Fluctuations and Averaging of Proton Chemical Shifts in the Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: The effects of motional averaging on the aromatic ring-current contribution to the proton chemical shifts in proteins are examined. Atomic trajectories obtained from a 96-ps molecular dynamics simulation of the bovine pancreatic trypsin inhibitor are used in conjunction with the Johnson–Bovey model of ring-current shifts to calculate the time evolution of the proton chemical shifts. Although large high-frequency fluctuations are observed (often greater than ±1 ppm), the average shift values in most cases are close to those obtained from the average structure; for some protons, significant differences are found. The calculated trends are used to probe the relationship between the average structure, atomic motions, and observed values of the proton chemical shifts. It is concluded that chemical shift values are in general most sensitive to the average structure of the protein and, because of the averaging involved, cannot be used directly to probe the short time structural fluctuations.

It is now established that protein molecules are not rigid but undergo significant fluctuations about an equilibrium structure; this subject has been recently reviewed in considerable detail (Gurd & Rothgeb, 1979; Karplus & McCammon, 1981). The fluctuations are revealed experimentally in studies of X-ray diffraction temperature factors, nuclear magnetic relaxation rates, fluorescence quenching, and depolarization and hydrogen-exchange kinetics. Theoretical predictions of the magnitude and time course of the fluctuations have been obtained from molecular dynamics simulations. For most proteins whose structures have been determined, however, only the average positions of atoms in the crystal are available. These positions have generally been obtained by refinement procedures which fit them to the X-ray data, subject to constraints on the bond lengths and angles. For several small proteins, X-ray diffraction, NMR,[1] and other techniques have begun to provide a more complete description of protein structure by supplementing the average atomic positions with information concerning the directions, magnitudes, and frequencies of the positional fluctuations (Karplus & McCammon, 1981).

A detailed description of the time dependence of the positions of the atoms is not yet available experimentally for any protein. The only source of such information is from molecular dynamics simulations, which utilize empirical energy functions and integrate the classical equations of motion for the atoms of the protein to determine the fluctuations on a subnanosecond time scale. A detailed study of the dynamics of the bovine pancreatic trypsin inhibitor (PTI) has been made (McCammon et al., 1977, 1979; Karplus & McCammon, 1979), and applications to other proteins, such as myoglobin (S. Swaminathan and M. Karplus, unpublished results), cytochrome *c* (Northrup et al., 1980), and lysozyme (B. D. Olafson, T. Ichiye, and M. Karplus, unpublished results) are in progress. The root mean square displacements of atoms obtained in these simulations are of the order of 0.5 Å and are consistent with temperature factors determined for protein crystals by using X-ray diffraction (Frauenfelder et al., 1979; Artymiuk et al., 1979). NMR parameters such as chemical shifts, coupling constants, and nuclear Overhauser effects are known from experimental studies on a variety of proteins to be very sensitive to the details of the protein structure (Roberts & Jardetzky, 1970; Wüthrich, 1976; Campbell & Dobson, 1979; Poulsen et al., 1980). It is therefore of interest to examine the possible effects of the predicted fluctuations on these parameters. In this paper we focus on the chemical shift values, using PTI as an example. Many of the proton resonances have been assigned and chemical shift values measured for this small protein (Dubs et al., 1979, and references therein).

There are a number of contributions to the chemical shift of a proton in a protein (McDonald & Phillips, 1967; Sternlicht & Wilson, 1967). The most fundamental effects depend on the chemical nature of the group containing the proton and are therefore relatively constant for protons in a given type of amino acid residue in a protein. Other contributions to the chemical shift arise from through-space interactions between a proton and the residues in its vicinity; these are of major interest in studies of the structures of folded proteins.

Comparisons of measured and assigned chemical shifts, relative to standard amino acid values, with calculations using static protein crystal structures have shown that the "ring-current shifts", which arise from the diamagnetic anisotropy of aromatic rings, often provide the dominant through-space contribution for protons bonded to carbon. For methyl groups in particular, there is good agreement between experiment and calculations which consider only ring-current effects (McDonald & Phillips, 1967; Dobson, 1977; Perkins & Wüthrich, 1979; Perkins & Dwek, 1980). A question examined in this study is whether the level of agreement limits the possible magnitude of fluctuations in the protein. In certain cases, for example, aromatic proton resonances, large discrepancies between experiment and the calculated shifts are observed. We determine in this paper whether the neglect of dynamical effects could be a contribution to this discrepancy. In addition, we examine the possibility that high-frequency fluctuations can be responsible for temperature dependences of chemical shifts (Perkins & Dwek, 1980).

We use a 96-ps molecular dynamics simulation of PTI (Karplus & McCammon, 1979) and calculate the ring-current

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[1] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; PTI, pancreatic trypsin inhibitor; ps, picosecond; rms, root mean square.

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contribution to the proton chemical shifts for a set of 653 structures obtained during the simulation. The magnitudes of the high-frequency variations in the shifts resulting from the positional fluctuations are examined. Since the NMR time scale is orders of magnitude slower than that of the calculated fluctuations, mean chemical shifts are determined by averaging over the values obtained at each point of the molecular dynamics trajectory. These mean shift values are compared with those calculated from the atom positions in the average structure obtained in the simulation; the latter corresponds to the structure which would be determined in an ideal X-ray diffraction experiment. Finally, these two sets of shifts are compared with those calculated from a set of average atomic coordinates subjected to energy minimization; in a certain sense this structure corresponds to the one which would be obtained from an X-ray refinement with bond length and bond angle constraints. The results of these comparisons provide the basis for an evaluation of the importance of dynamical effects on the ring-current contribution to proton chemical shifts in proteins.

The method used to calculate the chemical shifts from the molecular dynamics trajectory, the results and their analysis, and the conclusions are presented in this paper.

Methods

The atomic coordinates of PTI utilized in this investigation were those generated in a 96-ps molecular dynamics simulation equilibrated at 306 K (Karplus & McCammon, 1979). A time step of approximately 10^{-3} ps was used in the integration. Of the 10^5 coordinate sets generated in this way, every 150th set was selected for the chemical shift calculation; a total of 653 coordinate sets, one for every 0.147 ps, was used. The dynamical simulation was carried out by using extended atoms; in this approach hydrogen atoms are not explicitly included in the simulation, but their presence is accounted for by adjusting the mass and van der Waals radius of the directly bonded heavy atom. It has been shown by use of a protein potential in which all hydrogen atoms are treated explicitly that the energy-minimized structure is very close to that obtained with the extended atom model and that, in particular, the proton positions change by negligible amounts (D. J. States and M. Karplus, unpublished results). This result indicates that it is appropriate to use the extended atom model in the present calculation; the increase in computer time required for treating all 901 atoms vs. the 458 atoms in the extended atom model would not be justified. Solvent was neglected in the simulation, except that four water molecules which are well-defined in the 1.5-Å resolution crystal structure were included (Diesenhofer & Steigemann, 1975). The crystal coordinates were obtained from the Protein Data Bank at the Brookhaven National Laboratory (Bernstein et al., 1977). Details of the simulation have been reported elsewhere (Gelin & Karplus, 1979; McCammon et al., 1979).

For each coordinate set used in the chemical shift calculations, the 324 protons bound to carbon were generated in standard configurations with bond lengths of 1.08 Å and named according to convention (IUPAC–IUB Commission on Biochemical Nomenclature, 1970). The effects of methyl group rotation were simulated by averaging the computed chemical shift over the three constituent protons of the methyl group in its staggered configuration. For determination of the importance of other configurations to the chemial shift of a methyl group proton, two additional sets of configurations were generated by rotating the methyl groups by 40° and 80° from the staggered configuration. For protons that interact significantly with an aromatic ring, chemical shifts obtained by averaging over all three configurations differed by less than 6% from those computed from the staggered configuration. Consequently, shifts computed for the staggered configuration were used in this investigation.

Isotropic ring-current chemical shifts were determined by use of the Johnson–Bovey approximation (Johnson & Bovey, 1958). The requisite elliptic integrals were evaluated numerically by using a Chebyshev approximation which is implemented as part of the International Mathematical and Statistical Library (IMSL) subroutine package. The Johnson–Bovey ring-current shifts were multiplied by constant factors of 1.0 for phenylalanine and 0.94 for tyrosine, reflecting the intensities of their "ring currents" relative to that of benzene (Giessner-Prettre & Pullman, 1971); no histidine or tryptophan residues are present in PTI.

For the purpose of analysis, the computed shifts were partitioned into angular and radial contributions by means of a dipole approximation (Pople, 1956). This approximation has been shown to yield chemical shifts that are similar to those obtained from the Johnson–Bovey approximation for protons that are not very close to aromatic rings (Perkins & Dwek, 1980). The radial contribution is taken to be proportional to $1/r^3$, where r is the distance from the center of the aromatic ring to the proton. The angular contribution is taken to be proportional to $1 - 3\cos^2\theta$, where θ is the angle between a vector normal to the ring plane and a vector from the ring center to the proton.

Time average values of the functions of interest (atom positions, chemical shifts, $1/r^3$, and $1 - 3\cos^2\theta$) were computed by averaging the functions over the chosen subset of the full trajectory. The results for the average structure make use of the mean atomic positions computed from the same subset of coordinates.

Results and Discussion

Shifts Computed from the Simulation. Examination of the explicit time dependence of the chemical shifts computed from the individual coordinate sets of the dynamic simulation reveals large high-frequency fluctuations for many of the protons. Of the 324 protons examined, 78 protons experienced a range of chemical shifts exceeding 1 ppm; for 39 of these, the computed range exceeded 2 ppm and in one case ($H^{\beta 2}$ of Pro-2) was nearly 6 ppm. Representative examples of calculated shifts are shown in Figure 1, which make clear that major fluctuations arise from motions on the subpicosecond time scale, although longer time variations in the chemical shifts (on the order of several picoseconds) are also apparent in the plots. Data for a number of protons selected to illustrate general aspects of the results are listed in Table I.

In Figure 2 the root mean square (rms) shift fluctuations are plotted against the average shifts from the simulation. An indication of the large magnitude of the calculated fluctuations is the fact that the rms values are in general comparable with the average shifts. A significant fraction of the protons (~80%) are located relatively far away from any aromatic side chain and so have shift and fluctuation values clustered around the origin (±0.1 ppm). Figure 2 also shows that, in general, protons with large average shifts experience large fluctuations. There are exceptions; for example, $H^{\alpha 1}$ of Gly-37 and $H^{\beta 2}$ of Ala-48 (Figure 1c) have average shifts of 0.36 and 0.35 ppm, respectively, while the rms fluctuations are 0.07 and 0.09 ppm. Protons with small average shifts in general have small shift fluctuations, but there are instances in which large fluctuations are observed. An example is $H^{\beta 1}$ of Asp-24, which has an rms shift fluctuation of 0.48 ppm and an average shift of 0.09 ppm.

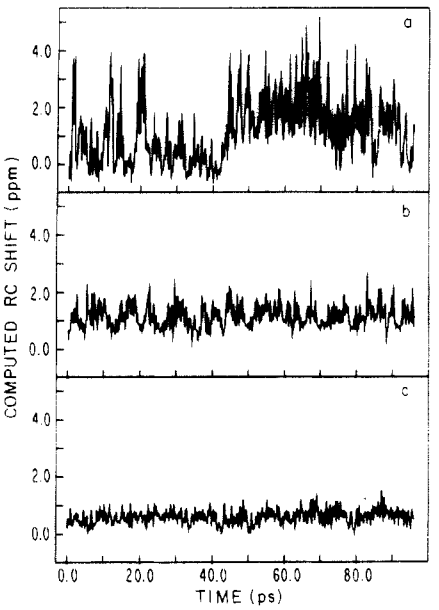

FIGURE 1: Computed ring-current shift fluctuations as a function of time for (a) Pro-9 $H^{\beta 2}$, (b) Gly-28 $H^{\alpha 2}$, and (c) Ala-48 $H^{\beta 2}$.

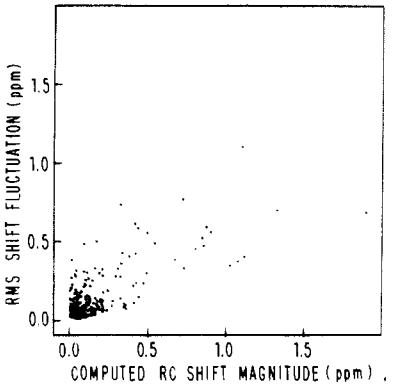

FIGURE 2: Root mean square shift fluctuations plotted against the magnitude of time average ring-current shifts. Each dot corresponds to one of the 324 protons examined.

Table I: Computed Ring-Current Shifts[a]

proton	$\langle\sigma\rangle$	σ_{av}	σ_c	σ_{rms}
Pro-9 $H^{\beta 2}$	1.100 (1.087)	1.749 (1.691)	2.060	1.10
Arg-20 $H^{\beta 1}$	1.324 (1.286)	1.688 (1.639)	0.363	0.70
Phe-22 $H^{\beta 1}$	−0.403 (−0.367)	−0.537 (−0.495)	0.040	0.22
Phe-22 $H^{\delta 1}$	0.170 (0.212)	0.093 (0.150)	0.018	0.50
Asn-24 $H^{\beta 1}$	0.088 (0.049)	−0.076 (−0.097)	0.343	0.48
Gly-28 $H^{\alpha 2}$	1.114 (1.075)	1.222 (1.143)	0.020	0.40
Cys-30 $H^{\beta 2}$	−0.409 (−0.292)	−0.453 (−0.315)	−0.177	0.11
Thr-32 $H^{\gamma 2}$	0.785 (0.804)	0.841 (0.865)	0.774	0.28
Gly-37 $H^{\alpha 1}$	0.358 (0.337)	0.385 (0.361)	0.330	0.07
Ala-48 H^{β}	0.558 (0.583)	0.580 (0.609)	0.407	0.25
Met-52 H^{ϵ}	−0.006 (−0.055)	0.001 (−0.050)	−0.027	0.08
Thr-54 $H^{\gamma 2}$	0.038 (−0.025)	0.046 (−0.025)	−0.353	0.13
Cys-55 $H^{\beta 2}$	0.898 (0.772)	0.997 (0.816)	1.456	0.56

[a] Chemical shifts are in parts per million. In this and the following tables, $\langle\rangle$ denote time average, and the subscripts av, c, and rms denote average structure, crystal structure, and root mean square fluctuation, respectively. Values in parentheses are the ring-current shift from the nearest ring.

In most instances the time evolution of the chemical shift of a proton exhibits random fluctuations about a single average value over the entire simulation, and a nearly normal distribution of the shifts is obtained; representative examples are shown in Figure 3, which makes clear the wide range of distributions. For a number of protons, the calculated shift fluctuates randomly about one average value for part of the trajectory and then is displaced so that it fluctuates about a different average value. The resulting distributions are generally distorted from a normal random distribution. An example is $H^{\beta 2}$ of Pro-9 (Figures 3a and 4a). In other instances the fluctuations in the shift change in magnitude while the average shift remains relatively constant. Examples of this type of behavior are the methyl protons of Thr-54 (Figure 4b) and $H^{\delta 1}$ of Phe-22 (Figure 5). These effects reflect conformational transitions in the region being examined which are rare events on the time scale of the protein trajectory. The physical basis for the shift transitions may sometimes be seen in the time course of nearby torsion angles. For Pro-9 the shift transitions at approximately 42 ps may be attributed to changes in the ψ torsion angle of Pro-8 (Figure 4a). This causes $H^{\beta 2}$ of Pro-9 to move toward the ring of Phe-33; their relative motion is discussed further under Analysis of Averaging Effects. For Thr-54, the changes in the magnitude of the computed shift fluctuations which occur at 55 and 78 ps are due to changes in the χ_1 torsion angle, which rotates the methyl group away from the ring of Phe-22 (Figure 4b). For Phe-22, the shift transitions are due to changes in both the χ_1 and χ_2 torsion angles of this residue (Figure 5). The change in χ_2 at 78 ps indicates a 180° flip of the ring, interchanging protons on opposite sides of the ring. For $H^{\delta 1}$, the small fluctuations and average shift value result from its increased distance from Phe-33; $H^{\delta 2}$, on the other hand, is brought closer to Phe-33 by the ring flip, and its shift fluctuations (which are not shown) become larger at 78 ps. The effects between 50

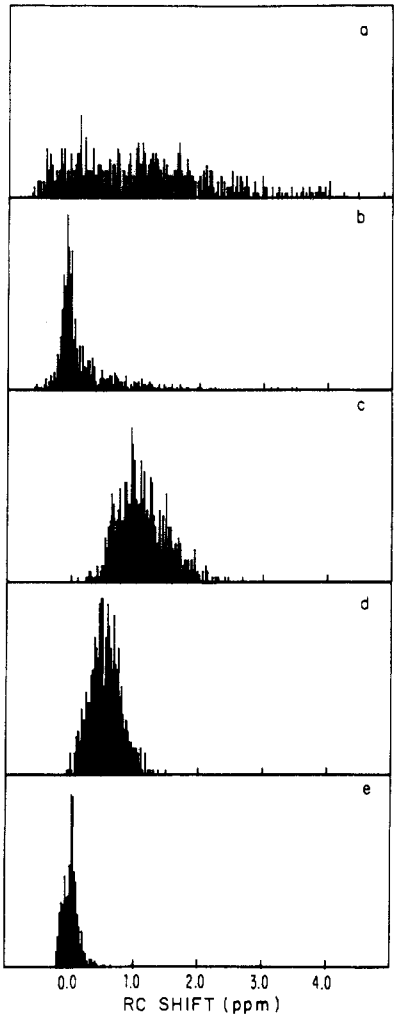

FIGURE 3: Distributions of the ring-current shifts, calculated from the 653 structures examined in this work, for (a) Pro-9 $H^{\beta 2}$, (b) Phe-22 $H^{\delta 1}$, (c) Gly-28 $H^{\alpha 2}$, (d) Ala-48 H^{β}, and (e) Thr-54 $H^{\gamma 2}$.

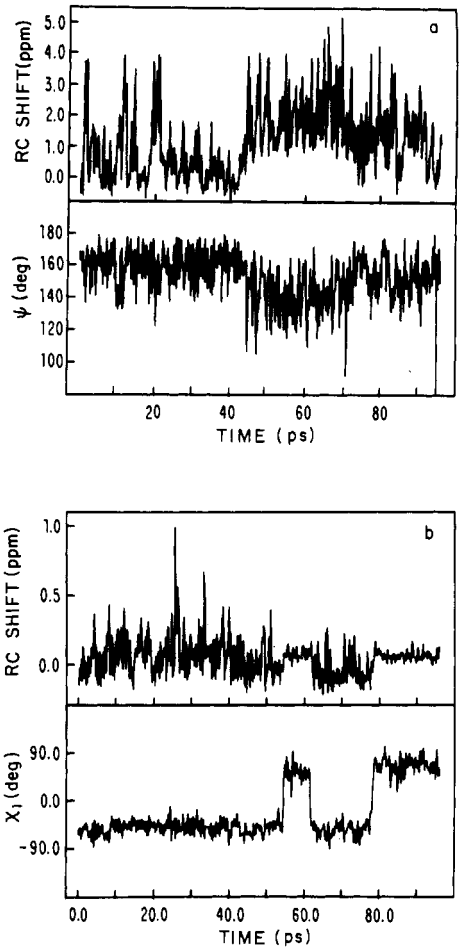

FIGURE 4: Comparison of fluctuations in ring-current shifts with fluctuations in specific torsion angles: (a) shift fluctuations of Pro-9 $H^{\beta 2}$ and fluctuations of the ψ torsion angle of Pro-8 and (b) shift fluctuations of Thr-54 $H^{\gamma 2}$ and fluctuations of the χ_1 torsion angle of Thr-54.

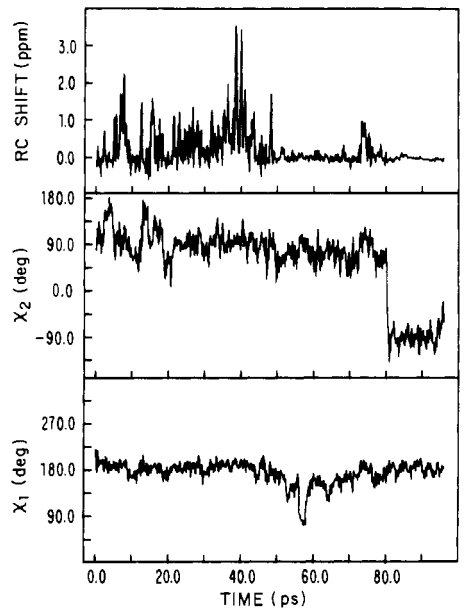

FIGURE 5: Comparison of the ring-current shift fluctuations of Phe-22 $H^{\delta 1}$ with the fluctuations of the χ_1 and χ_2 torsion angles of Phe-22.

and 72 ps arise because the ring rotates away from Phe-33 about χ_1, so that the ring-current shift due to Phe-33 becomes much smaller.

The variations in the shift fluctuations, resulting from rare conformational transitions such as ring flips, are illustrative

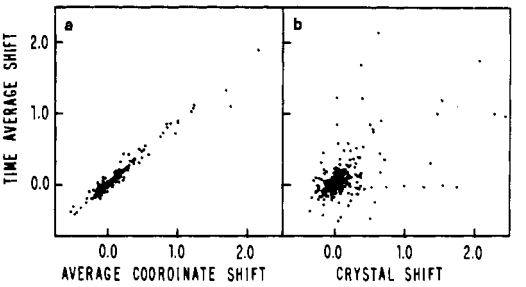

FIGURE 6: Plots of time average ring-current shifts against ring-current shifts calculated from (a) the average coordinates and (b) the crystallographic coordinates.

of what can happen in protein dynamics. Their effect in the present simulation is to yield chemical shift values which are incompletely averaged. In most instances, however, such "rare" events occur often enough so that they are averaged on the NMR time scale; an example of this is the observation in many proteins of a single resonance for protons on opposite sides of Phe and Tyr rings, made equivalent by rapid ring flips. Such rapid averaging is not always the case, however, for which Phe and Tyr ring protons again provide examples. When the rate of ring flipping is much slower than the chemical shift difference between the protons on opposite sides of the ring, separate resonances are observed. When the rate of ring flipping is comparable to the chemical shift difference, the rate can be determined from exchange effects in the spectrum. Several measurements of ring flipping rates in proteins have appeared in the literature (Campbell et al., 1975, 1976; Snyder et al., 1975; Wagner et al., 1976).

When the large range of chemical shift values computed for many protons from individual configurations of the simulation set is considered, it is important to compare the values of the shifts determined by averaging over the trajectory with those obtained from the average atomic coordinates. Although the same trajectory was used to evaluate the chemical shifts in the two cases, differences can arise because in one case the shift values are averaged over the set of coordinates and in the other the coordinates are averaged first and the shifts are then calculated from the average coordinates. Clearly, the first procedure yields the physically correct average, though the second corresponds to that used ordinarily, since only the average coordinates are available from an X-ray structure. Table I shows the results for a number of protons that have large shifts because they are close to aromatic rings. The two methods of computing shifts generally give similar results, despite the very large range of chemical shift values computed for individual configurations of the simulation. However, as can be seen from Table I (e.g., Pro-9 $H^{\beta 2}$, Arg-20 $H^{\beta 1}$), significant differences do occur. As expected, the differences are related to, but considerably smaller than, the rms fluctuations. Figure 6a shows a plot of the two sets of shifts; there are only 27 protons for which the difference is greater than 0.1 ppm. The figure shows a systematic trend toward larger shifts calculated from the average atomic positions than from the time average values of the shifts. The differences are largest for protons with the largest average shifts. A least-squares fit of the data in Figure 6a yields a slope of 0.84. In most cases the dominant contribution arises from the proximity of one aromatic ring (see Table I).

Analysis of Averaging Effects. For examination in detail of the important factors in the averaging of the ring-current shifts, the dipole approximation (see Methods) was used to partition the computed shifts into radial and angular contributions proportional to $1/r^3$ and $1 - 3\cos^2\theta$, respectively. Both radial and angular displacements of a proton with respect to

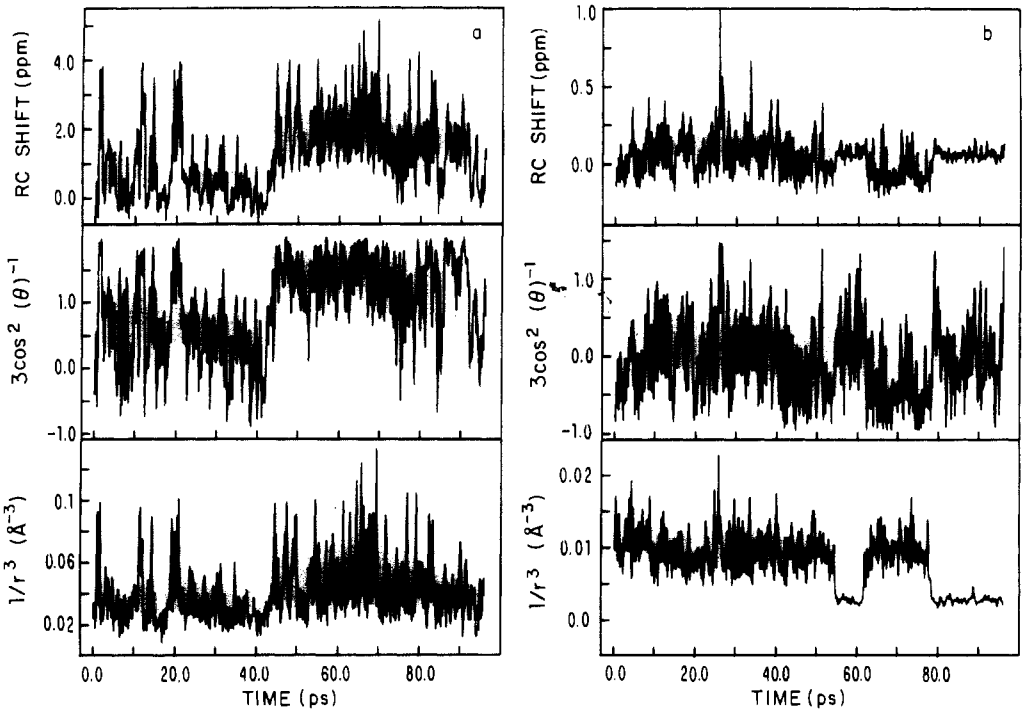

FIGURE 7: Comparison of fluctuations in ring-current shifts with fluctuations in the angular ($1 - 3\cos^2\theta$) and radial ($1/r^3$) contributions for (a) Pro-9 $H^{\beta 2}$ and (b) Thr-54 $H^{\gamma 2}$.

Table II: Radial Contribution to the Ring-Current Shift[a,b]

proton	$\langle r \rangle$	r_{av}	$1/\langle r \rangle^3$	$1/r_{av}^3$	$\langle 1/r^3 \rangle$
Pro-9 $H^{\beta 2}$	3.12	2.76	0.0329	0.0476	0.0379
Arg-20 $H^{\beta 1}$	3.14	2.97	0.0323	0.0332	0.0362
Phe-22 $H^{\beta 1}$	3.78	3.56	0.0185	0.0221	0.0197
Phe-22 $H^{\delta 1}$	4.81	4.33	0.0090	0.0123	0.0153
Asn-24 $H^{\beta 1}$	4.21	3.92	0.0134	0.0165	0.0165
Gly-28 $H^{\alpha 2}$	3.50	3.44	0.0233	0.0245	0.0252
Cys-30 $H^{\beta 2}$	4.25	4.18	0.0130	0.0136	0.0137
Thr-32 $H^{\gamma 2}$	3.73	3.63	0.0193	0.0216	0.0219
Gly-37 $H^{\alpha 1}$	4.98	4.96	0.0081	0.0082	0.0082
Ala-48 H^{β}	4.05	3.99	0.0150	0.0151	0.0182
Met-52 H^{ϵ}	8.51	8.04	0.0016	0.0020	0.0028
Thr-54 $H^{\gamma 2}$	5.63	5.53	0.0056	0.0063	0.0074
Cys-55 $H^{\beta 2}$	3.16	3.08	0.0317	0.0343	0.0338

[a] r (in angstroms) is the distance from the center of the nearest aromatic ring to the proton. [b] See footnote *a* of Table I.

an aromatic ring must be considered in order to account for the ring-current fluctuations. In Figure 7 the fluctuations of $1/r^3$ and $1 - 3\cos^2\theta$ for two protons relative to the closest aromatic ring are compared with the net chemical shift fluctuations. The fluctuations in either $1/r^3$ or $1 - 3\cos^2\theta$ alone are inadequate to describe the shift fluctuation. Particularly striking are the shift transitions (see above); e.g., for the methyl group of Thr-54, both $1/r^3$ and $1 - 3\cos^2\theta$ simultaneously show large "coarse grained" variations in the time period after 50 ps.

The radial contribution, $1/r^3$, to the chemical shift is considered first. In every case (Table II) the distance r between a proton and an aromatic ring has a larger time average value than the value obtained from the average coordinates. A corresponding effect, though smaller in magnitude, occurs in the determination of bond lengths from X-ray diffraction data, where bond lengths are underestimated if the anisotropic thermal motions of the atoms are not taken into account in the analysis (Busing & Levy, 1964). This result has been noted in earlier molecular dynamics simulations (McCammon et al., 1977). While the bond length shortening is only of the order

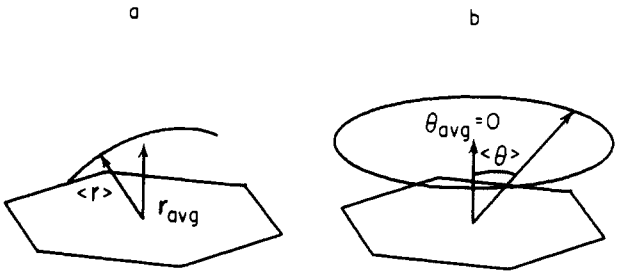

FIGURE 8: Illustration of angular and radial averaging. In (a) a proton moves with constant radius in an arc above the ring; this results in $\langle r \rangle > r_{av}$. In (b) a proton moves along a circle with constant azimuthal angle; this results in $\langle \theta \rangle > \theta_{av}$.

of 0.03 Å, the discrepancy in the average distance between a proton and an aromatic ring can be as large as 0.5 Å (see Table II). The largest discrepancies are expected to occur when highly correlated antiparallel displacements occur. An idealized example is the motion of a proton through an arc of constant radius above an aromatic ring; Figure 8a illustrates the effect of this kind of motion on the difference between the time average value of r, $\langle r \rangle$, and the value of r computed from the average coordinates, r_{av}.

Since $\langle r \rangle$ exceeds r_{av} in every instance, the value of $1/\langle r \rangle^3$ is always smaller than $1/r_{av}^3$ (Table II). However, the time average value of $1/r^3$, $\langle 1/r^3 \rangle$, is not equal to $1/\langle r \rangle^3$, because $\langle 1/r^3 \rangle$ is weighted toward the shortest distances, which tends to increase its value. The net result of these two effects in the averaging is that $\langle 1/r^3 \rangle$ tends to be rather close to $1/r_{av}^3$, and there is no trend in the differences between $\langle 1/r^3 \rangle$ and $1/r_{av}^3$. The largest difference listed in Table II is 0.01 for $H^{\beta 2}$ of Pro-9; this would lead to a chemical shift difference of approximately 0.3 ppm. For the methyl protons of Met-52, the two averages for the distances show a very large discrepancy. They are attributed to the large position fluctuations of this group; the rms position fluctuation of the methyl carbon is 3.5 Å during the simulation. Large position fluctuations alone are not, however, sufficient to produce large fluctuations in the radial contribution to the ring-current shift. In particular, the methyl

Table III: Angular Contribution to the Ring-Current Shift[a,b]

proton	$\langle\theta\rangle$	θ_{av}	$1-3\cos^2\langle\theta\rangle$	$(1-3\cos^2\theta)_{av}$	$\langle 1-3\cos^2\theta\rangle$
Pro-9 $H^{\beta 2}$	36.3	30.0	−0.95	−1.25	−0.88
Arg-20 $H^{\beta 1}$	27.4	19.7	−1.36	−1.66	−1.30
Phe-22 $H^{\beta 1}$	73.8	75.2	0.77	0.80	0.68
Phe-22 $H^{\delta 1}$	50.4	46.5	−0.22	−0.42	−0.25
Asn-24 $H^{\beta 1}$	58.3	59.1	0.17	0.21	0.02
Gly-28 $H^{\alpha 2}$	14.4	5.1	−1.81	−1.98	−1.76
Cys-30 $H^{\beta 2}$	78.2	79.2	0.87	0.89	0.83
Thr-32 $H^{\gamma 2}$	24.7	20.5	−1.48	−1.58	−1.40
Gly-37 $H^{\alpha 1}$	20.0	16.9	−1.65	−1.75	−1.62
Ala-48 H^{β}	28.3	26.8	−1.32	−1.37	−1.28
Met-52 H^{ϵ}	75.3	82.7	0.81	0.94	0.72
Thr-54 $H^{\gamma 2}$	52.3	57.1	−0.12	0.11	0.18
Cys-55 $H^{\beta 2}$	39.5	37.8	−0.79	−0.87	−0.77

[a] θ (in degrees) is the angle formed by a vector from the nearest aromatic ring center to the proton and a vector normal to the ring plane. [b] See footnote *a* of Table I.

group of Met-52 is more than 7 Å from the nearest aromatic ring, so that the rms chemical shift fluctuations are only 0.09 ppm.

A corresponding analysis can be carried out for the angular contribution to the chemical shifts (Table III). Since by definition the range of θ is limited to between 0° and 90°, protons which fluctuate across the plane ($\theta = 90°$) of an aromatic ring have a time average value of θ, $\langle\theta\rangle$, that is smaller than the value obtained from the average coordinates, θ_{av}. This behavior is found for many of the protons which are in the deshielding region ($\theta > 54.7°$) near an aromatic ring. The opposite effect is observed for the value of θ for protons which are in the shielding region ($\theta < 54.7°$) of a nearby aromatic ring. For many of these protons, $\langle\theta\rangle$ is greater than θ_{av} as a result of the coincidence of the polar axis and a vector normal to the ring plane. An extreme case, a proton moving with fixed angle θ about the polar axis above a ring, has θ_{av} equal to zero while $\langle\theta\rangle$ is equal to θ (Figure 8b).

The net effect of the fluctuations on the angular contribution to the ring-current shift is nearly always to reduce slightly the average shift relative to the value computed from the average structure (Table III). Because of the change of sign of $1 - 3\cos^2\theta$ at $\theta = 54.7°$, this trend is found for nearly all values of θ, despite the different averaging behavior of θ for protons near $\theta = 90°$ and those near $\theta = 0°$. The differences in the various averages of the angular contribution are much smaller than those of the fluctuations. Nevertheless, the decrease in the angular contribution is the major source of the small systematic differences between the average shift values and shifts computed from the average coordinates. For example, the largest differences between $\langle\sigma\rangle$ and σ_{av} are found for Pro-9 and Arg-20, and the angular contribution constitutes more than 80% of the differences.

It is interesting to note that an additional factor affecting the averaging of the chemical shifts arises from the correlation between r and θ values describing the position of a proton relative to an aromatic ring. Figure 9 contains a scatter diagram of the r and θ values for several protons with respect to the closest aromatic ring. When the distance between a proton and an aromatic ring is small, the correlation of motion between them can be considerable, as is revealed by the excluded region around the aromatic ring. For example, for $H^{\beta 2}$ of Pro-9, which is close to the ring, there is a clear correlation between r and θ; the smallest r values tend to occur for small values of θ, corresponding to an approach perpendicular to the ring plane. The effect of this correlation on the shift values is indicated by the difference between the value of $\langle(1 - 3\cos^2\theta)/r^3\rangle$ and $(1 - 3\cos^2\theta_{av})/r_{av}^3$ (Table III).

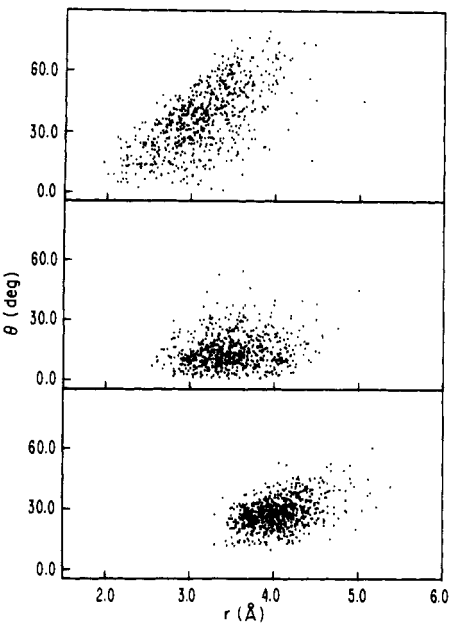

FIGURE 9: Plots of θ against r for (top) Pro-9 $H^{\beta 2}$ relative to Phe-33, (middle) Gly-28 $H^{\alpha 1}$ relative to Tyr-23, and (bottom) Ala-48 H^{β} relative to Tyr-21.

The observations, made earlier, that protons of Gly-37 and Ala-48 exhibit large average shifts while experiencing small shift fluctuations are also suggestive of correlated motion. For $H^{\alpha 1}$ of Gly-37, the rms fluctuation in the distance from the nearest ring (Tyr-35) is 0.26 Å, much less than the rms position fluctuation of the proton itself (0.83 Å). Similarly, for $H^{\beta 2}$ of Ala-48 the rms fluctuation in the distance from Tyr-21 is 0.28 Å, again much less than the rms position fluctuation (0.77 Å).

Further Aspects of Average Coordinates. Although the X-ray structure corresponds in principle to the average structure of the protein in the crystal, in practice differences are introduced by the refinement methods employed (Diamond, 1971). It is commonly assumed that the average structure may be described as a single configuration of the atoms with specified values for bond lengths and angles and appropriate values for the nonbonded contacts. The actual average structure obtained from a simulation need not correspond to such a physically reasonable structure. For examination of the magnitude of the differences in computed shifts between the average coordinates from the simulation and a single physically reasonable configuration close to this average structure, an energy refinement of the average structure was performed. This resulted in a rms change of the heavy atom coordinates by 0.17 Å and produced a structure of much lower energy. However, the computed ring-current shifts were only slightly altered from those obtained with the average structure. Only in 12 instances did the differences in computed shift exceed 0.1 ppm; these differences are not large enough to alter the trends already observed.

The question arises as to the method of generating average coordinates for protons, because they are not obtained directly from the X-ray analysis or included in the simulation. Two possible approaches were examined. In the first, average proton coordinates were obtained by averaging the proton coordinates generated for each of the configurations of the heavy atoms in the simulation. In the second, average proton coordinates were obtained by generating protons for the average heavy atom coordinates. The observed differences in chemical shifts obtained from the two sets of proton coordi-

nates were small, exceeding 0.1 ppm in only six instances. The protons with large shift differences were among the protons experiencing the largest shift fluctuations during the simulation; the largest difference, 0.185 ppm, was observed for $H^{\beta 2}$ of Pro-9.

Implications for Experimental Studies. It is not the purpose of this paper to compare experimental and calculated shifts. The dynamical simulations are such that the average structure deviates significantly from the X-ray structure (the rms difference between C^{α} coordinates is approximately 2.1 Å). The effect of this deviation on the calculated shifts is shown in Figure 6b. It is of interest in this regard that the largest differences between the shifts calculated from the simulation and the X-ray structure are found for protons which have large shift fluctuations during the simulation. Further, the calculated differences are generally smaller than the magnitude of the fluctuations (Table I).

The agreement between the time average shifts and shifts computed from the average structure, despite the small systematic differences, demonstrates the importance of the average structure in determining the observed shift values. For protons or other nuclei whose secondary shifts are dominated by the ring-current contribution, the chemical shifts computed from the X-ray structure of the protein are thus expected to agree well with experimental values, to the extent that the X-ray structure represents the average structure of the protein in solution. Only for a protein which undergoes frequent fluctuations of a much larger magnitude than those examined in this study are the discrepancies between the time average shifts and shifts computed from the average structure likely to be of significance.

The observed large shift fluctuations reflect the sensitivity of the shifts to slight perturbations in the structure and the fact that at room temperature a range of conformations in the neighborhood of the native structure is accessible to the protein. Thus it is apparent that changes in the average structure within the thermally accessible range as determined in this simulation could often yield calculated shifts in excellent agreement with experiment. That this is not always the case, however, is shown by the ring protons of the aromatic residues in PTI. Here shift fluctuations and shifts computed from both the present simulation and the X-ray structure are uniformly small, but a wide range of secondary shifts is observed experimentally (Perkins & Wüthrich, 1979). For these protons, perturbations in the average structure in the thermally accessible range would not change the computed ring-current shifts sufficiently to bring them into agreement with experiment. Thus, other contributions to the secondary shift may be dominant. There are contributions to the chemical shift, such as electric field (Buckingham, 1960; Sternlicht & Wilson, 1967) and local susceptibility anisotropy effects (Ando & Gutowsky, 1980), which have not been considered here. In general these other contributions are not sufficiently well understood to allow accurate calculations.

The near complete averaging of the shift fluctuations observed during the simulation suggests that high-frequency fluctuations of the type calculated here are not responsible for the experimental temperature dependence of many proton resonances in proteins. Instead, shifts which are highly temperature dependent are probably associated with regions of a protein in which the average structure changes with temperature; this is likely to be true for parts of the protein that have large calculated shift fluctuations. Alternatively, highly temperature dependent shifts may be observed for protons in regions undergoing very large fluctuations. The importance of such structural fluctuations has not been assessed in the present study.

Conclusions

Calculations based on a molecular dynamics simulation of PTI have revealed very large picosecond fluctuations (up to ±6 ppm) in the ring-current contribution to proton chemical shifts as a consequence of the atomic motions occurring in the protein interior. Nevertheless, the time average values of the ring-current shifts obtained from the simulation are close to those calculated from the average structure. Although systematic differences are present, they are small enough to suggest that the average structure can be used to predict chemical shifts in proteins. In most cases, the difference between the shifts calculated from the average or X-ray structure and those obtained from experiment is well within the range of the fluctuations in the shift resulting from the thermal motions. Thus small changes in the average structure, corresponding to the thermally accessible range, could result in exact agreement with experiment. The fluctuations are not large enough, however, to account for certain discrepancies, notably those of the aromatic protons. Here, contributions other than ring-current shifts are probably much more important.

The fluctuations in the shift values are shown to be the result of both radial and angular contributions, the behavior of either of the contributions alone being insufficient to account for the variations in the shift values. The principal source of the angular fluctuations appears to be torsional motion of the aromatic rings, while the radial fluctuations are frequently due primarily to motions of the groups containing the protons. The direction, although not the magnitude, of the slight systematic differences observed between time average values and values computed from average coordinates is due principally to the angular contribution.

The agreement between shifts computed from protein crystal structures and experimental shifts for several types of protons, particularly methyl protons, is shown by this study *not* to be evidence for the lack of fast motion in proteins, because fast motional averaging leads to chemical shifts which are not greatly different from those predicted for the average structure. This averaging also has relevance to the temperature dependence of chemical shifts in proteins. Since the average position of a proton relative to an aromatic ring, and not the shift fluctuations, is the principal determinant of the average shift, the temperature dependence of the shifts is likely to be due to the fact that the average structure of the protein varies with temperature; that is, the relative populations of different conformations within the thermally accessible range, each with significantly different shift values, depend on temperature. Illustrative of such changes are the conformational transitions and concomitant alterations in the average shift observed in the simulations. The results of this study do not support an interpretation that the temperature dependence is due primarily to the increase in torsional fluctuations of aromatic rings with increased temperature.

That the magnitudes of shift fluctuations obtained in the present study are not unrealistic is suggested by the similarity between the atomic rms displacements estimated from the X-ray temperature factors for PTI (J. Diesenhofer, private communication) and those calculated from the dynamical simulation. Large, low-frequency fluctuations, analogous to the transitions occasionally observed in the simulation, could also influence the chemical shifts. Experimentally observed chemical shifts are averaged on a millisecond time scale, so that fluctuations several orders of magnitude slower than those

observed in this study will be completely averaged in an NMR experiment. Their contribution to the average chemical shifts, which is not obtained from the present simulation, is, however, limited by the fact that such large fluctuations must be sufficiently rare that they do not significantly increase the temperature factors. An example is the flipping of aromatic rings, a process much slower than the time scale of this study yet which results in the complete averaging of chemical shifts for protons on opposite sides of most phenylalanine and tyrosine rings in proteins. This is a special case, since the two allowed orientations of the rings correspond to equivalent structures, so that the flipping transition does not result in an increase in the temperature factor.

The similarity between time average shifts and shifts computed from the average coordinates shows that the chemical shift is not a sensitive measure of protein dynamics on the picosecond time scale but reinforces the conclusion that it is a useful probe of local structure within a protein. The approach described above can be applied to the analysis of the effects of protein dynamics on other NMR parameters and on properties such as fluorescence depolarization. The effects of angular fluctuations have already been predicted to significantly affect ^{13}C relaxation rates (Levy et al., 1981), and the magnitude of the torsion angle fluctuations observed in the present work suggests that this should also be the case for vicinal (scalar) proton coupling constants. Of particular relevance to the present study are proton cross-relaxation rates which have been used to provide information about both internuclear distances and torsional fluctuations in proteins (Poulsen et al., 1980; Olejniczak et al., 1981). These, like chemical shifts, depend on both radial and angular fluctuations, and their relative importance is being investigated.

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