Accessing protein conformational ensembles using room-temperature X-ray crystallography

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Modern protein crystal structures are based nearly exclusively on X-ray data collected at cryogenic temperatures (generally 100 K). The cooling process is thought to introduce little bias in the functional interpretation of structural results, because cryogenic temperatures minimally perturb the overall protein backbone fold. In contrast, here we show that flash cooling biases previously hidden structural ensembles in protein crystals. By analyzing available data for 30 different proteins using new computational tools for electron-density sampling, model refinement, and molecular packing analysis, we found that crystal cryocooling remodels the conformational distributions of more than 35% of side chains and eliminates packing defects necessary for functional motions. In the signaling switch protein, H-Ras, an allosteric network consistent with fluctuations detected in solution by NMR was uncovered in the room-temperature, but not the cryogenic, electron-density maps. These results expose a bias in structural databases toward smaller, overpacked, and unrealistically unique models. Monitoring room-temperature conformational ensembles by X-ray crystallography can reveal motions crucial for catalysis, ligand binding, and allosteric regulation.

Macromolecular X-ray crystallographic diffraction experiments provide powerful insights into the relationship between structure and biological function. Although macromolecules populate vast ensembles of alternative conformational substates (1), crystallographic models depicting the major average conformation have provided foundational ideas about the mechanisms of biochemical reactions. By slowing radiation damage to the sample, crystal cooling has catalyzed a revolution in structural biology, enabling structure determinations from tiny crystals using bright synchrotron X-ray sources (2–4). It is estimated that more than 95% of the >65,000 crystal structures deposited in the Protein Data Bank (PDB) are based on cryogenic data (5).

Crystal cooling is generally thought to introduce little bias in the functional interpretation of structural results. Some investigators have suggested that the standard practice of plunging crystals into liquid nitrogen and collecting X-ray diffraction data at 100 K traps a representative set of conformations populated at room temperature (6, 7). In contrast, early structural comparisons by Petsko, Frauenfelder, and colleagues indicated that cooling myoglobin crystals causes a small reduction in the protein volume due to anisotropic displacements of atomic positions and subtle changes of contacts between α-helices (8). A landmark study of crystalline ribonuclease A (RNaseA) by Tilton, Petsko, and coworkers revealed diverse temperature-dependent changes in the average structure, ranging from shrinkage at low temperatures to increased loop disorder at 47°C (9). A recent analysis comparing 15 room-temperature and cryogenic crystal structures documented that cryocooling generally increases lattice contacts and reduces protein volumes but causes only small changes in crystallographic models (10).

In contrast to these small conformational perturbations, the dynamic characteristics of proteins in crystals are exquisitely sensitive to temperature (11, 12). After accounting for static structural differences between molecules, model errors, and crystal lattice defects in myoglobin crystals, Frauenfelder and Petsko demonstrated widespread temperature-dependent decreases in the harmonic atomic vibrations measured by B-factors. Based on the distribution of these differences, they proposed that minor conformational substates with alternative side-chain conformations are involved in transient ligand-entry pathways (13). In crystalline RNaseA in liquid solvent, a “glass transition” in the protein between 212 and 228 K reduces B-factors, increases the quality of core packing, and eliminates motions needed for inhibitor binding to the active site (9, 14). The cooling-induced reductions in B-factors suggest that cryogenic structures adopt less variable conformations.

Indirect measures such as B-factors or protein-volume changes, however, do not distinguish whether cryocooling simply reduces vibrational motions or biases the overall conformational ensemble. A theoretical analysis by Halle’s dynamic quenching theory, changes in the energy landscape upon cooling can cause specific changes in the relative populations of alternative conformations that are not apparent from B-factor analysis or the standard refinement of unique models. Consistent with this view, we found previously that catalytically essential, alternative conformations of the proline isomerase, CypA, detected in solution using NMR are populated in crystals at room temperature but absent at cryogenic temperature (16). This temperature-dependent difference suggested that functionally important mobile regions of proteins might be particularly susceptible to annealing into a unique conformation during the cooling process.

To test the idea that crystal cryocooling changes protein conformations, we examined 30 proteins for which high-resolution X-ray data were collected previously at ambient and cryogenic temperatures (17–19). To eliminate packing defects necessary for structural interpretation, we compared structural ensembles from cryo-cooled and room-temperature crystal structures. To do so, we have developed new computational tools for electron-density sampling, model refinement, and molecular packing analysis (20).

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temperatures. To access the conformational ensembles, we computationally sampled the electron-density maps using the programs qFit, to build and refine multiconformer models (17), and Ringer, to define unmodeled alternative side-chain conformations (18). These methods sample values of electron density that are usually ignored during model building to discover alternative conformations and distinguish these substrates from harmonic motions of the average structure. Our results show that crystal cryocooling systematically distorts the repertoire of accessible conformations. Conformational ensembles based on room-temperature data generally provide a fuller description of the physiological macromolecular mechanisms than traditional models based on cryogenic data.

Results

Automated Multiconformer Refinement Enables Temperature Comparisons. To compare conformational ensembles, we identified 30 proteins with available high-resolution (2.0 Å or better) X-ray diffraction data collected at ambient and cryogenic temperatures (Table S1). For 17 of the 30 pairs (including our data on CypA), the same investigators collected the X-ray datasets. Additionally, 20 of the 30 pairs were crystallized in nearly identical conditions (differing only slightly in concentrations of buffer, precipitant, salt, and cryoprotectant). The datasets have $R_{\text{merge}}$ values indicating no unusual radiation damage (Fig. S1), and we excluded data that were collected specifically to examine the effect of temperature on radiation damage.

To reduce biases introduced by different refinement programs and model builders, we rebuilt all 60 models using qFit (17), which automates the discovery and building of alternative poly-peptide conformations, allowing for coupled side-chain-backbone flexibility. The multiconformer qFit models were further refined with phenix.refine (19) to ensure that all models experienced similar restraints on geometry, B-factors and solvent, improving $R_{\text{free}}$ values by an average of 1.0% relative to the deposited structures. Because the mean resolution is high for both the room-temperature (1.44 Å) and cryogenic-temperature (1.30 Å) datasets, the estimated coordinate errors are low (0.05–0.22 Å with a mean of 0.13 Å). In all cases, the coordinate error is smaller than the rmsd for equivalent non-hydrogen backbone atoms (mean = 0.34 Å), suggesting that small but significant temperature-dependence differences exist between the models.

To further access conformational ensembles, we used the program Ringer (18). Ringer samples electron density around side-chain dihedral angles and identifies peaks representing discrete alternative conformations at levels of electron density traditionally considered to be dominated by noise. This information can provide an estimate of the distribution and relative populations of numerous unmodeled alternative side-chain conformations. The conformations identified using a combination of Ringer and manual model building agree generally with those identified using qFit (Fig. 1 and Fig. S1B).

Cryocooling Remodels Side-Chain Conformational Distributions. To test the idea that cryocooling affects more than the harmonic vibrations (Fig. S2A) of the conformational ensemble, we used Ringer to sample the electron density around all residues with a rotatable $\chi_1$ angle (4,008 residues from 30 proteins). For each residue, we calculated a $\chi_1$-correlation coefficient between the room-temperature and cryogenic electron-density distributions. The $\chi_1$-correlation coefficient establishes the conformational basis for differences in side-chain mobility with minimal dependence on map scale or resolution. If conformations are unperturbed and residues only experience a loss of thermal motion, then the electron-density distributions of the room-temperature and cryogenic datasets are highly correlated. At room temperature, for example, Asn106 in asparaginase has a lower electron density peak and wider edges that reflect increased thermal motion, which is well modeled by B-factors and yields Ringer plots with a correlation coefficient of 0.99 (Fig. 2Ai). Most residues (69.3%) have $\chi_1$ correlation coefficients greater than 0.9, which indicates the primary effect of cryocooling is to reduce thermal motion around conformations with similar populations at both temperatures (Fig. 2B).

However, Ringer plots with lower correlation coefficients highlight a variety of complex, cooling-induced changes to side-chain conformational distributions (Fig. 2A). For example, Leu98 from CypA (Fig. 2Aii) and Ser109 from superoxide reductase (Fig. 2Aiii) demonstrate alternative conformations that have reduced populations at cryogenic temperatures. Additional residues with Ringer correlation coefficients below 0 indicate that cryocooling induces a new conformation that was not significantly occupied at room temperature. For example, trypsin Val53 in the complex with bovine pancreatic trypsin inhibitor (BPTI) switches from the 180° rotamer to the previously unoccupied ~60° rotamer (Fig. 2Aiv). Using a conservative correlation cutoff of 0.85 to determine if cryocooling changes the side-chain conformational distribution (by reducing the populations of alternative conformations or switching rotamers), we found that 18.9% of all residues are remodeled at $\chi_1$ by cryogenic data collection (Fig. 2B). In agreement with the idea that solvent interactions are altered at cryogenic temperatures (9, 14), residues at structurally unconstrained solvent-accessible sites are most susceptible to these effects. However, cryocooling remodels the $\chi_1$ angle for 7.8% of all buried residues (Fig. 2B). Because differences in the models precluded simple comparisons of the Ringer correlations at other side-chain dihedral angles, these results represent a lower limit on the effect of cryocooling on side-chain conformations that can be detected in the electron density.

To assess the effects of cryocooling at higher $\gamma$-angles and account for main chain variations, we examined alternative conformations from the qFit models. For each residue at each $\gamma$-angle, we calculated the maximum absolute change in rotamer occupancy between the room-temperature and cryogenic ensembles. Importantly, this measure can compare any combination of multi-conformer or single-conformer residues. We found that 13.1% of residues have at least a 20% change in occupancy at $\chi_1$...
Solvent and Lattice Compression Remodels Surface Residues. To examine whether the driving forces that remodel side-chain ensembles are consistent with previous studies of the temperature dependence of crystal structures (10, 20), we measured changes of unit-cell volume, protein volume, and lattice contacts. In our dataset, 27 of 30 crystal unit-cell volumes contract upon cryocooling (mean contraction = 3.4%), and the unit-cell volumes generally shrink more than the protein volume (mean contraction = 1.4%) (Fig. S4). Although most atoms move toward the center of mass upon cryocooling, isotropic compression alone is insufficient to account for the coordinate differences, as previously observed for myoglobin (8) and alpha-lytic protease (6). Increases in protein volume can occur by the formation of new lattice contacts (Fig. S3), resulting in expulsion or thermal contraction of solvent (20). To assess the effects on lattice contacts, we measured occupancy-weighted non-hydrogen atomic contacts at a 3.5-Å distance cutoff (Fig. S4). Increases in lattice contacts (mean = +17%) suggest that cryocooling creates a larger contact area by reducing the solvent channels. Thus, cryocooling increases the crystalline nature of the protein, changing the relatively loose association between molecules at room temperature into a more stable lattice that buries more surface area. This lattice stabilization, combined with reduced thermal motion at lower temperatures, might explain improvements in diffraction resolution observed at cryogenic temperatures. Alternatively, cryocooling in unsuitable solvents can cause dramatic losses in diffraction quality if...
Quantitative Improvements in Protein Packing Remodels Buried Residues. Changes to crystal contacts and surface mobile loops, however, do not explain why buried residues change in response to crystal cryocooling. Because cryocooling decreases the protein volume without changing the number of atoms, we reasoned that a major effect would be to change the quality of packing. To test this hypothesis, we used RosettaHoles2 (21), which calculates the distribution of small interior packing defects and large voids. Our results confirmed that the cryogenic structures have superior packing compared to the isomorphous high-resolution room-temperature structures (Fig. S4B). The rubredoxin models from joint neutron and X-ray studies, where the incomplete deuteration of the cryogenic structure may be incorrectly modeled during refinement (22), were the only exception to this trend. A possible caveat to this pattern is that the cryogenic structures are generally determined at higher resolution, and high-resolution refinement can produce a better description of packing. However, even for the five structures where the room-temperature data were collected to higher resolution, the cryogenic structures demonstrate superior packing.

To examine how this loss of packing defects relates to function, we examined the spatial distribution of temperature-dependent packing defects in CypA. Several packing defects (indicated by letters in Fig. 3B) occur in both the cryogenic and room-temperature models, which may enable functional protein flexibility. For example, Defect A, which is present at both temperatures, abuts a loop that participates in a conformational exchange process observed by NMR (23). Mutations that change the local packing surrounding this void impact the loop conformation and modify the affinity of primate CypA homologs for the HIV capsid (24).

In addition to the cavities present at both temperatures, the room-temperature structure contains several small packing defects (indicated by numbers in Fig. 3B) that are absent at cryogenic temperatures due to the compression of the protein. Three temperature-dependent small cavities occur adjacent to residues with alternative conformations that are essential for catalysis (16) (Fig. S5).

The Room-Temperature Ensemble Reveals Fluctuations in H-Ras. In CypA, crystal cryocooling preferentially biases alternative side-chain conformations responsible for conformational exchange detected by NMR relaxation experiments (16). A coordinated analysis of X-ray and NMR data uncovered a correspondence between the regions of the protein that are sensitive to cooling and those that are mobile in solution. To test the generality of this idea, we analyzed the small GTPase H-Ras bound to the GTP-analog, GMPPNP, by comparing high-resolution room-temperature (1.31 Å) and cryogenic (1.26 Å) X-ray data (25) to NMR relaxation dispersion experiments (26).

Experimental and computational studies (27, 28) have established the basis for long-range conformational coupling and the effects of effector proteins in the GDP- to GTP-bound allosteric switch in small GTPases such as Ras. NMR studies of H-Ras (26) suggested an additional intrinsic conformational rearrangement within the GTP-bound state (Fig. 4A). However, the high-resolution crystal structure of H-Ras bound to GMPPNP paradoxically revealed a single conformation, which is not poised for hydrolysis. This catalytically incompetent conformation left unresolved the basis for the fluctuations observed by NMR. Because this conformational exchange process is not structurally defined, it is unclear if these motions promote interactions such as binding to effectors such a GTPase Activating Protein (GAP) (29) or populate catalytically competent conformations.

In the ensembles of H-Ras:GMPPNP, the catalytically incompetent conformation dominates at both cryogenic and room temperatures. Unexpectedly at room temperature, we detected an alternative, catalytically competent Gln61 conformation at low values of electron density below the 1σ level traditionally used for model building (Fig. 4B and C). In the minor conformation of the active site, Gln61 switches rotamers and positions a water molecule that is poised to attack the terminal phosphate (Fig. 4C). This new Gln61 rotamer resembles the active conformation stabilized by the Ras-GAP complex with the transition state analog, GDP-AlF₃ (29, 30) (Fig. 4B and Fig. S6A).

In agreement with the NMR relaxation data (26), many residues surrounding Gln61 and extending to helix 3 show temperature-induced changes in side-chain conformational distributions (Fig. 4C and Fig. S6B). Temperature-dependent differences in these areas are apparent in electron-density maps calculated in different ways and are not likely due to changes in radiation damage or global crystal quality (Fig. S7). One network identified by NMR is centered on His94 in helix 3 (Fig. 4A). Strikingly, the room-temperature electron-density map revealed at low levels of electron density alternative conformations in residues along helix 3—including two His94 conformations—whereas the cryogenic map contains only a single conformation (Fig. S4). The minor substates observed at room temperature encompass a subtle repositioning of helix 3 associated with the new rotamer distributions of His94 and neighboring residues.

To understand how H-Ras moves from a diverse ensemble at room temperature to a more unique structure upon cooling, we

Fig. 4. Cryocooling quenches an intrinsically flexible network in H-Ras. (A) Residues with detectable conformational exchange by NMR (26) (brown, orange, and red) mapped onto the structure of GMPPNP-bound H-Ras (PDB ID code 5P21). Residues colored red were fit to a collective exchange rate, and residues colored brown were too broadened to permit classification into a collective exchange process. Gln61 is thought to position a catalytic water molecule (H₂O) intrinsically within the hydrolysis of GTP. (B) Models built into room temperature (red and orange), but not the cryogenic (blue), electron-density maps reveal two conformations of Gln61. Gln61 fluctuates to a catalytically competent conformation resembling the GDP-AlF₃ transition state complex stabilized by GAP binding (magenta). Electron-density maps are shown at 1.0 (blue) and 0.3 (cyan) σ contours. The X-ray datasets are derived from a single study of H-Ras crystals grown in a single condition. (C) Ringer electron-density sampling reveals Gln61 and surrounding residues in the active site display conformational differences between the room-temperature structure (red) and cryogenic structure (blue).
examined changes in packing. At the end of helix 3, a cavity collapses upon cryocooling, which causes changes in relative populations of alternative conformations of the surrounding residues (Fig. 5 B and C). The movement of helix-3 residues Arg97, Glu98, and Lys101 into the cavity at cryogenic temperatures causes the small backbone rearrangements that stabilize the single rotamer of His94. Collapse of the cavity propagates through a local backbone shift, which reduces side-chain disorder. In agreement with the NMR results, this side-chain disorder traverses the protein through residues that are flexible in solution to the active site 20 Å away (Fig. 4/4 and Fig. S6B). Thus, unlike the jigsaw-puzzle-like packing of the cryogenic structure, the room-temperature ensemble reveals alternative conformations across a network that corresponds structurally with the conformational exchange processes observed by NMR.

Discussion
By defining previously undetected protein conformational ensembles rather than unique structural models, our analysis suggests that the nearly universal practice of cryocrystallography shifts the intrinsic populations of conformers. Pervasive and specific modifications to protein side-chain distributions occur upon crystal cooling. These biases across 30 pairs of structures are associated with compression of the protein, increases in lattice contacts, and reduction of thermal motion. For CypA and H-Ras, crystal cooling restricts coupled motions precisely in flexible regions detected in solution using NMR. These patterns are unlikely to arise from differences in radiation damage between room temperature and cryogenic data collection.

Because alternative conformations reflect nearly iso-energetic substrates (31), changes in the energy landscape induced by cooling can readily shift populations in the ensemble. Low-entropy, low-entropy conditions at low temperatures change solvent inter-

actions, reduce packing defects, and redistribute alternative conformational populations. Our results support the theoretical prediction that crystal cooling is too slow to trap the equilibrium side-chain distributions at both solvent-exposed and buried positions of proteins (15). Consistent with Halle’s dynamic quenching theory (15), cryocrystallography causes anisotropic and idiosyncratic effects on the contraction of each protein and the quality of local packing (Fig. 3). These tendencies, including the tightness of core packing and reduced protein volume, may percolate throughout computational biophysics, where accuracy is often judged by recapitulating features of high-resolution cryogenic X-ray structures.

Understanding how individual proteins and solvents respond to crystal cryocooling may help reconcile the static images derived from traditional X-ray crystallography experiments with the dynamic view of proteins from solution NMR experiments (32) or comparisons of multiple independent crystal structures (33). X-ray crystallography is intrinsically an ensemble measurement, even though unique models are normally used to communicate the results of X-ray diffraction experiments. By detecting minor populations at electron-density levels often thought to be dominated by noise, recently developed methods including qFit and Ringer enhance access to alternative conformations from individual crystals. Ensembles detected in room-temperature electron-density maps of CypA (16) and H-Ras (Figs. 4 and 5) structurally define important, functional, conformational exchange processes that also occur in solution.

Our analysis suggests that crystal cryocooling restricts H-Ras from sampling conformations that could promote GTP hydrolysis. Electron-density maps calculated with data obtained at room temperature reveal hidden alternative conformations in which Gln61 populates the catalytically competent conformation. The discordance between the rate of NMR fluctuations (micro-second-millisecond) and the rate of turnover (minutes-hours) suggests that this conformational transition does not limit the rate of intrinsic GTP hydrolysis. The presence of multiple independently correlated processes, indicated by NMR and our analysis of the X-ray data, implies that the inefficiency of intrinsic hydrolysis may result from uncorrelated motions around the catalytic center. Alternatively, the conformational plasticity of Gln61 may facilitate a conformational selection mechanism of GAP binding to the catalytically competent conformations. This model makes the prediction that perturbations along the network, such as binding of effectors or mutations along helix 3, could increase conformational coupling to alter the functional properties of H-Ras. Indeed, adventitious Ca$^{2+}$ and acetate binding in the allosteric cavity stabilizes the active conformation of the Gln61 network in crystals (34) (Fig. S6C), and a mutation that adds hydrophobic bulk to this cavity (Lys101Leu) switches cellular morphologies associated with Ras activation of the Raf kinase (35). Raf interacts with H-Ras near the active site over 20 Å away from the site of the mutation, functionally linking an expanded cavity to the conformation of the active site (Fig. S6C). NMR measurements indicate that mutations that disrupt hydrogen bonds along this network alter interactions between H-Ras and the GTP-analogue (36). In addition, mutations of H-Ras paralogs that abut or are located on helix 3 (Arg68Thr, Asp69Asn, Met72Leu, Asp92Asn, Asp92Tyr, Arg97Gly) have been identified in cancers (37), supporting the idea that the network is critical for the function of H-Ras (Fig. S6C).

Our results suggest that X-ray crystallography—along with simulations (38), sequence analyses (39), and NMR experiments (40)—can offer increasing access to conformational ensembles required for protein functions. Ringer and qFit are best employed using X-ray data to at least 2.0 Å resolution, and almost half of recent PDB depositions meet this threshold. In the cases we have examined, linking ensemble analysis to the solution distribution of substrates requires room-temperature X-ray data. However, in recent years, room-temperature X-ray data collection has
become quite rare, and cryogenic structures dominate the PDB. In our experience, many crystals that diffract to high-resolution at cryogenic temperatures can be used to collect comparable room-temperature data at a synchrotron. Our results suggest that effort should be put toward the redeployment of data collection techniques at room temperature with careful attention to standardizing practices that mitigate radiation damage. A combined strategy of ensemble analysis and room-temperature X-ray data collection can be applied to many proteins to define conformational substates linked to ligand binding, catalysis, and allosteric regulation.

Materials and Methods

Structure factors in cif format were downloaded from the PDB and converted to mtz format with phenix cif_as_mtz (19). When no \( R_{\text{free}} \) set was deposited or could be extracted, we chose a test set using the standard parameters in phenix.refine (19). Structures were rebuilt using qFit (17) and then refined for our purposes or could be extracted, we chose a test set using the standard parameters in phenix.refine (19). Structures were rebuilt using qFit (17) and then refined for five additional cycles in phenix.refine, with occupancy refinement and automated solvent picking. For all models, we included riding protein hydrogen atoms in refinement. At a c.l.-Å resolution were refined with anisotropic B-factors for all nonsolvent and nonhydrogen atoms. Unit-cell volumes were calculated with mathtools, coe from CCP4 (41). Protein volumes, calculated, with 3V (42), and packing scores, calculated with RosettaHoles2 (21), are based on all matching nonhydrogen atoms of the primary structure. Residues with side-chain-up half-sphere exposure (radius = 13 Å) greater than 24 were considered buried (43). All structural figures were prepared with PyMOL.

Ringer analyses of 1 electron density were performed by sampling from the major conformation in unfilled cross-validated, maximum-likelihood-weighted, 2mFo-DF maps created with phenix.maps with a grid set to 1/5 of resolution. Although the maps are from isomorphous crystals at high resolution, to reduce the effect of map scaling and resolution, we used the correlation coefficients of Ringer electron density distributions in 10° increments to determine the behaviors of side-chain conformational distributions. Multiconformer residues from qFit models were analyzed by summing the occupancy of each alternative conformation into the nearest rotamer bin (60°, 180°, 300°), for individual y-angle analysis or into the nearest rotamer (44) assigned by phenix.ratyalyze, for the analysis across all angles. The maximum absolute differences in rotamer occupancy between the room-temperature and cryogenic ensembles range from 0, indicating that the conformational distribution is identical, to 1, indicating that there is no agreement in rotamer populations. The average maximum rmsd between room temperature and cryogenic models increased by 0.11 Å (\( p = 1.7 \times 10^{-10} \)) for all residues, 0.09 Å (\( p = 3.6 \times 10^{-10} \)) for buried residues, and 0.12 Å (\( p = 3.0 \times 10^{-10} \)) for solvent-exposed residues. A two-sided paired t-test was used to determine significance.

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