii) a single set of chemical shifts, but (iii) potentially two (or more) sets of restraints, and (iv) two (or more) sets of atomic coordinates. In the case of multiple coordinate sets derived from slowly exchanging systems, there is again (i) a single set of data, but (ii) multiple sets of chemical shifts, one associated with each member of the slowly exchanging system, as well as (iii) potentially two (or more) sets of restraints, and (iv) two (or more) sets of atomic coordinates. This data organization required for representing multiple-state NMR structures is not currently supported by the public biomolecular structure databases.

Generalization to other experimentally- and computationally-generated multiple-state models

The issues of representing single-state and multiple-state ensembles also impact the representation of biomolecular structures based on cryoEM, X-ray crystallography, FRET, chemical cross-linking data, and other experimental methods. Issues of data structures needed to represent these models and data are analogous to those discussed above for solution NMR data, but are beyond the scope of this perspective. Ensuring consistency in representing multiple conformational states modeled from various experimental

and computational methods is critical for developing integrated structural biology methods and advancing dynamic modeling techniques.

Future outlook

The representation and management of multiple conformational models of biomolecules is very important for understanding their dynamic behavior and how these dynamics influence their biochemical functions. Standardized ontologies and formats for representing diverse conformational ensembles are crucial for effective communication and integration of structural data across the scientific community. Recent advances in experimental and computational methods, including machine learning (ML), provide exciting new opportunities for modeling and characterizing multiple conformations. Experimental distance restraint data can also be used as input for training ML-based structure prediction methods [70] and will certainly have an impact on ML-based methods for modeling multiple conformational states. As combined experimental and modeling methods develop, models of conformational ensembles of proteins and nucleic acids will enable biochemical, biophysical, and biological studies, and the ability to consistently represent and archive information about conformations in dynamic equilibrium will facilitate research and enhance our understanding of biomolecular function.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT3.5 to improve manuscript readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

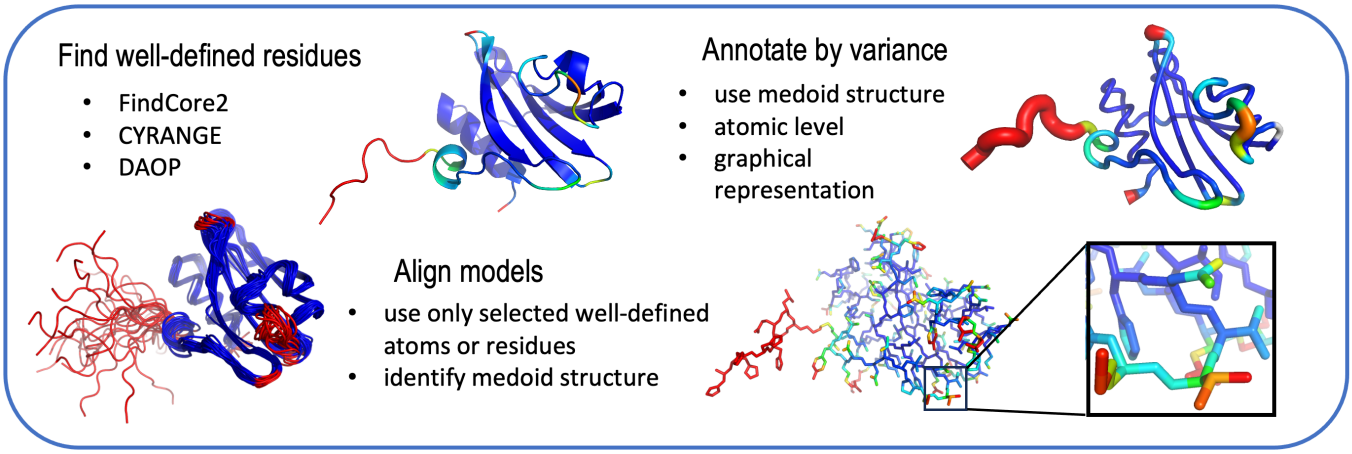

Fig. 1. Schematic representations of a "single-state" ensemble of coordinates. PDB entries for NMR structures typically consist of an ensemble of ~20 structures obtained through restrained modeling with NMR data. "Well-defined" parts of the structure can be determined using conventions encoded in programs like CYRANGE [67], FindCore2 [66], and dihedral angle order parameter DAOP [63,64] softward, and colored to indicate well-defined (blue) and not-well-defined (red) residues (left side, pdb 2kcd). The PDBStat program [63] provides tools to create an image of the protein annotated with this information about model convergence. The medoid structure, determined by aligning models using well-defined heavy (or backbone N, Cα, C') atoms, is the first entry in a new ensemble coordinate file in conventional (or mmCIF) PDB format. This file includes per-residue tags (q=1 for well-defined residues, q=0 for others) stored in the occupancy field. In addition, atom-specific coordinate variances are determined from the average atomic root mean-squared fluctuation (RMSF) across the structural ensemble [65,66,71], and reported in the B-factor field as effective "NMR B-factors", representing positional uncertainties across the ensemble. These PDB (or mmCIF) format files can then be used to visualize well-defined regions (left) or atomic variance (shown schematically in three ways, top and right side) using programs like PyMOL. Representations showing atomic variance by coloring or by scaling the size of the ribbon are shown. For multiple domain structures, variance matrix analysis is used to parse the coordinates into well-defined units, which are then analyzed separately [65-67].

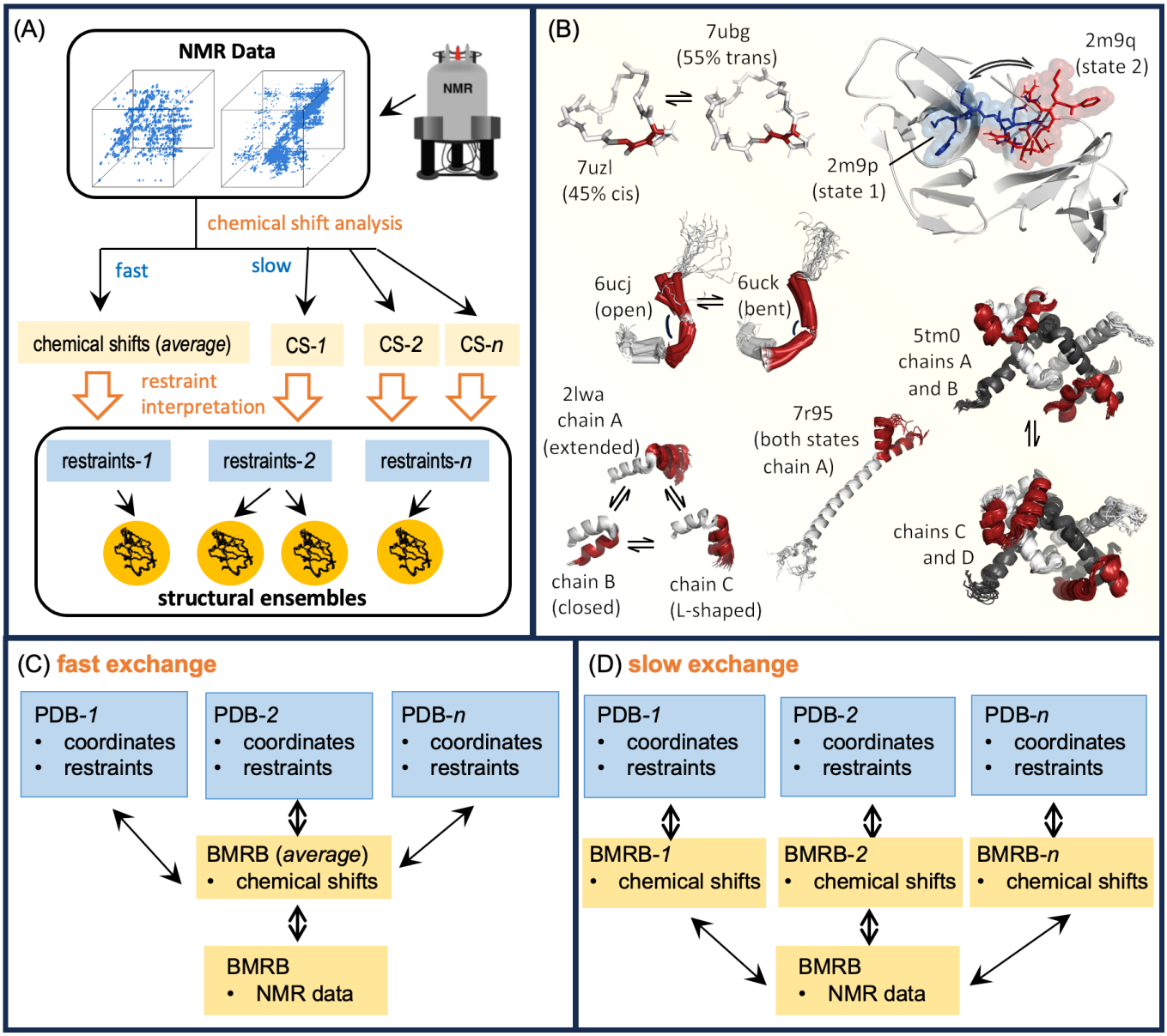

Fig. 2. Schematic of NMR data deposition pipeline for proteins reported to have multiple-state structures

(A) The process involves data collection and chemical shift analysis resulting in one or more sets of chemical shifts. NMR data interpretation is then used to derive one or more sets of restraints used to model one or more structural ensembles. (B) Representative examples of two-state ensembles including: 9-residue Rosetta-designed cyclic peptide D9.16 (pdb IDs 7ubg and 7uzl) [22], inhibitor-bound dengue virus NS2B/NS3 protease (2m9p and 2m9q), pro-islet amyloid polypeptide in detergent micelles (6ucj and 6uck) [72], *E. coli* tryptophan repressor (two states combined as pdb 5tm0) [47], and the membrane-bound SARS-CoV-2 spike protein HR1 ectodomain (two states combined as 7r95) [73]. Also shown is the three-state ensemble of influenza hemagglutinin fusion peptide A (combined as 2lwa) [74] (C and D). Schematic representation for the data organization required for deposition of multiple-state structures into the PDB and BMRB: (C) Fast exchange between the conformers yields population-averaged chemical

shifts, resulting in one or more sets of restraints and corresponding PDB coordinate sets. (D) Slow exchange between conformers leads to distinct chemical shifts, with multiple chemical shift entries from a single NMR dataset that are then used to calculate multiple sets of PDB coordinate ensembles. As in the fast exchange case, a single set of chemical shifts arising from one slow-exchange ensemble could generate multiple restraints, leading to multiple coordinate sets, depending on the data analysis method. Multiple coordinate sets may also be generated from a single restraint set in certain cases.

Table 1. Databases of multiple conformational states of proteins and nucleic acids, and other key resources.

Database	url
ACMS: Provides a detailed description of the alternate conformations of various residues for more than 60,000 high-resolution crystal structures.	http://iris.physics.iisc.ac.in/acms
AlphaFold Protein Structure Database: Provides over 200 million protein structure predictions.	https://alphafold.ebi.ac.uk
Binding MOAD - Mother of All Databases: A subset of the Protein Data Bank (PDB), with many high-quality structures of ligand-protein complexes.	http://www.bindingmoad.org
Biological Magnetic Resonance Bank (BMRB): Archive of biological NMR data.	https://bmrb.io
CoDNaS 2.0: A comprehensive database of protein conformational diversity.	http://ufq.unq.edu.ar/codnas
D3PM: A comprehensive database for protein motions, including changes with ligand binding.	http://www.d3pharma.com/D3PM/index.php
DNAproDB: Web-based database and structural analysis tool designed to access and visualize structural data of DNA–protein complexes.	https://dnaprodb.usc.edu
Electron Microscopy Data Bank (EMDB): A public repository for electron cryo-electron microscopy maps and tomograms of macromolecular complexes and subcellular structures.	https://www.ebi.ac.uk/emdb
EM Data Resource: A unified data resource for 3-Dimensional Electron Microscopy (3DEM) structure data archiving and retrieval.	https://www.emdataresource.org
ESM Metagenomic Atlas: Atlas of 772 million predicted metagenomic protein structures	https://esmatlas.com
MultiComp: A database for exploring multiple conformations of membrane proteins.	https://multicomp.nibiohn.go.jp
Nucleic Acid Data Bank (NDB) and Nucleic Acid Knowledgebase: A data resource for experimentally determined structures containing DNA and RNA nucleic acid polymers and their biological assemblies.	http://nakb.org
wwPDB worldwide Protein Data Bank: Primary international data repository for protein and nucleic structures.	https://www.wwpdb.org

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