Polar Hydrogen Positions in Proteins: Empirical Energy Placement and Neutron Diffraction Comparison

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ABSTRACT   A method for the prediction of hydrogen positions in proteins is presented. The method is based on the knowledge of the heavy atom positions obtained, for instance, from X-ray crystallography. It employs an energy minimization limited to the environment of the hydrogen atoms bound to a common heavy atom or to a single water molecule. The method is not restricted to proteins and can be applied without modification to nonpolar hydrogens and to nucleic acids. The method has been applied to the neutron diffraction structures of trypsin, ribonuclease A, and bovine pancreatic trypsin inhibitor. A comparison of the constructed and the observed hydrogen positions shows few deviations except in situations in which several energetically similar conformations are possible. Analysis of the potential energy of rotation of Lys amino and Ser, Thr, Tyr hydroxyl groups reveals that the conformations of lowest intrinsic torsion energies are statistically favored in both the crystal and the constructed structures.

Key words: protein structure, empirical energy, energy minimization, molecular dynamics

INTRODUCTION

Empirical energy functions are now being widely used for structural and dynamic studies of macromolecules.[1-3] The initial calculations in this area made use of an extended atom model[4,5]; that is, all hydrogen atoms were treated as part of the heavy atoms to which they are attached, and the heavy atoms had their parameters adjusted to take account of the inclusion of the hydrogens. More recently, it has become feasible to refine the methodology by treating the polar hydrogens explicitly (i.e., to include them in the calculations on the same level as the heavy atoms).[6,7] This is of importance in the correct treatment of the hydrogen bonding, which plays an important role in determining the properties of these systems. The aliphatic hydrogens are still treated as parts of extended atoms in most cases, although programs[8,9] exist for macromolecules that include all hydrogen atoms. Comparison calculations suggest that the inclusion of all aliphatic hydrogens is not essential at the present level of accuracy. They appear to be significant in obtaining the correct shape for aromatic residues in proteins and for the nucleic acid bases, and, of course, they have to be included if their spe-

cific motional properties are of interest. A case in point is provided by methyl group rotations, which are of interest in nuclear magnetic resonance (NMR) spectra but cannot be treated by an extended atom model; a myoglobin simulation including all leucine and isoleucine methyl group hydrogens has been performed.[10]

Since X-ray structural data for proteins at the present level of resolution do not locate hydrogen atoms, it is necessary to have a procedure for constructing them from the heavy atom coordinates to obtain a starting structure for calculations. Although this is no problem for peptide NH hydrogens, which are essentially fixed by the heavy atom geometry, as are most aliphatic hydrogens other than the methyl group hydrogens, there is no unequivocal way of orienting polar hydrogens with dihedral angle rotational degrees of freedom (i.e., Ser, Thr, Tyr, Lys), as well as the hydrogen atoms associated with water molecules included in the calculation. One way of proceeding is to make use of high-resolution neutron diffraction structures.[11-13] As an alternative, we describe here a method for constructing initial positions for the polar hydrogens by a combination of search and energy calculation techniques. A description of the method is given. Applications of the method are made to three proteins (ribonuclease A, trypsin, and bovine pancreatic trypsin inhibitor [BPTI]) for which X-ray and neutron diffraction data are available.

In addition to its interest for the more accurate application of empirical energy function methods, the present analysis provides information concerning hydrogen atom positions that supplement previous work on main-chain and side-chain heavy atoms. For side-chain heavy atoms, it has been shown that the dihedral angle distributions found in proteins approximate those expected for isolated dipeptide side-chains; that is, in the protein a position near the isolated dipeptide minima is often selected for each χ value.[14-17] The possible significance of this for the folding process has been discussed.[16] It has also been noted in a detailed study of the side-chains in BPTI that there are significant exceptions to the above

rule;[16] i.e., local backbone or neighboring residue positions can perturb the observed minimum from any of those expected for the isolated system.

MATERIALS AND METHODS

Given the heavy atom coordinates, construction of all hydrogen positions of the protein is performed by a three-step, iterative procedure. In the first step, any crystallographically or otherwise defined water molecules are represented by uncharged oxygen atoms with the standard van der Waals parameters. The protein or nucleic acid hydrogens are classified according to their bonding environment. The following classes represent all of the possibilities that have to be considered: a) hydrogen bound to a donor with at least two heavy donor antecedents with known coordinates (e.g., peptide hydrogens, histidine imidazole hydrogens, aliphatic CH_2 hydrogens), b) hydrogen bound to a donor with only one known donor antecedent and no other atoms bound to it (e.g., [η-H] group of a tyrosine residue), c) hydrogen bound to a donor with only one known donor antecedent and one additional hydrogen (e.g., [η-H_2] group of arginine), d) hydrogen bound to a donor with only one known donor antecedent and two additional hydrogens (e.g., [ϵ-H_3] group of lysine or a methyl group).

The attribute known in the above usually refers to a heavy atom donor antecedent whose coordinates are determined by X-ray crystallography. However, in what follows it may also refer to a particular hydrogen whose position is known by some other means, e.g., from neutron diffraction. Any remaining hydrogen positions are then constructed using the following scheme.

In the initial step, all hydrogens belonging to class a are placed by using equilibrium bond lengths, bond angles, and dihedral angles. This problem is sometimes overdetermined, and an averaging over the placements corresponding to all combinations of bond angles is performed; e.g., in the case of the N-H group of a peptide bond there are two bond angles C-N-H and C^α-N-H, which, together with the (improper) dihedral angle C-C^α-N-H, are each sufficient to determine the position of the NH hydrogen.

In the next step the hydrogen positions characterized by classes b–d are constructed. All of these classes have in common that there is a dihedral degree of freedom in placing the hydrogen (e.g., a rotation around the C^ξ-N^ξ bond of lysine). To find an energetically favorable conformation, the dihedral angle with respect to the antecedent-donor symmetry axis is modified in small steps. In case of symmetry in the donor group the full 0°–360° range of the dihedral angle can be reduced, e.g., the threefold symmetry of a lysine [ξ-H_3] donor group allows a reduction to a 0°–120° range. In the calculations an angle grid of a 10° was found to be satisfactory. For each dihedral angle the hydrogens of the donor are placed according to their equilibrium bonding geometry, and the rela-

tive energy of the corresponding conformation is evaluated. The bonding geometry of the hydrogen donor for cases b and c is planar and for case d is tetrahedral. The energy is determined by using a hydrogen bond potential, a van der Waals term, an electrostatic term, and proper dihedral term as described in reference 1. Improper dihedral terms have been left out as they are constant during the dihedral angle rotation. Since only one or a small number of atoms is being moved, the calculation is very fast. The conformation corresponding to the dihedral angle with the lowest total energy is taken, and the hydrogens of the donor group are placed according to this conformation. This procedure is performed in the order given by the residue sequence of the macromolecule. Hydrogens that have not yet been constructed are not included in energy evaluations, and those that have been constructed are included.

After construction of all hydrogens of the protein or nucleic acid the water hydrogens are constructed. The water molecules are represented by the ST2 model.[18] However, the algorithm is general and can be used with any explicit hydrogen water model, such as T1P3P.[19] To avoid a dependence on the input sequence the water molecules are ordered with respect to the minimum distance of the water oxygen to any protein atom; water molecules near the protein are treated first. The following possibilities are distinguished: a) water molecules able to form two hydrogen bonds to known acceptor atoms in the vicinity of the water molecule, b) water molecules able to form only one hydrogen bond to a known acceptor atom, c) water molecules not able to form any hydrogen bonds to known acceptor atoms. A known "acceptor atom" can be another water oxygen atom or any protein hydrogen bond acceptor atom, where the position is known from the X-ray structure or other considerations. Most water molecules correspond to class a, but classes b and c occur for situations where only very few water oxygen positions are known. In case a, a rotation of the water molecule in the plane defined by the two nearest acceptor atoms is performed, and the hydrogens of the water molecule are placed according to the minimum energy conformation. In case b one hydrogen is placed between the water oxygen and the acceptor atom. The water molecule is then rotated around this hydrogen bond axis, and the other hydrogen is placed in the minimum energy conformation. The energy expression used consists of the van der Waals, electrostatic, and water model-specific interactions.[1,19] Finally, the water molecules of case c are placed in a standard way (H_1 on x-axis, H_2 in the positive x,y quadrant). The above three-step procedure is referred to simply as hydrogen construction.

After all hydrogen positions have been determined by the hydrogen construction, a second pass is performed in which the minimum energy conformation for each donor group and water molecule is reevaluated. This time all hydrogens are included in the
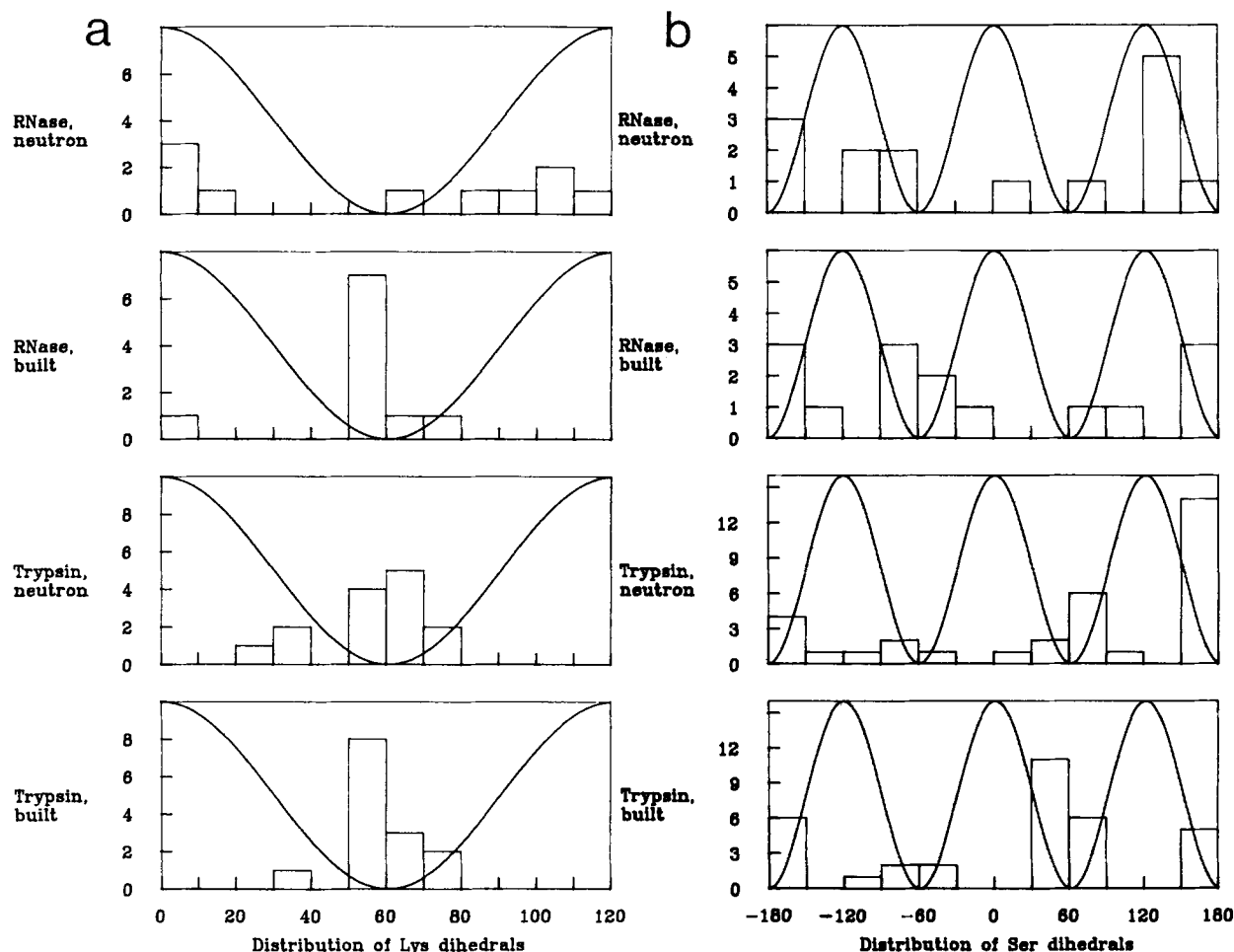
Fig. 1. Distribution of dihedral angles for the antecedent bond of the donor groups of (a) Lys (ζ-H$_3$), (b) Ser (γ-H), (c) Thr (γ-H), and (d) Tyr (ϵ-H); the distributions are shown for the neutron diffraction structures and the hydrogen-constructed (2nd-iteration) structures of trypsin and ribonuclease. The solid curves indicate the dihedral potential energy on an arbitrary scale.

interaction energy terms. No distinction is made between hydrogen and deuterium atoms. Since this type of sequential procedure can have a built-in bias, a useful check is to start constructing the protein hydrogens at two different points, e.g., from the N-terminal and C-terminal ends.

RESULTS

The three molecules studied in detail are bovine pancreatic ribonuclease, MIP (monoisopropylphosphoryl)-inhibited bovine trypsin, and BPTI. For bovine pancreatic ribonuclease A the structure solved by A. Wlodawer et al.[11] in a joint neutron and X-ray refinement at 2.0 Å resolution was used. There are 128 known water molecules (D$_2$O) in the structure. The protein is composed of 124 residues with 245 polar hydrogen (deuterium) atoms. For MIP-inhibited trypsin the neutron diffraction structure solved by A. Kossiakoff[13,20] at 2.2 Å resolution was used. The pro-

tein contains 223 residues with 384 polar hydrogen (deuterium) atoms. The structure includes 30 ordered water molecules (D$_2$O). Only the oxygen positions of the water molecules were available. For BPTI the neutron diffraction structure solved by Wlodawer et al.[12] in a joint neutron and X-ray refinement at 1.8 Å resolution was used. The protein contains 58 residues with 112 polar hydrogen atoms. Of the 63 ordered water molecules only the four internally bound ones were considered in this work. In the following the three systems are simply referred to as ribonuclease, trypsin, and BPTI.

The construction of the protein heavy atoms was carried out according to the procedure described in "Materials and Methods." Starting with the heavy atom positions from the neutron diffraction data (including water oxygen atoms), the polar hydrogens were constructed. Two successive iterations for the construction of hydrogen positions were performed.

c

RNase, neutron

RNase, built

Trypsin, neutron

Trypsin, built

-180 -120 -60 0 60 120 180

Distribution of Thr dihedrals

d

RNase, neutron

RNase, built

Trypsin, neutron

Trypsin, built

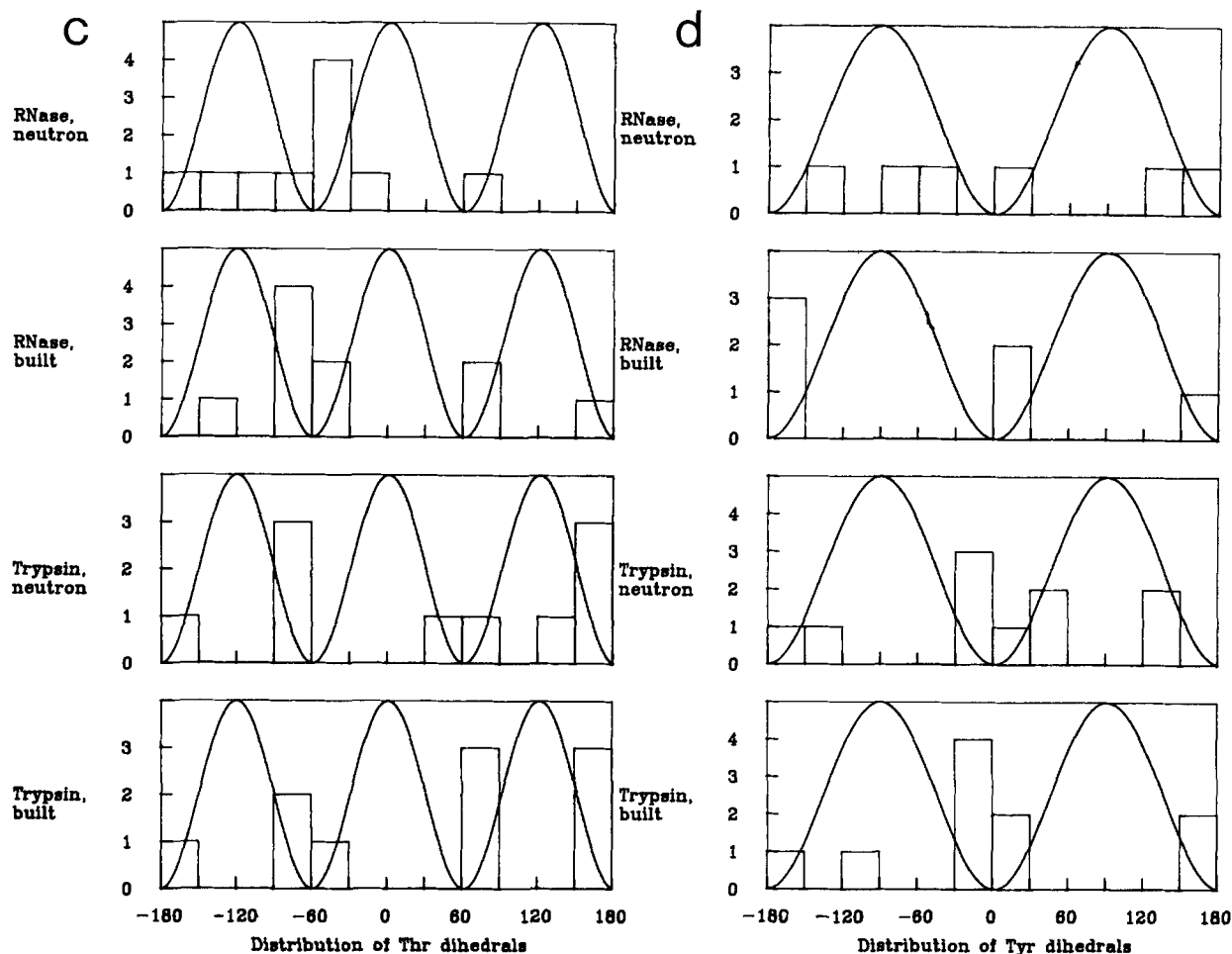-180 -120 -60 0 60 120 180

Distribution of Tyr dihedrals

Figure 1 continued

The coordinates after the second iteration were taken as a reference structure for comparison with the neutron diffraction structure assignments. As a consistency test a third iteration was carried out. In addition, a global hydrogen energy minimization of the second iteration structure was performed by employing a conjugent gradient technique.[21,22] During this minimization the heavy atom positions were held fixed, and the geometry of the water molecules was kept rigid by using SHAKE.[23,24] The structure was minimized until the energy difference between successive steps was less than 10^{-4} kcal/mol. This difference corresponds to the accuracy limit of the performed calculations; test runs using double precision on the VAX 11/780 showed no significant modifications when performing further minimization cycles. In the following we refer to hydrogen atoms regardless of whether they are actually identified as hydrogen or exchanged deuterium atoms in the neu-

tron diffraction experiments. The averages of root-mean-square (r.m.s.) deviations between hydrogen positions for various structures of trypsin, ribonuclease, and BPTI are shown in Table I. The relatively small deviations between the second and the third iteration (see rows "2nd–3rd" in Table I) indicate that the algorithm reaches self-consistency except for Thr residues in ribonuclease; in this case, several alternative conformations are possible (for a specific example of alternative conformations, see below). For most practical purposes, two iterations of the hydrogen construction will be sufficient. We have, therefore, restricted ourselves to analyze the structures obtained after two iterations.

Global hydrogen position minimization after the second iteration produced only minor modifications of the hydrogen positions except for water molecules (Table I). The deviations for water molecules are due to the fact that the hydrogen construction method
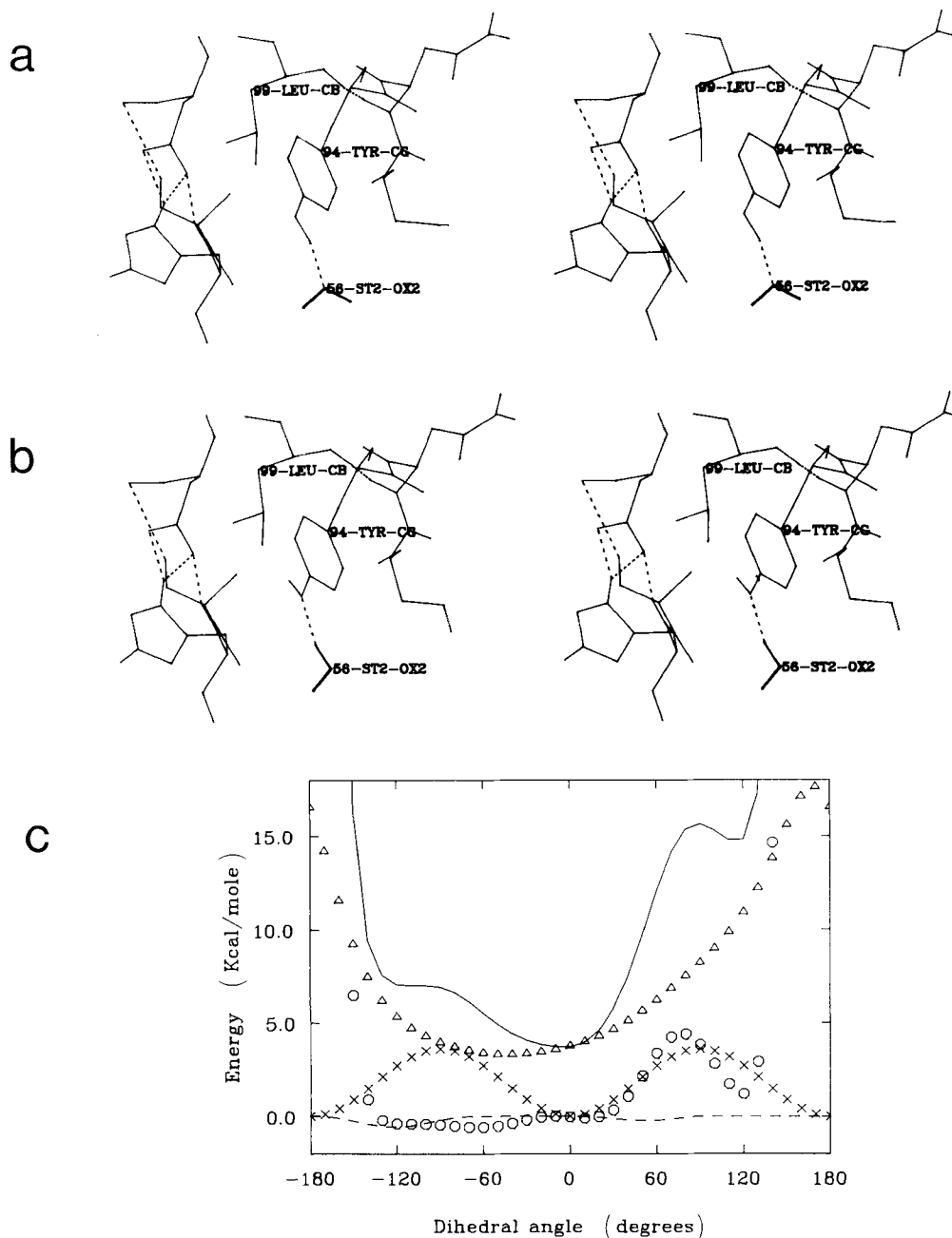
Fig. 2. Tyr-94 (γ-H) donor group of trypsin. **a:** Stereo picture of the environment within 5 Å of the O^γ atom of the structure obtained after two iterations of hydrogen construction. **b:** Stereo picture of the environment within 5 Å of the O^γ atom as determined by the neutron diffraction data. The protein hydrogen positions were obtained from the neutron diffraction structure, and the missing water hydrogen positions have been determined by hydrogen construction. Hydrogen bonds are indicated if the following criteria are met: the heavy donor-acceptor distance is less than 4.0 Å, and the donor-hydrogen-acceptor angle is less than 60° (the definition of the angle is such that a linear hydrogen bond corresponds to 0°). **c:** Partial energies as a function of dihedral angle; "—" total energy, "– – –" hydrogen bond energy, "O" van der Waals energy, "x" dihedral energy, "△" electrostatic energy.

performs only a limited planar search for each water molecule defined by the water oxygen and two nearby acceptors and, therefore, does not include out-of-plane conformations, which might have a lower energy.

Deviations between the constructed hydrogen positions and the neutron diffraction assignments are shown in rows "2nd-Neut" (Table I). Significant deviations are present for Lys, Ser, Tyr, Thr residues, water molecules, and the N-termini. The deviations for Asn, Gln, and Arg residue hydrogens are small since they are treated as almost fixed in crystallographic refinement and in empirical potential energy

TABLE I. r.m.s. Differences of Hydrogen Positions†

	Fixed‡	N-ter NH_3	Lys ζ-H_3	Ser γ-H	Asn $\delta2$-H_2	Gln $\epsilon2$-H_2	Arg η-H_2	Tyr η-H_2	Thr $\gamma1$-H	Oh2 H_2
A. Trypsin										
No.	226	3	42	33	32	20	8	10	10	60
1st–2nd	0.00	0.00	0.00	0.37	0.00	0.00	0.04	0.06	0.10	0.04
2nd–3rd	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.01
2nd–Mini	0.06	0.09	0.04	0.07	0.04	0.05	0.04	0.11	0.06	0.35
2nd–Neut	0.09	0.76	0.19	0.89	0.04	0.05	0.02	0.64	0.34	–*
B. Ribonuclease										
No.	131	3	30	15	20	14	16	6	10	256
1st–2nd	0.00	0.00	0.08	0.24	0.00	0.07	0.00	0.08	0.78	0.18
2nd–3rd	0.00	0.00	0.02	0.08	0.00	0.00	0.00	0.00	0.41	0.03
2nd–Mini	0.07	0.04	0.13	0.11	0.06	0.07	0.04	0.05	0.14	0.56
2nd–Neut	0.10	0.49	0.60	0.98	0.11	0.10	0.08	0.60	1.12	1.20
C. BPTI										
No.	59	3	12	1	6	2	24	4	3	8
1st–2nd	0.00	0.00	0.00	1.16	0.00	0.00	0.00	0.90	0.00	0.04
2nd–3rd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2nd–Mini	0.08	0.05	0.03	0.08	0.05	0.03	0.04	0.05	0.07	0.20
2nd–Neut	0.11	0.81	0.25	0.71	0.11	0.09	0.08	0.81	0.19	0.35

†r.m.s. differences in Å averaged over all hydrogen atoms of the specified donor groups; "i-th" refers to i-th iteration of the hydrogen construction; "Mini" refers to the structure obtained by global hydrogen position minimization, and "Neut" refers to the appropriate neutron diffraction structure.

‡Including peptide group hydrogens, His δ1, His δ2, Arg ε, and Trp ε.

*There were no assignments for water hydrogens in the trypsin neutron diffraction structure.

functions; e.g., the dihedral angle barriers in the empirical potential energy function are relatively high (8.2 kcal/mol) compared to lower barriers for the Lys (0.6 kcal/mol), Tyr (1.8 kcal/mol), Ser (0.5 kcal/mol), and Thr (0.5 kcal/mol) residues. The small deviations for hydrogens without a degree of freedom (e.g., peptide group hydrogens, His δ1, and His ε2 hydrogens) are due to slightly different equilibrium parameters in the energy function and in the crystallographic refinement of the neutron diffraction structures. There are differences for the N-termini in trypsin and BPTI. In the neutron diffraction structure of trypsin the geometry of the NH_3-group was found to be highly distorted from ideal tetrahedral geometry, which is probably due to an artifact in the refinement program used. In the neutron diffraction structure of BPTI the N-terminus has ideal tetrahedral geometry, but the conformation is different from that one obtained through hydrogen construction.

The r.m.s. differences in Table I correspond to dihedral angle differences of the donor groups with rotational degrees of freedom. The percentage of dihedral angles for all three proteins that deviate by less than 20° is 40%, and for those that deviate by less than 40° it is 62%. The results for Ser residues is significantly worse than for the other flexible residues; i.e., the percentage of Ser residues having a deviation of greater than 60° is 47%. There appears to be no significant correlation between these devia-

tions and solvent accessibility, temperature factors, energy barriers, or proximity to water molecules. However, the temperature factors of all Ser residues of the trypsin neutron diffraction structure are quite large. Also, when hydrogens are built using the neutron results and the structure is locally minimized (i.e., the energy of each donor group is minimized individually while keeping the rest of the structure identical with the neutron diffraction structure) the resulting dihedral angle values are in better agreement with the constructed structures than the original neutron diffraction structure.

Distributions of dihedral angles for the neutron diffraction structures and the hydrogen-constructed structures of trypsin and ribonuclease are shown in Figure 1a–d. It appears that most angles are located near the minima of the dihedral potential energy indicated in Figure 1a–d. The neutron diffraction structure of ribonuclease has the most violations from this rule, e.g., the distribution of dihedral angles is almost random for Lys and Thr residues. This result suggests that the ribonuclease neutron diffraction structure hydrogen positions are less refined than in the other neutron diffraction structures.

In the following we discuss specific examples of the differences between the neutron diffraction and the hydrogen-constructed structures. In the hydrogen-constructed structure for Tyr 94 of trypsin, the η hydrogen is placed with a dihedral angle of $\chi = 10°$,
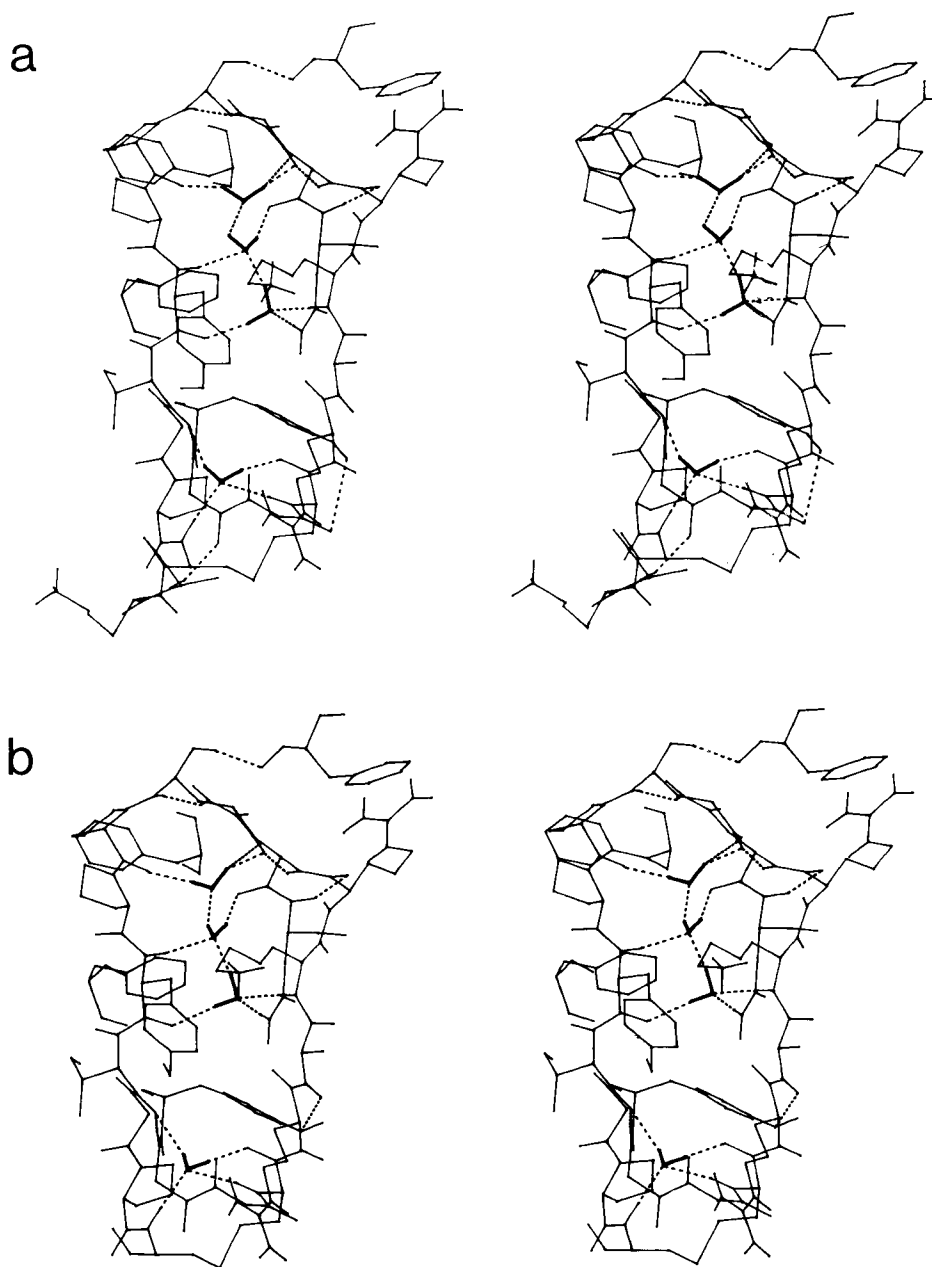
a

b

Fig. 3. Stereo pictures of the 5 Å environment of the four ordered water molecules in BPTI. a: The hydrogen positions of the protein and the water molecules were obtained after two iterations of hydrogen construction. b: The hydrogen positions of the protein and the water molecules were obtained from the neutron diffraction structure.[12] Hydrogen bonds are indicated if the following criteria are met: the heavy donor-acceptor distance is less than 4.0 Å and the donor-hydrogen-acceptor angle is less than 60° (the definition of the angle is such that a linear hydrogen bond corresponds to 0°).

and it donates no hydrogen bonds (Fig. 2a). The O^η oxygen is accepting a hydrogen bond from water 56. In contrast, the neutron diffraction structure (Fig. 2b) shows an alternative conformation where the η hydrogen of Tyr-94 points toward a lone pair of water 56. The latter conformation is in fact more favorable by about 0.4 kcal/mol (not shown). The reason that the hydrogen-construction method picked the first conformation is that during the positioning of the Tyr η hydrogen the water is only represented by an un-

charged oxygen atom, and, at this stage, the conformation in Figure 2b has a somewhat lower energy. This forces the subsequent placement of one of the hydrogens of water 56 such that it points toward the tyrosine O^η. This conformation represents a local minimum at 10° of the dihedral angle energy (Fig. 2c) and is stable enough not to be changed during further iterations or minimizations. The maximum at χ = 180° reflects a close contact between the hydrogen and the hydrogen of water 56. Outside this repulsive

region the energy profile is mainly determined by the electrostatic energy and modulated by the dihedral angle energy. The neutron diffraction conformation and the hydrogen-constructed conformation are alternatives with a small energy difference.

Figure 3 shows the environment of the four crystallographically ordered water molecules in BPTI with the hydrogen positions constructed (Fig. 3a) and obtained from the neutron diffraction data (Fig. 3b). The agreement between constructed and observed positions of the water hydrogens is good; in particular, all hydrogen bonds are conserved. The r.m.s. difference of the water hydrogen positions is 0.35 Å (Table I).

DISCUSSION

The hydrogen construction method presented in this work produces a good initial structure for further investigations including polar hydrogens. It should be particularly useful for molecular dynamics runs where the protein and water hydrogens undergo significant motion and a full global optimization is not necessary. The method can be described as a sequence of locally confined energy minimizations by which any close contacts between hydrogens are avoided.

The proximity of water molecules to the protein determines the order in which water molecule hydrogens are assigned. Water molecules near the protein are constructed first. In this way a structured water shell is built up around the protein. The conformational search for this water shell is very fast but also fairly limited. This explains some of the adjustments of the water molecules during simultaneous energy minimization of all hydrogen atom positions (Table I). The protein hydrogens are placed in a stable conformation, which remains unchanged during further minimization; i.e., the method produces an initial structure already near a local energy minimum of each protein hydrogen. The hydrogen construction reached self-consistency after two to three iterations for the systems studied. The computing time is small compared to global hydrogen position minimization.

It appears that in most cases the dihedral angles of the hydrogen donor groups are placed near the torsional energy minima. This is true not only for the hydrogen-constructed structures but also for the neutron diffraction structure assignments. The relatively small energy contribution from the torsional potential obviously selects allowed angle ranges around the minima. Nonbonded interactions such as electrostatic or solvent-protein interactions then determine which dihedral energy minimum has a lower energy. This compares well with results obtained for protein main and side chain atoms.[14–17] The mutual interaction between two or more protein and/or water hydrogens produces many possible hydrogen conformations or substates of the protein-water system. The energy differences between those substates appear to be small (under 0.5 kcal/mol); i.e., transitions between these substates could occur under physiological conditions and would be sampled in molecular dynamics studies.

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