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Hydroxyl hydrogen conformations in trypsin determined by the neutron diffraction solvent difference map method: Relative importance of steric and electrostatic factors in defining hydrogen-bonding geometries

(neutron diffraction/^2H_2O-H_2O solvent difference maps)

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ABSTRACT **Neutron diffraction maps have been used to assign the rotor conformations of the hydroxyl hydrogens in trypsin. Knowledge of these conformations is used to assess the relative importance of steric and electrostatic effects in conferring the H-bonding geometries of these groups. A general finding was that most hydroxyl groups are rotationally ordered with their highest populated conformation near the low-energy staggered orientation. For the low-energy conformers (−60°, 60°, 180°) of serine and threonine, the trans (−180°) position is most highly populated followed by +60°. In trypsin, only 1 of 24 serines was found in the −60° conformer. Serine hydroxyls preferentially act as H-bond acceptors and rarely are observed as H-bond donors alone. Threonines were found to be more likely than serines to participate in two H bonds; tryosines were found to prefer to act as donors. In H-bonding situations in which there was incompatibility between the energies defining the barrier to rotation and the local electrostatics, the electrostatic criteria dominated. Overall, the findings support a model of H bonding where there exists strong inherent complementarity between the low-energy hydroxyl orientations and the local electrostatic environment.**

The tertiary structure of a protein is defined by an elaborate combination of steric and electrostatic forces involving both protein–protein and protein–solvent interactions. Among the principal structural elements defining this structure are H bonds, interactions whose energies are both distance and direction dependent and are modulated by the dielectric character of the surrounding medium. The nature of the H bond has been extensively studied, and although it is fairly well understood in quite simple systems (1), in proteins, the heterogeneity of the interactions and the complex nature of the effective local dielectric make their geometries considerably more difficult to predict quantitatively.

Crystallographic studies have established several general stereochemical properties of H bonding in proteins (ref. 2 and references therein). In general, it appears that the H bond can be affected by a number of factors distinct from those immediately associated with the participating donor and acceptor groups, such as electrostatic effects and steric constraints of neighboring groups. In fact, the folded structure of a protein appears to be the result of a number of energetic compromises, some competing and others reinforcing, with the resulting balance being just enough to keep the molecule in its folded state (3–5).

Among the important types of H-bonding groups is the hydroxyl group, which facilitates bonding by acting as either a donor or an acceptor group or both. Because of this property, serine, threonine, and tyrosine can be found in diverse electrostatic environments. As a means for gaining further understanding of the relative importance of H-bond formation in the folding of proteins, hydroxyl groups offer a unique opportunity because they have a degree of rotational freedom that allows them to align themselves to minimize the energy of their H-bonding interactions. Opposing free rotation of the hydroxyl rotor is an inherent energy barrier reflecting the steric repulsion of adjacent bonded atoms. These values range from 1.3 kcal/mol for serine and threonine to 3.5 kcal/mol for tyrosine (1 cal = 4.184 J) (6). Thus, in the cases in which the orientation defining the strongest H-bond formation differs from that of lowest steric repulsion, the observed rotomer conformation indicates which effect predominates and to what extent.

To determine the conformation of a hydroxyl group requires that both the oxygen and hydrogen atoms be accurately located. By x-ray diffraction, the oxygen position of well-ordered hydroxyl groups can easily be determined, and in some cases the hydrogen position can be inferred by the character of the H-bonding partners. In many instances, however, these assignments are ambiguous and hydrogens with unusual or unexpected stereochemistry will not be identified. Except for relatively small proteins in which NMR techniques are applicable, direct assignment of hydrogen positions in proteins can best be made by neutron diffraction (Fig. 1), because the scattering potential of atom types depends on the character of the atomic nucleus (8), not the number of electrons, as is the case in x-ray diffraction.

Information of the rotomer orientations of each well-ordered hydroxyl group has been used to address several structural issues for which neutron data are uniquely applicable. The specific issues addressed relate to the relationship between the preferred hydroxyl orientations and their local electrostatic and steric environments. In particular, it was of interest to determine whether those hydroxyl groups involved in hydrogen bonding have geometries preferentially defined by the energy associated with the group's intrinsic barrier to rotation or by the H-bond distance (or other electrostatic criteria). How many situations exist in which both the conformational energy and the electrostatic field strength are complementary? Conversely, can conformations of high energy be rationalized by opposing electrostatic or steric effects? In addition, since the chemical character of a phenolic hydroxyl is somewhat different from a serine or threonine hydroxyl, it was of interest to ascertain whether

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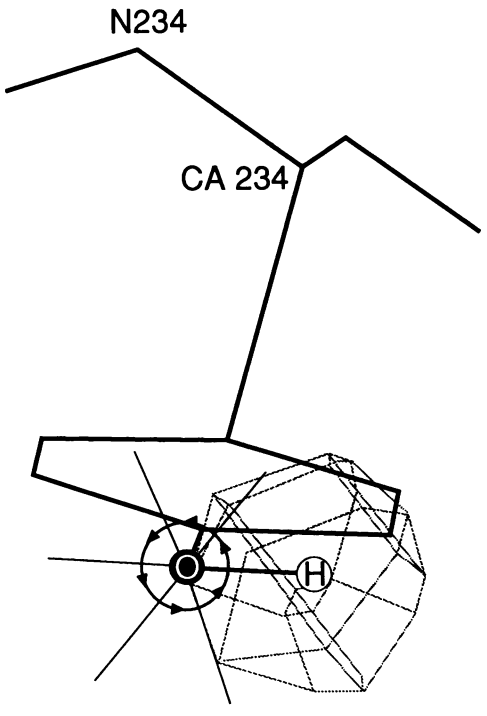

FIG. 1. Hydroxyl hydrogen (H/^{2}H) difference peak for Tyr-184. Method to determine hydroxyl rotomer orientation uses density from solvent difference map sampled at defined intervals (10° in this analysis) around the hydroxyl rotor axis. The density at the sampling points is approximated by using a 27-point interpolation scheme (7), which, in the grid size of the maps used, corresponds to a 3.7-Å^3 volume.

there were corresponding differences in donor/acceptor preference between the hydroxyl types.

METHODS

Determination of Hydroxyl Orientations. The positions of hydroxyl hydrogens were determined by using ^2H_2O-H_2O neutron solvent difference maps (9, 10). In practice, these difference maps (or H/^{2}H exchange maps) are obtained by comparing the diffracted intensities from a crystal containing H_2O as the major solvent constituent to a crystal in which ^2H_2O is exchanged for H_2O. Because H and ^{2}H have very different scattering properties (−3.8 fermi for H, +6.7 for ^{2}H) (7), their differences [+6.7 − (−3.8) = 10.5 fermi]‖ are accentuated to give an accurate and nearly unbiased representation of exchangeable hydrogen sites and water structure.

The neutron data on an unexchanged (H_2O) and an exchanged (^2H_2O) crystal were collected at the Brookhaven High Flux Beam Reactor with crystals in both cases of ≈3 mm^3. The exchanged crystal was soaked in a ^2H_2O mother liquor for ≈1 year to exchange all waters of crystallization, labile side chain hydrogens, and most of the amide peptide hydrogens. The two trypsin structures were refined independently to give *R* factors of 0.191 at 1.8 Å resolution for the ^2H_2O structure and 0.193 at 2.1 Å for the H_2O structure. A detailed description of the data collection procedures and structure analysis for the ^2H_2O structure has been reported elsewhere (11).

‖A fermi is a measure of the scattering capacity of a type of atom (such as hydrogen, nitrogen, or oxygen) toward neutrons and is similar to an x-ray form factor. Fermis are in units of scattering length, 10^{-13} cm.

Because the solvent difference map method requires an equivalent set of exchanged and unexchanged data, the analysis was limited to the 2.1-Å resolution of the H_2O data. The method of identification and refinement of H/^{2}H exchange density is a multistep procedure that has been described in detail elsewhere (9). Twelve cycles of solvent difference map refinement gave an *R* factor of 0.142. It is worthwhile to note that an important characteristic of the solvent difference map method is that it is considerably less affected by phasing errors than conventional difference maps.

The rotational orientation of each hydroxyl hydrogen was determined by sampling the difference map in 10° intervals around its rotor axis [defined by the C-$O_{(hydroxyl)}$ bond] at the stereochemically appropriate position expected for a bonded hydrogen (bond length, 1.05 Å; bond angle, 109°) (Fig. 1). The observed densities plotted as a function of their rotation angle give density profiles of the type shown in Fig. 2. The degree of order of the hydroxyl groups can be approximated by the shape of their profiles. The breadth of these peaks is also a function of the resolution of the data used in the analysis and the order of the parent oxygen onto which the hydroxyl hydrogen is bonded. By using this approach, a large percentage of the hydroxyl conformations in the protein trypsin have been assigned and are used as the basis of the analysis presented here.

RESULTS AND DISCUSSION

Analysis of Hydroxyl Rotor Plots. The general character of the rotomer plots indicates that a majority of the hydroxyl hydrogens are rotationally ordered in the time averaged sense. The degree of order correlates with whether the hydroxyl is H bonded (as either a donor or acceptor) and to a lesser extent with the temperature factor of the parent hydroxyl oxygen. There are several instances in which the oxygen is highly ordered, as judged by its atomic temperature factor ($B < 15$ Å^2), yet the hydrogen density is too weak to allow assignment of the orientation. This is presumably because the hydrogen adopts several different conformations. On the other hand, in some cases the hydrogen adopts a single rotomer state allowing the orientation to be assigned with reasonable accuracy (±30°) even though the hydroxyl oxygen is thermally disordered ($B > 30$ Å^2).** This is a consequence of the fact that the scattering from an exchanged hydrogen is almost twice that of the oxygen, making its position more readily distinguishable from the general noise features in the map. As best as can be determined, all hydroxyl groups with the exception of Ser-54 are fully exchanged. The Ser-54 hydroxyl is embedded in a β-sheet linking O-43 and N-55; the hydroxyl hydrogen is ≈80% exchanged.

The pattern of the rotor profiles defining hydrogen orientations for threonine and serine groups shows a clustering around the low-energy staggered positions [+60°, 180°, −60° (±20°)] (Fig. 2 *a–c*). Of the three predominant conformers, the trans (180°) position is the highest populated (46%) followed by the 60° conformer (38%). Only 1 of the 24 well-ordered serines (Ser-84) has a hydroxyl hydrogen that adopts a conformation close to −60°. Although the trypsin findings presented here are derived from a limited data base, the absence of the −60° conformation for serines is statistically significant. For the serine and threonine groups, no apparent correlation was found between the hydroxyl oxygen conformation, as measured by its χ_1 torsion angle, and the observed orientation of the hydroxyl hydrogen.

All the tyrosine side chains in trypsin are reasonably well-ordered as are their hydroxyl hydrogens. The preferred rotor conformers of tyrosine hydroxyls are 0°, 180°, reflecting

**Orientations were assigned to Ser-61, -84, -127, and -147 from the position of the hydroxyl hydrogen.

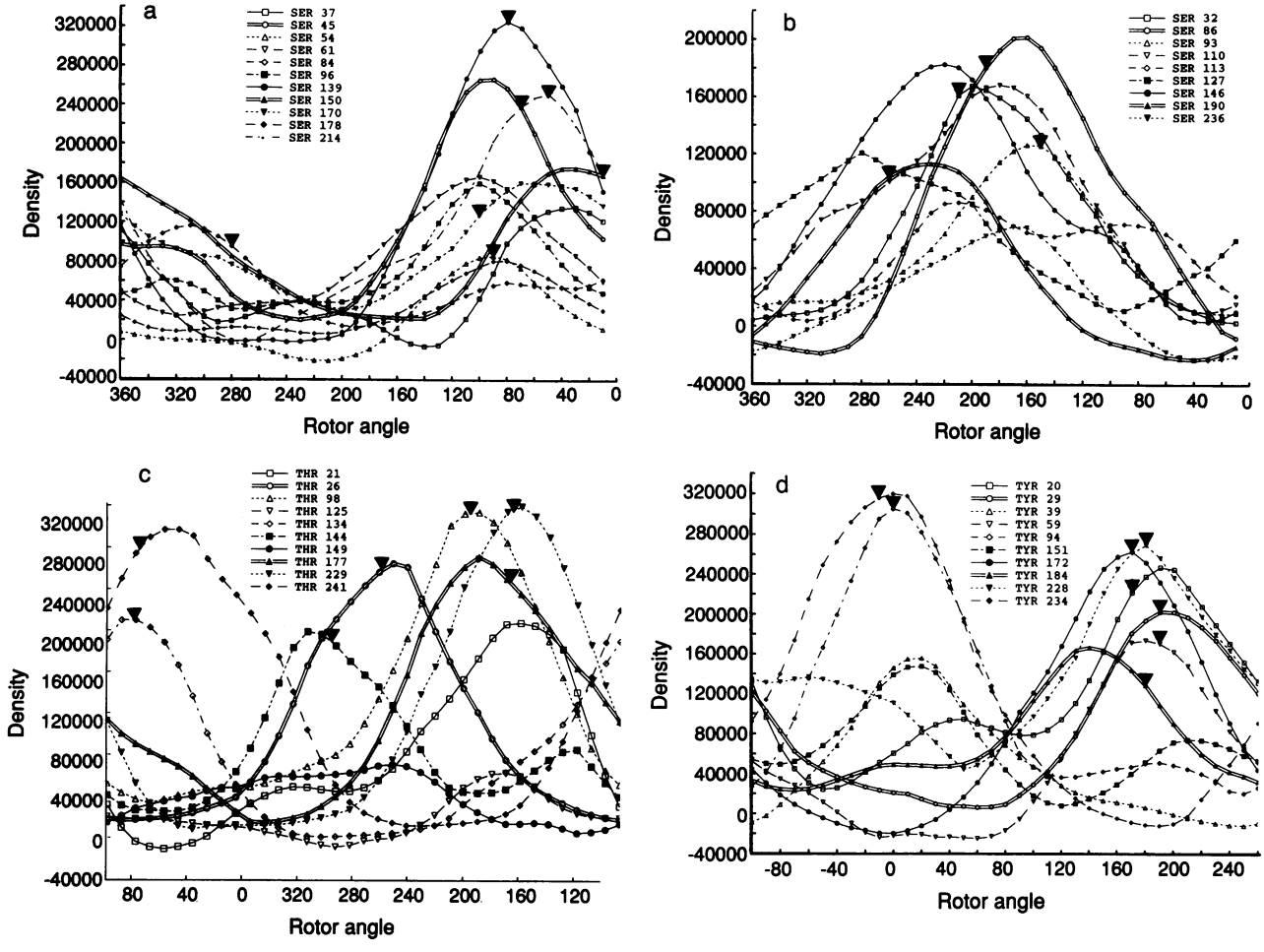

FIG. 2. Rotor density profiles indicating the density observed at specific rotation angles for the well-ordered hydroxyl groups. Low energy positions for threonine and serine are (−60, +60, 180), for tyrosine (0, 180°). Serines are divided into two groups to facilitate interpretation. (*a* and *b*) Serines. (*c*) Threonines. (*d*) Tyrosines. For hydroxyl groups that H bond as donors, the rotor angle coinciding with the shortest distance to an acceptor group is highlighted by an arrowhead.

the high energy barrier (3.5 kcal/mol) created by the π electron system of the phenol ring (6). The average deviation observed for these conformers in the 9 tyrosines in trypsin is 8.7°. Although in general these groups adopt one or the other conformation exclusively, there is evidence of bimodal character in the density distribution for Tyr-20 and Tyr-94 (Fig. 2*d*). In both cases, the predominant conformer forms a H bond through the hydroxyl hydrogen to a water(s); there is no apparent H-bond partner for the hydroxyl oxygen in either case. A 180° rotation of the O-H orientation would reverse the H-bonding pattern of the hydroxyl. In this orientation, the H-bonding capacity of the oxygen could be satisfied through an interaction with the water without requiring a spatial disruption of the water structure. Because this water is at the surface and interacts with no other protein group, its conversion from being the H-bond acceptor in the predominant hydroxyl conformer to being a donor in the minor conformer could be accommodated without causing indirect effects. In another study, the bimodal character of Tyr-94 was predicted based on the water's ability to interchange donor/acceptor roles and other electrostatic criteria (12).

H-Bonding Trends. The H-bonding donor/acceptor preferences of the three hydroxyl types appear to be somewhat different. Of the 25 well-ordered serine hydroxyl groups, 24 of the O^{γ} oxygens participate in H bonds as acceptor groups, while only 12 of the hydroxyl hydrogens act as donors. In 11 of the 12 cases in which they act as donors, the hydroxyl groups also participate in H bonding in an acceptor capacity. Thus, in those instances in which the hydroxyl is involved in a single H bond there is a strong preference (11 to 1) for the interaction to be through the oxygen rather than the hydrogen.††

These observed donor/acceptor preferences for serines differ somewhat from the general trend reported by Baker and Hubbard (2). Based on data compiled from the structures of 13 proteins (trypsin included), they found that, when considering H-bonding interactions between the hydroxyl and other protein atoms exclusively, the serine hydroxyls acted primarily as donor groups (67% of the time). Part of this discrepancy is derived from the fact that in the Baker–Hubbard analysis, water interactions were not included, while in this analysis many of the included interactions involve bound water molecules. In fact, for the serines in trypsin there is a clear pattern that shows bound water molecules associate preferentially with the O^{γ} (18 instances of H bonding to O^{γ} compared to 5 to the hydrogen). Even though water molecules bound to hydroxyl oxygens may form strong H bonds, due to the rather diffuse nature of the oxygen lone pairs (13) they can appear to be only partially ordered in density maps. Because ^2H_2O-H_2O difference maps are considerably more sensitive (a water at 10–20% occupancy can be identified) than x-ray difference maps, more

††A recent neutron analysis in this laboratory showed that there is not a similarly strong preference for hydroxyls in subtilisin to act as acceptors rather than donors.

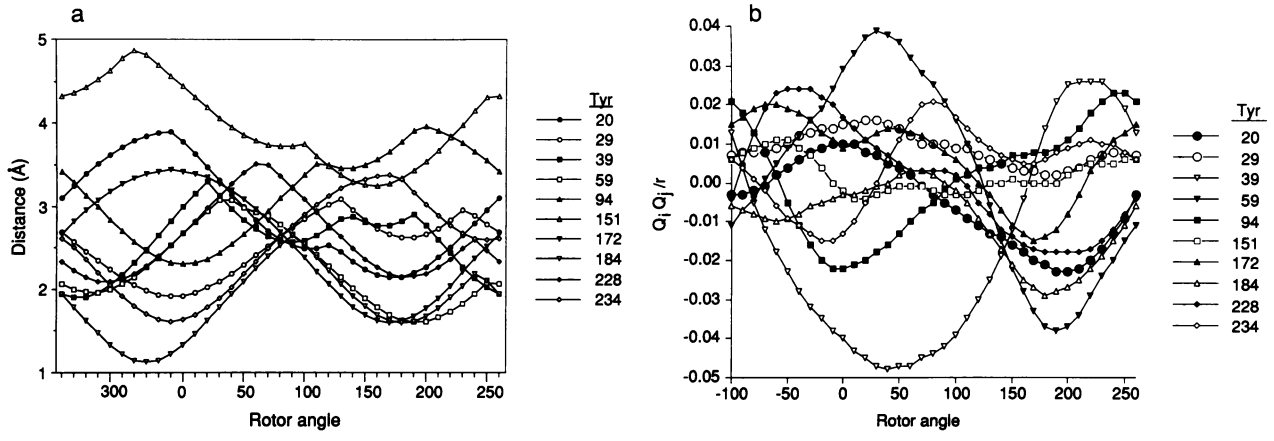

FIG. 3. (*a*) Distance of closest approach of tyrosine hydroxyl hydrogens to potential acceptor ligands as a function of rotation angle. Note the bimodal character of several tyrosines (residues 29, 184, and 228) and apparent short H-bond distances. This is due to situations where the O^{η} is H bonded to a water; water peaks result from the scattering of hydrogens (deuteriums) alone and thus are found closer to acceptor ligands than are oxygens in the x-ray case. (*b*) Values of electrostatic potentials centered at the hydroxyl hydrogen position for the tyrosine residues calculated as a function of rotation angle. Electrostatic potential was calculated by using $\Sigma\, Q_iQ_j/R$; where $Q_{(i,j)}$ are the partial charges of atoms i and j. Partial charges were taken from ref. 6. All atoms within a 6-Å sphere of the hydroxyl hydrogen were included. In cases in which there were interactions with water, the orientation of the water was assigned to complement the donor/acceptor character dictated by the orientation of the hydroxyl.

coordinated water molecules were included in this data base than were available from the x-ray structure (14).

In the H bonding of the threonine hydroxyl groups, there are 8 cases in which the group acts as an acceptor and 6 in which it is a donor. Consistent with the findings for the serines, the H-bond donor capacity is strongly coupled to its also being an acceptor (6 of 6 cases). However, compared to serines, threonines display a stronger tendency toward having their hydroxyl involved in two H bonds.

The tyrosines differ considerably in their H-bonding properties from those observed for the serine and threonine hydroxyl types. The phenolic hydroxyl of the tyrosines shows a distinct preference to function as H-bond donors (8 of 10). Only one (Tyr-39) acts solely as an acceptor, while two are involved as both a donor and an acceptor. The hydroxyl of Tyr-151 is unique in the trypsin structure in that it is the only hydroxyl group located in a buried environment with no protein ligands or water in its vicinity. The cost in energy of burying a hydroxyl in this highly shielded environment is likely to be significant and since it is not conserved in similar serine protease enzymes, its presence in bovine trypsin is difficult to rationalize.

Hydroxyl Orientations: Steric vs. Electrostatic Effects. To evaluate how H-bonding distances (and consequently H-bonding energy) change as a function of the hydroxyl orientation, the distance of approach of the hydroxyl hydrogen to a potential H-bond acceptor was evaluated for each group as a function of its rotation angle (Fig. 3*a*). In those instances in which acceptor groups were within H-bonding distance, the curves were generally characterized by broad smoothly varying minima. A rotation of 45° either side of the minimum changes the apparent H-bond distance by only ≈0.25 Å; a 20° rotation changes the bond distance by <0.10 Å. Given that the experimental accuracy of the coordinates used in this analysis is slightly better than 0.15 Å, deviations from the minimum H-bonding distance are considered significant only when they exceed 20°.

A similar curve to that described above is seen when the electrostatic potential calculated for the hydroxyls is plotted as a function of the rotor conformations (sampled at 10° intervals) (6) (Fig. 3). It is emphasized that this calculation is only meant to identify trends and is nonrigorous since it does not consider dielectric or other shielding effects. The breadth of the peaks defining the maximum and minimum of the curve indicates that the electrostatic effects are also described by a slowly changing function. Overall, the trends in the energy profile correlate well with the observed hydroxyl conformation. It is interesting to note that the two hydroxyls with the lowest calculated energy (Tyr-39 and -59) are not the highest ordered based on respective peak heights, while Tyr-29 and -151, which show little energy preference, are as well-ordered as Tyr-39 and -59. This suggests that electrostatics alone do not dictate rotomer order.

In Fig. 4, the deviations from the shortest H-bond distance of the 18 most highly ordered hydroxyl groups are plotted against the observed departure of the corresponding rotor orientation from a staggered (−60°, 180°, +60°) (0°, 180° for tyrosines) conformation. The findings indicate that a majority of H-bonding distances are very near their minimum possible value (<0.2 Å). By and large the rotor conformation that defines the minimum H-bond distance is close to a staggered orientation; the optimum orientations based on H-bonding distance are highlighted in the rotomer profile. Note, however, that the several hydroxyl groups that deviate significantly from staggered (Ser-45, Ser-54, Thr-26, Thr-134) have rotor conformations that minimize H-bonding distance. Thus, when there exist H-bonding situations in which the bonding distance and the barrier to rotation of the hydroxyl hydrogen are not correlated, the distance criterion appears to take precedence.

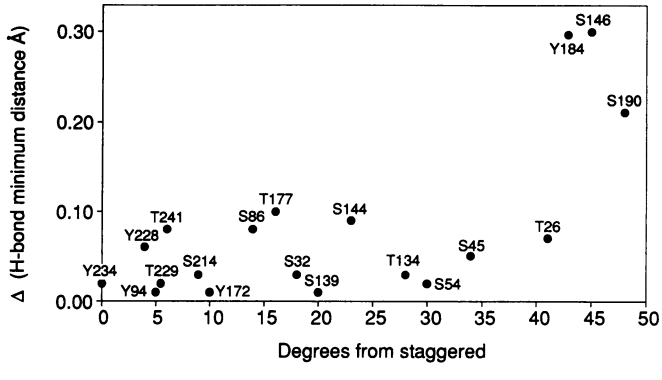

FIG. 4. Difference (in Å) between the observed H-bond length and the minimum calculated value for the hydroxyls that act as donors plotted against their deviation from staggered. Note that most H bonds are within 0.15 Å of a minimum even though some are significantly perturbed from staggered.

For hydroxyl groups that are involved in two H bonds, the angle between the hydroxyl hydrogen and the atom acting as the donor to the hydroxyl oxygen is 139° ± 21°.‡‡ The relatively large spread seen in this angle indicates that there is no strong preference for a single donor–acceptor geometry. Furthermore, the value of 139° for this angle indicates that the H-bonding ligand is not pointed directly at an oxygen lone pair, but more toward a point bisecting the lone pairs, slightly favoring one over the other. This is consistent with other observations and theory that imply that, due to the diffuse nature of the lone pairs, directionality of the acceptor is somewhat less important than that of the donor (13).

Using more sophisticated computational methods than were used here, Brünger and Karplus (12) have calculated the positions of all the hydroxyl rotors in trypsin based on steric and electrostatic factors. They found that the lowest energy rotomers generally conformed to staggered conformations and were in basic agreement with the previously published hydroxyl coordinates from this laboratory. The hydroxyl coordinates presented here also agree reasonably well; however, their overall accuracy is considerably higher than that reported previously. This is especially important in establishing the significance of deviations from the norm.

Groups Deviating from the General Trends. As shown in Fig. 4, there are four groups that are perturbed out of the staggered conformation by 40° or more. These groups were examined in detail to ascertain the stereochemical basis for the observed deviations. All four groups act as both a H-bonding donor and acceptor. The conformations of the Thr-26 and Ser-146 hydroxyl groups, relative to the peptide main chain, are similar and are characterized by their hydroxyl oxygen H bonding to its own backbone amido peptide nitrogen. This type of back-bonding to the main chain results in a poor H-bond geometry, but there clearly exists some interaction because the O^{γ} to HN distances are <2.5 Å, considerably less than the normal van der Waals distance. The interaction of the O^{γ} with the main chain nitrogen greatly restricts the rotational freedom of the hydroxyl hydrogen. For the hydroxyl of Thr-26 to attain a staggered conformation, while still remaining H bonded (to O-23), requires the hydrogen to HN distance be reduced to 1.8 Å, a situation that is sterically unacceptable. However, in the observed conformation, the hydroxyl rotomer almost exactly coincides with the position that maximizes the H-bond interaction in the context of the steric restrictions presented by the main chain conformation. Ser-146 has similar limitations with regard to its rotomer conformations. In the three possible staggered positions either there are severe steric overlaps or the H-bonding interactions are reduced or eliminated.

In the case of Ser-190 the perturbation appears to be affected more by electrostatic factors than those described above. Ser-190 is in the substrate binding pocket of the enzyme and H bonds to three groups—as an acceptor to an internal water and as a donor to a second internal water and the hydroxyl O^{η} of Tyr-228. At rotomer angles near either 180° or 60°, the donor–acceptor geometries of the serine interchange, which would effectively require a compensatory reorientation of the internal waters. This presumably would present an energetically unfavorable situation because one of the water molecules, 312, acts as an acceptor to the amide peptide nitrogen of residue 214 and the second internal water. These water molecules are highly conserved in the trypsin family of enzymes (15), as are their H-bonding interactions, and thus their orientations would not be expected to be alterable. At the other staggered conformer (−60°) the hydrogen is sterically impeded by the HN of the main chain. Although the observed conformation of 120° is of maximum energy, it appears to be the best compromise to maintain all H-bonding interactions.

The displacement of 40° from the low energy conformer of the Tyr-184 hydroxyl is not easily rationalized on simple steric or electrostatic grounds. Based on electrostatic criteria, the rotomer angle should be much closer to the low energy conformer (180°) than is observed. The H bond to the carbonyl oxygen of 159 in the low-energy conformer is perhaps somewhat short (1.6 Å, hydrogen to oxygen) and there is a van der Waals close contact to the main chain C^{α}, but a rotation of no more than 20° (not the 40° that is observed) out of plane would be required to relieve the close contacts. The hydroxyl oxygen also H bonds to a water; however, the geometry of this interaction does not appear to be significantly altered between the observed position and the position of the predicted low-energy conformer. Consequently, it is not apparent, based on steric and electrostatic grounds, why there is such a marked departure (1.2 kcal/mol) of the hydroxyl rotomer from ideality.

The findings from the neutron solvent difference map data have shown that the orientation of hydroxyl hydrogens is not exclusively determined by H-bonding geometries—other electrostatic and steric factors also contribute. Although in some instances steric and electrostatic effects perturb hydroxyl orientations out of low-energy conformations, overall the findings present a consistent picture of the inherent complementarity between the low-energy hydroxyl conformations and their stereochemical environment. The observations presented here, correlating steric and electrostatic influences on hydroxyl conformations, should prove useful as a guide to define, from the complicated mixture, the relative importance of the several of the types of forces involved in protein packing.

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‡‡Angle defined by torsion angle between H-$O_{(hydroxyl)}$–electron pair–donor.