order of magnitude of these calculated times is confirmed by the dielectric relaxation data, which gives relaxation frequencies at ~200 and 700 MHz for Hf ATP at pH 9. If the attenuation of the angular correlation is due solely to time-dependent processes, the change with pH could imply either faster rotational diffusion or a speedier electronic rearrangement after the β decay. On the addition of the viscosity increasing agents, up to 4% PAA and also a PAA gel, the attenuation coefficient goes to zero within the accuracy of the measurements. This result is incompatible with the expected change in the rotational motion when the viscosity is increased, and suggests that the strong attenuation is due to nuclear decay aftereffects. Dielectric relaxation data, however, show no significant change in the observed relaxation frequencies for Hf ATP at pH 9 upon the addition of 2% PAA; this might suggest the importance of group motion within the molecule. Angular correlation and dielectric relaxation measurements are continuing in these systems.

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DIFFRACTION ANALYSIS OF MOTION IN PROTEINS

Wayne A. Hendrickson and John H. Konnert, *Laboratory for the Structure of Matter, Naval Research Laboratory, Code 6030, Washington, D. C. 20375 U.S.A.*

BACKGROUND

Analysis of the x-ray or neutron diffraction from crystals gives only an average picture of the contents of a crystallographic unit cell, the average being over a long time (usually many hours) and the entire lattice. However, this picture can rather accurately define not only the mean atomic positions but also the distribution of displacements from rest positions. These displacements may include components from three main sources: (*a*) thermal vibration, both of individual atoms and of rigid groups, (*b*) dynamic disordering among thermally accessible conformational states, and (*c*) static variations among the structures within different unit cells, either due to lattice imperfections or because of conformational heterogeneity that is frozen in at the experimental temperature. Thus the accumulated spoor of atomic motions is accessible to the diffraction experiment even though the dynamic pathways are not.

Proteins and other biological macromolecules are typically rather flexible and the lattice contacts in crystals of these large molecules are usually much less restrictive than are those in the tightly knit crystals of small molecules. This often results in extensive atomic motion and disorder. Atomic displacements cause a decrease in coherent scattering that is exponentially related to scattering angle and to the breadth of the distribution of displacements. Consequently the diffraction intensities from proteins usually diminish rapidly with scattering angle and this greatly limits the extent of measureable data.

The intensity of each diffraction spot depends in a known way on the distributions, for all atoms, of the components of displacement normal to the Bragg plane of the reflection. Hence in the course of the refinement of an atomic model against diffraction measurements one can determine the characteristic parameters (often called thermal parameters) of the distributions of atomic displacements. The function used to model these distributions can be formulated at various levels of sophistication (1). However, the number of variables increases greatly when anisotropic and anharmonic effects are considered. Large displacements are likely to be anisotropic and anharmonic; but if displacements are large, measurable data are few. Thus just where higher order effects are most pronounced they tend to become indeterminate.

STEREOCHEMICAL RESTRAINTS

The motions and disorder in a typical protein crystal engender a paucity of data that limits the resolution of electron-density maps and, thereby, the accuracy of initial atomic models. This also adversely affects the observations-to-parameters ratio. These limitations together with the sheer size of the computational problem have tended to frustrate attempts to refine these models. We have developed a new refinement method that copes with these problems by imposing stereochemical restraints to overcome the paucity of data and by using conjugate-gradient procedures to make the problem computationally manageable (2, 3).

The stereochemical restraints take cognizance of the vast knowledge accumulated about the chemistry and geometry of the components from which macromolecules are built. Known features include bond lengths and angles, planarity of certain groups, chirality of asymmetric centers, nonbonded contacts, and restricted torsion angles. Stereochemical knowledge can take the form of an expected value for some feature, a bond length for example, and the variance of the distribution of values it might take. A protein model can be simultaneously refined against the diffraction data and these stereochemical observations.

Stereochemistry has implications for thermal parameters as well as for positional parameters. The rms displacements from mean atomic positions in macromolecules are high, usually several tenths of ångströms; whereas the variation with time in covalently bonded distances is generally not greater than a few hundredths of ångströms. Variations in distances associated with bond angles is also quite limited. Thus atomic motions must be highly correlated. Qualitatively, if an atom undergoes large displacements from its mean positions then certain other stereochemically related atoms must undergo similarly large, concerted displacements. The thermal parameters that describe atomic displacements should be made to agree with known stereochemistry. We have imposed this stereochemistry by restraining the variances of time-dependent interatomic distance distributions to suitably small values (4).

The variance in a bond length due to dynamic fluctuations can be calculated from the mean-square displacement $\overline{u^2}$ of the bonded atoms provided that a particular joint distribution of displacements is assumed. If this correlation of atomic motions is described in a manner

analogous to "riding motion," then the fluctuation variance V in an interatomic distance d_{ij} is

$$V_\nu = (\Delta_\nu^2 \cos^2\theta + \Delta_\nu^4 [\tfrac{1}{2} \sin^4\theta - 3 \cos^2\theta \sin^2\theta]/d_{ij}^2),$$

where $\Delta^2{}_\nu = |\overline{u^2}_{\nu,i} - \overline{u^2}_{\nu,j}|$ and θ is the angle between the bond direction and a specific direction of motion ν. The most restrictive condition arises when ν coincides with the interatomic vector, but relative displacements in any direction increase the variance. In the case of isotropic motion the overall variance reduces to $V = \Delta^2 = |B_i - B_j|/8\pi^2$.

REFINEMENT APPLICATIONS

One of the first applications to include thermal factor restraints was an isotropic refinement of yeast phenylalanyl tRNA (4). The rms variances obtained for distance fluctuations ranged from $(0.05)^2$ for bond distances to $(0.11$ Å$)^2$ for bonding angle distances. Despite this close conformity of thermal factors to known stereochemistry, atomic displacements exhibit large and meaningful variations suggestive of rigid body motions. The inclusion of this thermal factor treatment in the refinement also greatly improved the clarity of difference maps and permitted the identification of bound spermine molecules and magnesium hydrate ions (5).

The careful refinement of *S. griseus* protease A (SGPA) also included isotropic thermal restraints (6). The R-value is 0.14 at 1.8 Å resolution with excellent geometry. Variations in the resulting thermal values correlate with enzymatic function. Stretches of polypeptide chain that comprise the substrate binding region of SGPA have some of the largest atomic displacements in the molecule. Moreover, displacements for these regions in three equally well refined enzyme-substrate complexes of SGPA have decreased substantially whereas thermal values elsewhere are comparable to the native values (M. N. G. James, personal communication).

A restrained anisotropic refinement of the carp calcium-binding protein has shown that meaningful refinement of anisotropic thermal parameters can be made even at 2 Å resolution (4). The results confirm the expectation that the vibrational behavior of atoms in protein molecules can be highly anisotropic. In this case the average fluctuation variance was held to $(0.03$ Å$)^2$. Tighter restraint is possible here since the anisotropic model is more realistic. An anisotropic refinement of bovine pancreatic trypsin inhibitor (7) is in progress.

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