Crystal Structure of Tetradeca-(3-Fluorotyrosyl)-Glutathione Transferase

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The substitution of unnatural amino acids into proteins has long been recognized as a valuable technique for structural and mechanistic investigations of enzymes, truly bringing the study of macromolecules into the realm of physical organic chemistry. Unnatural amino acids can be incorporated site-specifically by the Schultz methodology[1] or, in special cases, globally by heterologous expression of the protein in the presence of the unnatural amino acid. The latter technique is now commonly used for the incorporation of selenomethionine for use as an isomorphous heavy atom derivative in the solution of protein crystal structures.[2]

Fluorinated aromatic amino acids such as 3-fluorotyrosine (3-FTyr) and 5- and 6-fluorotryptophan are among the few unnatural amino acids that can be incorporated into proteins by microorganisms at reasonable levels of enrichment. This fact has been exploited for some time in ^{19}F NMR spectroscopic investigations of the microenvironments of tyrosyl and tryptophanyl residues in proteins.[3] More recently, 3-FTyr has been used as a mechanistic probe of enzymes that utilize a tyrosyl hydroxyl group in catalysis.[4,5] The mechanistic perturbation is generally assumed to derive solely from the more acidic hydroxyl group of 3-FTyr.[6] Although fluorine is often considered an isosteric replacement for hydrogen, the real situation is more complex. An aromatic C−F bond is 0.3 Å longer and has a bond dipole opposite that of a C−H bond. In addition, fluorine is a weak hydrogen-bond acceptor. In spite of their widespread use as environmental and mechanistic probes, there exists no information concerning the structural consequences of the substitution of fluorinated aromatic side chains in a macromolecular context.

We recently demonstrated that global incorporation of 3-FTyr into a GSH transferase alters the position of the proton in the ground state changing the catalytic mechanism from electrophilic stabilization (Tyr6O−H···$^-$SG) of the nucleophile to general base catalysis (3-FTyr6O$^-$···H−SG).[5] The change in mechanism is accompanied by a 10-fold decrease in catalytic efficiency. Whether this decrease is related entirely to the intrinsic difference in mechanism or to unknown structural alterations deleterious to catalysis is not clear. To investigate the structural consequences of the replacement Tyr with 3-FTyr at the active site and elsewhere in the protein, we have solved the crystal structure of the $(3\text{-FTyr})_{14}$-enzyme at a resolution of 2.2 Å.[7]

The structure is the first of a protein in which Tyr residues are quantitatively replaced by 3-FTyr and provides a library of 14 (28 crystallographically distinct) microenvironments from which the nature of the interactions of the fluorine atoms with the rest of the protein can be evaluated. There are several possible effects of fluorine substitution on the behavior of tyrosyl side chains in addition to that expected from the lower pK_a of the hydroxyl group.[5] The single fluorine breaks the 2-fold symmetry of the aromatic ring such that one conformer, the other conformer, or neither conformer may be favored by steric or electronic factors such as hydrogen bonding.

Twenty-four of the 28 3-FTyr residues in the asymmetric unit exhibit electron density consistent with a single conformer with respect to the location of the fluorine atom. This common situation is best exemplified by the $2F_o - F_c$ electron density map for the two interacting core residues 3-FTyr32 and 3-FTyr61 (Figure 1). Only two residues (3-FTyr154 in subunits A and B) show clear evidence of occupying two conformers at this resolution, as illustrated in Figure 2. The remaining two 3-FTyr residues at position 115 in both subunits are in regions of poor electron density and exhibit no $F_o - F_c$ peaks for the fluorine atoms.

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(6) For example, the pK_a of the hydroxyl group of 3-FTyr6 is 7.5 as compared to 10.0 for Tyr6 in the native enzyme (see ref 5).

(7) Tetradeca(3-fluorotyrosyl)-GSH transferase (>95% 3-FTyr), (ref 5) 10 mg/mL, containing, 2 mM of the product (9*R*,10*R*)-9-(*S*-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene, 0.3% *n*-octyl-*β*-D-glucopyranoside, 10% polyethylene glycol (PEG) 3000, 1 M LiCl buffered with 50 mM MOPS (pH 6.8), crystallized in the space group $P2_12_12$ from hanging drops equilibrated at 22 °C against wells containing of 22−26% PEG 3000, 1 M LiCl buffered with 50 mM MOPS (pH 6.8). A single crystal with dimensions of 0.2 × 0.2 × 0.5 mm was mounted on a nylon loop, flash frozen and maintained at −170 °C during the entire data collection. X-ray diffraction data were collected using a Rigaku R-axis IIc image plate set 120 mm from the crystal and a Rigaku RU-200 rotating copper anode source operating at 5 kW (100 mA, 50 kV). Exposures of 30 min/degree of oscillation range were used. The raw data were processed and scaled using the HKL software package (ref 8). The molecular replacement structure solution was found using the protein atom coordinates of 3GST (ref 9) as the search model and the AMoRe package (ref 10). After initial simulated annealing with X-PLOR (ref 11), additional refinement was carried out using the TNT refinement package (ref 12). The complete model of the product in the active site was built into the $F_o - F_c$ map contoured at 3σ after one round of least-squares refinement. The F atoms identified in the $F_o - F_c$ map, contoured at 3σ, were then incorporated on the tyrosines. The refinement continued with the addition of water molecules until the final structure converged with good geometry and a crystallographic *R* factor of 0.179 on all data between 2.2 and 20.0 Å. Subunits A and B in the asymmetric unit have an RMS deviation on C$^\alpha$ of 0.44 Å. The coordinates have been deposited with the Brookhaven Protein Data Bank under file name 3FYG. Certain commercial equipment, instruments, and materials are identified in this paper to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument, or equipment identified is the best available for the purpose.

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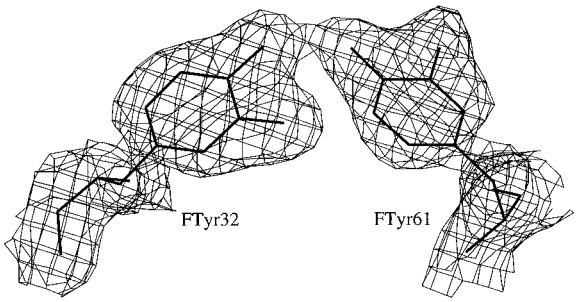

Figure 1. Portion of the final $2F_o - F_c$ electron density map contoured at 1σ illustrating the interaction of 3-FTyr32 and 3-FTyr61 in subunit A.

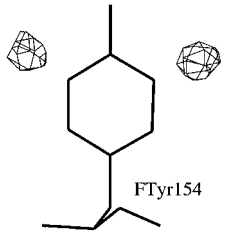

Figure 2. The $F_o - F_c$ electron density map for 3-FTyr154 contoured at 4σ indicating two peaks of excess electron density for the fluorine associated with two conformers of the side chain. Both 3-FTyr154A and 3-FTyr154B were eventually refined with an occupancy of 0.5 for the fluorine atom in the two positions.

Do the fluorine atoms participate in hydrogen-bonding interactions with other groups? The crystallographic evidence suggests that the fluorine atoms act as hydrogen-bond acceptors from both -NH and -OH donors. Fifteen of the 28 fluorine atoms are within hydrogen-bonding distance of one more potential donors, with 10 of the 15 close to water molecules. In the majority of cases, the hydrogen-bonding pattern of a 3-FTyr residue is identical in both subunits. For example, both the fluorine atom and hydroxyl group of 3-FTyr32 (Figure 1) are within hydrogen-bonding distance (2.9−3.1 and 2.6−2.8 Å, respectively) of the hydroxyl group of 3-FTyr61 in both subunits. Here it appears that the hydroxyl group of 3-FTyr61 acts as a hydrogen-bond donor since it has no other potential hydrogen-bonding partners. Other potential hydrogen bonds involving fluorine atoms include 3-FTyr160A and NE2 of His14A (3.2 Å), 3-FTyr196A and NE of Arg201A (3.2 Å), 3-FTyr202A and OG of Ser204A (2.7 Å), and 3-FTyr202B and NE2 of His14B (2.6 Å). Although the close proximity of a fluorine to a potential hydrogen-bond donor does not prove that a hydrogen bond exists, the preponderance of donors observed near fluorine atoms in the structure suggests that these interactions occur with reasonable frequency.

Does the introduction of fluorine alter the conformation of the 3-FTyr side chain? A survey of the side chains indicates that, on average, χ_1 and χ_2 change very little (ca. $\pm 6°$ and $\pm 10°$, respectively) in the fluorinated enzyme.[13] There is one very notable exception: the side chain of 3-FTyr6 located in the active site (Figure 3). In this instance the fluorine atom is nestled very close to the sulfur of the thioether product such that the side chain is rotated and the hydroxyl group is pushed 0.3−0.6 Å further away from the sulfur than in the native structure. The alternative conformation in which the fluorine is distal to the sulfur results in an unfavorable steric interaction with the carbonyl oxygen of Arg10.

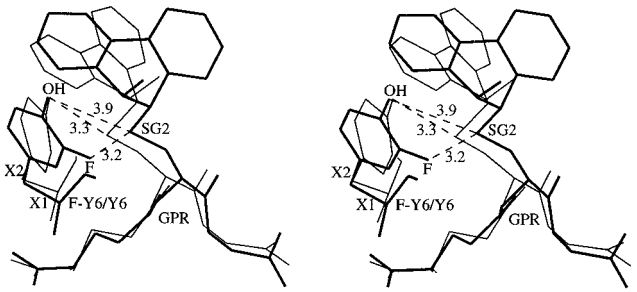

Figure 3. Stereoview of a comparison of the interactions of 3-FTyr6 and Tyr6 with the sulfur in the product (9*R*,10*R*)-9-(*S*-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene (GPR) in the mutant (thick line) and native (thin line) structures of subunit A. Interaction distances are given in angstroms. The differences in χ_1 and χ_2 in the native and mutant structures are $+9°$ and $-42°$, respectively.

The surprising proximity of the fluorine of 3-FTyr6 to the sulfur has significant implications for catalysis by the mutant enzyme. The sulfur of GS^- bound at the active site of the native enzyme is located in the plane of the aromatic ring in van der Waals contact with the edge and within hydrogen-bonding distance of the hydroxyl group of Tyr6.[14] The on-edge interaction of the thiolate with the quadrapole of the aromatic system is a favorable one with respect to electrostatics ($C^{\delta-}$—$H^{\delta+}\cdots{}^-SG$).[15] Moreover, the orientation of the sulfur is unchanged in the uncharged thioether product complex.[8] If the conformation observed in the product complex of the mutant enzyme accurately reflects the structure of the $(3\text{-FTyr})_{14}$-enzyme·GSH complex, then the ionization of the thiol on the way to the transition state for reaction would be less favorable due to the repulsive charge−dipole interaction between the C−F bond and the thiolate ($C^{\delta+}$—$F^{\delta-}\cdots{}^-SG$). In addition, any increase in the distance of the 3-FTyr—$O^-\cdots$H—SG hydrogen bond due to the close steric interaction between the fluorine and sulfur would tend to make the 3-fluorotyrosinate anion a less effective general base for forming the transition state.

We conclude that the influence of the fluorine atom on the catalytic efficiency of $(3\text{-FTyr})_{14}$-GSH transferase may be more complex than simply lowering the pK_a of the active site hydroxyl group. The structural results further suggest that due consideration be given to the proximity of the fluorine atom and the hydroxyl group of 3-FTyr-containing enzymes, particularly where the hydroxyl group is implicated in catalysis.

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(13) It should be pointed out that the position of the -OH group is extremely sensitive to changes in χ_1 and relatively insensitive to changes in χ_2 due to the coaxial relationship of the CB−CG and CZ−OH bonds.

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