Hydrogen atoms in proteins: Positions and dynamics

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Hydrogen atoms constitute about half of the atoms in proteins. Thus they contribute to the complex energy landscape of proteins [Frauenfelder, H., Sligar, S. G. & Wolynes, P. G. (1991) *Science* 254, 1598–1603]. Neutron crystal structure analysis was used to study the positions and mean-square displacements of hydrogen in myoglobin. A test of the reliability of calculated hydrogen atom coordinates by a comparison with our experimental results has been carried out. The result shows that >70% of the coordinates for hydrogen atoms that have a degree of freedom is predicted worse than 0.2 Å. It is shown that the mean-square displacements of the hydrogen atoms obtained from the Debye–Waller factor can be divided into three classes. A comparison with the dynamic mean-square displacements calculated from the elastic intensities obtained from incoherent neutron scattering [Doster, W., Cusack, S. & Petry, W. (1989) *Nature* 337, 754–756] shows that mainly the side-chain hydrogen atoms contribute to dynamic displacements on a time scale faster than 100 ps.

Hydrogen atoms constitute nearly half of the atoms in proteins. They mediate hydrogen bridges and take part in the nonbonding interactions such as electrostatic and van der Waals forces. They are essential for the stabilization of the defined three-dimensional structure of proteins and thus contribute to the complex energy landscape of proteins (1). Hydrogen atoms in polarized bonds play critical roles in enzymatic catalysis. They are involved in hydrogen bonds in the substrate-binding process and are essential in proton-transfer reactions during the catalysis. The knowledge about protonation states of amino acid residues in the active center of proteins is crucial for an understanding of reaction mechanisms. For example, the question about which of the two amino acids, Asp-102 or His-57, in trypsin is the origin of the catalytic proton was answered by neutron crystallography (2). In many cases, the identification of protonated amino acids in the catalytic center would allow a decision between two or more competing reaction models (3). In addition, the knowledge of hydrogen positions in nonpolarized bonds (C–H bonds) outside the active center leads to an understanding of the dynamic properties of proteins. The conformational flexibility of the protein matrix is essential for enzymatic catalysis and for biological molecular activity in general.

Nowadays, ultrahigh-resolution x-ray diffraction using third-generation synchrotron sources, sensitive area detectors, and cryoprotection of the protein crystals allow ordered hydrogen atoms to be located (e.g., refs. 4–6). Even protonation states of amino acid side chains can be determined with adequate high-quality x-ray data sets (7). However, other examples exist. In myoglobin, even the use of synchrotron radiation yielding resolutions better than 1.15 Å did not give the hydrogen positions (8). Neutron scattering allows experimental determination of hydrogen positions [see, e.g., the pioneering works of Schoenborn (9, 10)]. The advantage of neutrons is that hydrogen atoms can be determined even at a resolution <2 Å with good accuracy (11–13). Neutrons are scattered by hydrogen atoms as strongly as by other atoms of the protein. The isotopes ^1H and ^2H (D) scatter differently. ^1H has a negative scattering length ($b = -0.374 \cdot 10^{-12}$ cm), ^2H scatters similarly to a carbon atom ($b = 0.667 \cdot 10^{-12}$ cm and $0.665 \cdot 10^{-12}$ cm, respectively). Because of the absence of radiation damage, neutron structure determinations

can be carried out at physiological temperature, which is often impossible if synchrotron radiation is used. This capability is of high value, for example, in the study of enzymatic processes. Polar hydrogen atoms, like nitrogen-bound hydrogen in peptide bonds, can be exchanged by deuterium if the protein crystal is soaked in a D_2O buffer. This exchange is a common practice in neutron-diffraction experiments to reduce the background from incoherent scattering of ^1H , especially from the crystal water. Because of the high scattering length of ^2H , it is possible to determine the protonation states of amino acids in the active center or the orientation of water molecules even above 2-Å resolution (16, 17). In a scattering density map the nonexchanged ^1H atoms are visible as negative density peaks, whereas all other atoms are depicted by positive density. H/D exchange data contain information about solvent accessibility and thus flexibility of the protein structure. Furthermore, these data yield information about which hydrogen atoms are available, for example, for catalytic functions in enzymes. Recent progress in instrumentation (14, 18, 19), especially the use of large neutron-imaging plate area detectors, will widen the number of proteins accessible to this technique by relaxing the need for very big crystals. For the experiments we used myoglobin because this protein is stable and extremely well characterized. By soaking crystals in D_2O buffer, the crystal water and most of the polar hydrogen atoms were replaced by D. The structure refinement included all hydrogen positions (13). At a resolution of 1.5 Å, a comparison of calculated and experimentally determined hydrogen positions becomes possible.

Besides the structure, the dynamics of protein molecules is of high importance for the function. Mössbauer absorption spectroscopy was the first method to determine dynamic mean-square displacements (20–23), which have been compared with total mean-square displacements from x-ray structure analysis. (For a recent comparison, see ref. 24.) Later, the incoherent neutron scattering on ^1H became a powerful tool for the investigation of protein dynamics (25, 26). A neutron structure at high resolution allows the determination of the mean-square displacements of individual hydrogen atoms. A comparison with dynamic mean-square displacements determined by incoherent neutron scattering allows separation of contributions on different time scales.

Materials and Methods

Metmyoglobin, where a water molecule is bound at the heme iron (Fe(III)-high spin), was crystallized from the buffer 50 mM $\text{KH}_2\text{PO}_4/2.9$ M $(\text{NH}_4)_2\text{SO}_4$ at pH 6.8 in the space group $P2_1$ with $a = 64.53$ Å, $b = 30.87$ Å, $c = 34.87$ Å, and $\beta = 105.7^\circ$. We used a crystal of $2.5 \times 2.5 \times 1.0$ mm³ soaked in deuterated buffer for >10 years. Data were collected at room temperature at the monochromatic crystallography beam line BIX-3 at the JRR-3M reactor at the Japan Atomic Energy Research Institute (19) at a wavelength of 2.35- to 1.5-Å resolution. (For details of the data acquisition, see ref. 13.) Integration and further data reduction were carried out with DENZO, SCALEPACK (27), and TRUNCATE

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 1L2K).

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Table 1. Summary of crystallographic data

Data measurement	
Source, instrument	Nuclear reactor JRR-3M, BIX-3
Wavelength, Å	2.35
Crystal space group	$P2_1$
a, b, c , Å	64.53, 30.87, 34.87
β , °	105.7
Resolution, Å*	22.0–1.5 (1.55–1.50)
Unique reflections*	19,135 (1,458)
Redundancy*	2.9 (2.1)
R_{merge} , %*†	10.3 (24.9)
Completeness, %*	87.9 (67.0)
$I/\sigma(I)$ *	6.3 (2.7)
Refinement	
Resolution, Å	22.0–1.5
Total number of reflections	19,063
Reflections used for the refinement	17,755
Reflections used for cross-validation	1,308
R factor‡	0.201
$R_{\text{free}}^{\ddagger}$	0.238
rms deviations from ideality	
Bond length, Å	0.005
Bond angle, °	0.99

*Values in parentheses are for highest-resolution shell.

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$.

‡ $R_{\text{cryst}} = \sum_{hkl} |F_{\text{obs}} - F_{\text{calc}}| / \sum_{hkl} F_{\text{obs}}$ and $R_{\text{free}} = R$ factor calculated for test set of reflections not used in refinement (6.9% comprising 1,308 reflections).

(28). The refinement was carried out with X-PLOR (29) (versions 3.1 and 3.851) and CNS (30), where the atomic form factors have been replaced by the neutron-scattering lengths. An x-ray structure at a resolution of 1.5 Å was used as a starting model. Deuterium atoms in amino acid side chains were included into the model only if a significant density feature was present. Occupancies for the backbone amide hydrogen atoms were refined (H/D exchange). Coordinates and diffraction data have been deposited in the Protein Data Bank (PDB ID code 1L2K).

To check the integrity of the sample after the D₂O-soaking process, we compared our refined structure with the 1.2-Å x-ray structure of metmyoglobin at room temperature (31) (PDB ID 1BZ6). The calculated rms distance is 0.10 Å for main-chain atoms (C α , N, C) and 0.40 Å for all protein atoms, including the heme group. The rms distances between these structures from independent structure determinations are surprisingly small. The rms distance for main-chain atoms is within the overall coordinate error of our structure determination. The D₂O-soaking process did not influence the protein structure.

It has been found that the experimentally obtained B factors from the neutron measurement differ by a constant offset from the values of the x-ray structure analysis as described by Ostermann *et al.* (13), which can be attributed to the background estimation in the integration of the reflection intensities. Thus, a constant value of 0.067 Å² was added.

The quality of the data can be inferred from Table 1. Although the crystal is large, a satisfying value of R_{merge} is obtained. Moreover, the values of I/σ , and the completeness and the redundancy, are of good quality. As shown in Table 2, the refinement includes 2 times more parameters than x-ray structure analysis at the same resolution because all atoms are visible. The final R factor of 20.1% and free R factor of 23.8% reflect the care to avoid overfitting during the structure-refinement process. The error estimate from the calculation according to Luzzati yields 0.18 and 0.21 Å for a σ_A plot.

To test the quality of theoretically predicted hydrogen positions we produced a “predicted structure.” Starting from the experimentally obtained coordinates of all atoms, we first re-

Table 2. Atom types included in the refined model

Atom type	Number
² H atoms	194
¹ H atoms	1,143 (30 in the heme)
¹ H in methyl groups	282
Total number of hydrogen atoms	1,337 (30 in the heme)
Non-hydrogen atoms (C, N, O, S, and Fe)	1,247
Total number of atoms	2,584

Solvent molecules are not included here. The 146 hydrogen atoms bonded to the backbone N are counted twice (¹H and ²H atoms) because of the occupancy refinement.

moved all hydrogen atoms. Their coordinates were subsequently rebuilt with the program X-PLOR (29) (version 3.851) by using the “hbuild” command (32). Energy minimization of the hydrogen positions with fixed heavier atoms was carried out with CNS (30) by using van der Waals and electrostatic interaction with the standard parameters for hydrogen atoms. For these interactions, a switch function with a cutoff of 12 Å, starting at 11 Å, and a dielectric constant of 1 has been used. The predicted structure allowed us to calculate “predicted” density maps, ρ_{predic} , which were then compared with ρ_{exp} , which was calculated from the original neutron structure including the experimentally determined hydrogen positions.

Results and Discussion

Position of the Hydrogen Atoms. As an example, Fig. 1 *a* and *b* shows the neutron-scattering densities at two different residues, together with the structure model. Note the clear negative scattering density at the positions of the ¹H atoms in the methyl groups. For a comparison of the experimentally obtained hydrogen positions with the theoretically predicted ones, the difference $\Delta\rho = \rho_{\text{exp}} - \rho_{\text{predic}}$ was calculated in real space. The experimentally determined B factors were used to calculate the map for the predicted model. Thus, the differences are automatically weighted by the accuracy of the experimentally determined position. If the position of a hydrogen atom is rather uncertain as expressed by a high B factor, even a predicted position that differs from the experimentally determined position is not visible in the difference map. As an example, Fig. 1 *c* displays the difference density for the methyl hydrogen atoms (¹H) of Leu-32. The calculated hydrogen positions are displaced 0.76 Å from the experimental ones. In Fig. 1 *d*, an example of a polar hydrogen is shown where the calculated coordinates are wrong by 0.2 Å. In comparison with the experiment, the calculation shifts the D into a position where the hydrogen is closer to the plane of the peptide bond.

Fig. 2 shows a backbone picture of myoglobin with the differences $\Delta\rho$ between the experimentally obtained and the predicted scattering densities corresponding to displacements of >0.1 Å. The displacements of the $\approx 1,000$ covalently bound ¹H atoms and that of the 170 polar ²H atoms are depicted separately. The results are striking. In the interior, the position of a large number of covalently bound hydrogen atoms, particularly from the methyl groups, is predicted incorrectly. The positions of the polar hydrogen atoms in the interior are well predicted, whereas the calculation yields worse results at the surface. Large differences are also observed at threonine and serine residues as discussed by Brünger and Karplus (32).

The results are summarized in Fig. 3 as rms differences between predicted and experimentally determined hydrogen atom positions. Note that of the $\approx 1,200$ hydrogen atoms in myoglobin, nearly 700 have no degree of freedom. Therefore, an accurate prediction of their positions is trivial (see Table 2). For the nonexchangeable ¹H in methyl groups the rms differences

Fig. 1. (a and b) $2F_{\text{exp}} - F_{\text{calc}}$ scattering densities of Leu-32 (a) and hydrogen bridge (arrow) between Pro-100 and Tyr-103 (b) [dark gray, positive density (1.5σ); light gray, negative density (-1.5σ)]. (c and d) $\Delta\rho$ maps. (c) Density disappears at light gray and reappears at dark gray contours. (d) Density disappears at dark gray and reappears at light gray contours. Pictures were generated with the program o (35).

show a broad distribution. To reveal possible differences for the exchangeable hydrogen atoms, the energy restraint for the hydrogen to lie within the peptide plane was lowered for the refinement of the experimental data. The effect of the planarity restraint on the prediction is evaluated by the use of the standard value and the lowered value as displayed in Fig. 3b.

Without experimental data, the prediction with a low restraint is doubtful, because the corresponding distribution is wider. In comparison with the methyl groups, the histogram for the backbone hydrogen atoms is rather narrow. Deviations occur mainly in bifurcated hydrogen bonds, in loop regions, or where hydrogen bonding with the solvent is observed. The percentage

Fig. 2. Comparison of experimentally determined and calculated hydrogen positions. Shown are $\Delta\rho$ maps (green, positive; red, negative). (a) Only differences for ^1H atoms. (b) Only differences for ^2H atoms. Pictures were generated with the program o (35).

Fig. 3. Histogram of the rms differences (rmsd) between the coordinates of experimentally determined and predicted hydrogen atoms for hydrogen atoms in methyl groups (a), and for the exchangeable backbone hydrogen atoms. (b) Filled histogram, weak planarity restraint; unfilled histogram (first column), standard planarity restraint.

of hydrogen atoms that are located in methyl groups and differ >0.2 Å exceeds 70%.

Mean-Square Displacements and Dynamics. It makes no sense to discuss mean-square displacements for individual hydrogen atoms. Instead we tried to separate classes. In the histograms of mean-square displacements in Fig. 4a the difference between main-chain and side-chain hydrogen atoms is demonstrated. Fig. 4b differentiates hydrogen atoms in methyl groups and lysine groups. Accordingly, one can divide the hydrogen atoms into different classes. The displacements of the hydrogen atoms in the main chain are centered at $\langle x^2 \rangle = 0.18$ Å² [compare $\langle x^2 \rangle = 1/3 \langle r^2 \rangle = 0.18$ Å² as average of the non-hydrogen backbone atoms (24)]. Hydrogen atoms in the side chains show a larger diversity and can be subdivided into main-chain-like, methyl-like, and lysine-like. The latter set contains most of the hydrogen atoms in long side chains and represents the highest $\langle x^2 \rangle$ values. All histograms can be fitted simultaneously by a sum of three Gaussians with different centers and areas (see Fig. 4c). The results are compiled in Table 3. The relative amounts of atoms belonging to each Gaussian is consistent with their structural characterization.

We can now compare these three distributions of mean-square displacements with $\langle x^2 \rangle$ values, obtained from incoherent scattering of neutrons. Remember that the crystallographic data stem from coherent processes and are sensitive to static and dynamic displacements. In contrast, incoherent neutron scattering records only dynamic processes typically faster than 100 ps, depending on the energy resolution of the spectrometer. There-

Fig. 4. Mean-square displacements of hydrogen atoms (only ¹H). (a) All atoms (open bars), bonded to main-chain atoms (hatched bars), and hydrogen atoms in side chains (filled bars). (b) All hydrogen atoms in side chains (open bars), hydrogen atoms in methyl groups (filled bars), and lysine groups (hatched bars). (c) Least-squares fit (solid line) of the histogram for all hydrogen atoms (diamonds) with three Gaussian distributions (dashed lines).

fore, a time-independent contribution has to be taken into account in the comparison

$$\langle x_j^2 \rangle_{\text{cryst}}^n = \langle x_j^2 \rangle_{\text{fast}}^n + \langle x_j^2 \rangle_{\text{slow}}^n \quad [1]$$

The index “cryst” indicates values from the present structure determination at room temperature. The indices “fast” and “slow” differentiate between displacements that are recorded (fast) and that are not recorded (slow) by incoherent neutron scattering because of the energy resolution. Fast processes represent dynamic motions, whereas slow processes seem to be

Table 3. Gaussian distributions

	Counted from structure	Gaussian 1	Gaussian 2	Gaussian 3	Sum within Gaussians
Position, Å ²		0.182	0.246	0.333	
Width, Å ²		0.034	0.033	0.092	
All ¹ H	997 (194)	292	380	334	1,006
Main chain	161 (146)	122	26	11	159
Side chain	836 (48)	170	354	323	847
Methyl groups	282	21	226	40	287
Lysine side chains	152	0	4	140	144

First two rows, parameters for the Gaussian distributions. Following rows, number of hydrogens counted from the known molecular structure of myoglobin, attributed to the three Gaussian distributions, and within the sum of the Gaussians. The hydrogen atoms of the heme are included in the numbers for all ¹H and for the side-chain ¹H atoms. Numbers for ²H atoms are given in parentheses.

static for the incoherent neutron scattering. The index j labels the hydrogen atoms according to the three classes.

For a comparison with the incoherent neutron-scattering data on myoglobin of Doster *et al.* (25), we used the expression

$$S[q, 0] = \sum_{j=1}^3 c_j \cdot \exp[-q^2 \langle x_j^2 \rangle_{\text{fast}}^n]. \quad [2]$$

The weight factor, c_j , for the contribution of atoms of class j is taken from the area of the Gaussian j (see Table 3). $\langle x_j^2 \rangle_{\text{fast}}^n$ remain as free parameters for a fit of $S[q, 0]$ to the experimental data in ref. 25. The average mean-square displacement of the hydrogen atoms can be obtained from the slope of $\ln S[q, 0]/q^2$ for $q = 0$ (25). It can also be calculated by

$$\langle x^2 \rangle = \sum_{j=1}^3 c_j \cdot \langle x_j^2 \rangle_{\text{fast}}^n. \quad [3]$$

The data for $S[q, 0]$ and the average mean-square displacement at each temperature were fitted simultaneously. The weight for the fit to $S[q, 0]$ and $\langle x^2 \rangle$ was adjusted equally.

Results are shown in Fig. 5*a* for different temperatures. The contributions of the three different hydrogen classes are shown as dashed lines for the 320 K data in *Inset*. The result for the average mean-square displacement $\langle x^2 \rangle$ in comparison with the data of Doster *et al.* (25) is shown in Fig. 5*b* *Inset*. Smith *et al.* (33) have shown by an analysis of molecular dynamics trajectories that the description of $S[q, 0]$ by a sum of Gaussians similar to Eq. 2 is in qualitative agreement with the data.

The obtained dynamic mean-square displacements as a function of temperature are given in Fig. 5*b*, together with the mean-square displacement derived from the structure analysis. On a time scale faster than 100 ns, the main-chain hydrogen atoms contribute only slightly to the dynamic displacements, the mean-square displacements are “static.” Distribution 3 with the highest mean-square displacement is mainly dynamic and the static part is negligible. For the methyl-like hydrogen, the static and dynamic contributions are nearly equal.

Conclusion

Even given a good atomic resolution data set, x-ray crystallography may not yield the positions of all protein-bound hydrogen atoms. The calculation of hydrogen positions may give imprecise results especially for methyl groups. Structure determination by neutrons could be very helpful to control the quality of the calculations of hydrogen positions and to improve the force fields used in the modeling process. A detailed analysis of the Debye–Waller factors provides insight into the mechanism of protein

dynamics. Hydrogen atoms can be grouped into three classes of different mobility. The classes can be structurally attributed. Above the dynamic transition temperature, the mean-square

Fig. 5. (a) Fit of the elastic intensity from incoherent neutron scattering with the model of three different classes of hydrogen atoms. Data are taken from Doster *et al.* (25). Δ (largest intensity values), 202 K; $\circ$, 242 K; $\square$, 277 K; ∇ , 320 K. (*Inset*) Dashed lines, contributions of the three classes at 320 K. (b) Open symbols, temperature dependence of the dynamic mean-square displacement determined by the fits as shown in a. Δ , main-chain-like; $\square$, methyl-like; $\diamond$, lysine-like; filled symbols, the displacements $\langle x_j^2 \rangle_{\text{cryst}}^n$ determined from the structure analysis. (*Inset*) $\circ$, mean-square displacements taken from ref. 25; solid line, harmonic part of the mean-square displacements taken from ref. 25; $\blacksquare$, average mean-square displacements from Eq. 3.

displacements measured by incoherent neutron scattering are dominated by the side chains, whereas the main chain stays rather rigid on the time scale faster than 100 ps. As Mössbauer spectroscopy shows, their motion occurs on a slower time scale essentially between 1 ns and 300 ps (22, 24, 34). Highly resolved neutron-structure determinations of proteins, incoherent neutron scattering, and Mössbauer spectroscopy on iron-containing

proteins are complementary methods to study the protein dynamics and contribute to a better understanding of the energy landscape of a protein.

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