Getting protein solvent structures down cold

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At the heart of biochemistry are molecular interactions with solvent and hydrogen bonds. Yet, experimental placement of hydrogen atoms is supported only in a limited number of cases with x-ray crystallography, the preeminent technique providing atomic resolution molecular models for proteins 40 kDa and larger. This lack of experimental evidence leads to ambiguity in explication of enzymatic mechanisms and uncertain fits when trying to match ligand-binding sites. It is the experimental determination of hydrogen atom position, especially labile hydrogens, that is fueling the resurgence of neutron protein crystallography (1–3). Newly commissioned and planned facilities, combined with improved detectors and the use of a broader spectrum of available neutrons, are increasing the number of structural problems that can be solved with neutron diffraction (4–6). In this issue of PNAS, Matthew Blakeley, Joseph Kalb (Gilboa), and John Helliwell collaborate with Dean Myles to extend the group's previous x-ray and room-temperature neutron diffraction studies (1, 7) of the jack bean protein, Con A, with a low-temperature neutron diffraction study completed by using the quasi-Laue diffractometer (LADI) at the Institut Laue-Langevin (ILL) in Grenoble, France (8). The outcome of this research is a further extension of the range of protein crystallographic problems addressable with neutron diffraction.

Neutrons are scattered by the atomic nucleus, and x-rays are scattered by the orbiting electrons. As a consequence, the scattering from any nucleus is within the same order of magnitude regardless of atomic Z number, making neutrons unparalleled for imaging lighter atoms in the presence of heavier atoms (9). Although the number of ultra-high-resolution x-ray diffraction studies of proteins has been increasing, the number of crystals that diffract to 1.1 Å, the minimum resolution needed for hydrogen atom placement in a macromolecular diffraction data set, is a limited subset of all protein crystals. Neutron diffraction supports hydrogen atom modeling at much lower resolution; Blakeley *et al.* were able to assign hydrogen positions with 2.5-Å data. As can be seen in Fig. 1, labile hydrogens are more likely to be modeled with neutron data than with the best x-ray data (8, 10). For all crystallographic experiments, the lower the diffraction data measurement temperatures, the lower the atomic thermal parameters. Lower thermal parameters enhance the likelihood of a hydrogen atom within the molecule being visible in density maps. However, even with bonding atom B factor values up to 18 Å^2, nearly 100% of hydrogens are still observable in the neutron structure; in the x-ray structure the number of observed hydrogens approaches zero. The two plots are not strictly equivalent. The plot of Howard *et al.* (10) reports the percentage of x-ray-observed hydrogen atoms as a function of the B factor of the protein atom to which they are bound. In contrast, the neutron data refer to the solvent shell, and these solvent atoms tend to have higher B factors than do the protein atoms they surround. The observation of so many heavy water (D_2O) deuterium atoms is therefore particularly striking.

Neutron beamlines for protein crystallography are currently operating in Japan (biological crystallography stations BIX 3 and BIX 4 at the Japan Atomic Energy Research Institute) and France (LADI at ILL) and at the Protein Crystallography Station of the Los Alamos Neutron Science Center spallation source at the Los Alamos National Laboratory. New protein crystallography beamlines and detector upgrades have been proposed at ILL, at the spallation neutron sources in Japan (Proton Accelerator Research Complex, J-PARC), in the United Kingdom (Target Station 2 of ISIS), and at Oak Ridge National Laboratory (Macromolecular Neutron Diffraction Station at the Spallation Neutron Source, www.pns.anl.gov/instruments/mandi/mandi.html). At all such facilities, four key areas have been identified as necessary adjuncts to fuel successful widespread access to neutron protein crystallography: dedicated deuteration facilities for production of perdeuterated protein (the exchange of all hydrogen atoms with deuterium isotope); improved means of growing large, perfect crystals for diffraction; the development of software for molecular refinement of x-ray diffraction data with neutron diffraction data (X + N data refinement) from the same crystal; and rational and reliable means of flash-cooling large crystals for neutron diffraction studies. The experiments described in the paper of Blakeley *et al.* are excellent first steps in the extension of low-temperature studies to high-resolution neutron protein crystallography.

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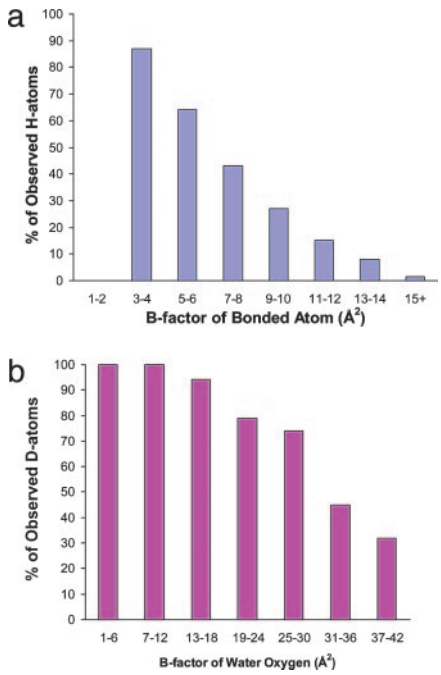

Fig. 1. Likelihood of seeing hydrogen atoms based on the B factor (a measure of atomic thermal motion and disorder) of the bonded atom. (*a*) Plot of observed hydrogen atoms of aldose reductase based on 0.66-Å-resolution x-ray diffraction data (10). (*b*) Plot of observed deuterium atoms in the solvent shell of Con A based on 2.5-Å-resolution low-temperature neutron diffraction data from Blakeley *et al.* (8).

Because of the reduced flux from neutron sources compared with x-rays, every enhancement of diffraction is critical (11). One means of boosting the signal is to replace hydrogen with the deuterium isotope, by exchanging the crystal solvent or protein perdeuteration. Hydrogen causes incoherent scat-

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tering, increasing the background and reducing the signal. A minimum of 10^{15} ordered unit cells are needed at most neutron sources for a high-resolution protein crystallography experiment (8). This number can be reduced by 2 orders of magnitude if the protein in the crystal is perdeuterated; it would then be similar in size to many standard sized x-ray diffraction crystals. Another means of enhancing diffraction is to minimize atomic thermal parameters by lowering the temperature of data measurement (usually to 100 K or lower). This technique is a common practice in x-ray crystallography because lower temperature can also mitigate the effects of radiation damage. Neutrons, in contrast, induce no radiation damage in the molecules of the diffracted crystal. Flash-cooling the larger crystal needed for neutron diffraction studies requires plunging the crystal into cryogen; a cold stream of gas will not reliably provide a cooling rate sufficient to preclude ice formation. However, as Blakeley *et al.* show in figure 2 of their paper, plunging into liquid N_2 for cooling can cause destruction of the lattice near initial contact with the cryogen. Blakeley *et al.* attribute the loss of diffraction to the Leidenfrost effect with the high initial crystal temperature causing vaporization at the crystal surface. Alternatively, this damage at the leading edge could be mechanical disruption due to the initial contact between the crystal and the liquid, analogous to the forces seen on the front and sides of a car traveling down the highway. Based on this paper, crystals flash-cooled in liquid cryogen should be measured furthest from the point of initial contact. With the lattice lost in a portion of the crystal, the number of ordered unit cells is reduced with concomitant loss of diffraction intensity.

With current expertise, flash-cooling large crystals will not work with every protein. So far, the experiments that have succeeded are with crystals of lower solvent content and solvent channel size than is the norm for proteins (8, 12). Our own preliminary studies with flash-cooling large D-xylose isomerase crystals by plunging into liquid N_2 has shown that larger crystals need significantly higher concentrations of cryoprotectant to prevent ice nucleation and crystallization during cooling. This increased concentration of cryoprotectant can destabilize the lattice degrading the crystal structure. A more general problem in protein cryocrystallography is the infiltration of cryoprotectant into the binding site, replacing solvent or the desired substrate. More work is needed to predict the cryoprotectant requirements and a flash-cooling strategy.

In light of the difficulties in flash-cooling large crystals for neutron diffraction, are low-temperature neutron experiments justified when there is no radiation damage? Yes, because there are greater benefits than lowering the atomic *B* factors. Here, again, Blakeley *et al.* have led the way by initially measuring neutron data at room temperature, followed by low-temperature neutron data measurement and then by x-ray data measurement. Such experiments may provide the only means of comparing the great majority of x-ray structures measured at low temperature with room-temperature conditions more closely mimicking physiology. In Con A, differences are seen in the orientation of water molecules between room temperature and low temperature. Blakeley *et al.* rightfully suggest that this could have broader implications in structure-based ligand design. Other candidates for low-temperature neutron studies will include proteins with relatively ephemeral crystals that degrade at room temperature, and crystals of freeze-trapped intermediates. In addition, the measurement of x-ray and neutron diffraction data from the same crystal will increase the number of observations for each molecular parameter, increasing the reliability of the model produced from combining both data sets. At present, software does not exist to do this, but with increasing opportunities to measure both neutron and x-ray data from the same crystal, molecular models of proteins that include experimentally determined hydrogen positions should prove commonplace.

I thank Drs. Kristin Kirschbaum, Juliette Devos, and Constance Schall for editorial assistance. This work was supported, in part, by National Aeronautics and Space Administration Grant NAG8-1838.

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