TITLE: Mechanistic Inferences from the Crystal Structure of Fumarylacetoacetate Hydrolase with a Bound Phosphorus-based Inhibitor.

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Abbreviations:
AP, acetopyruvate; $n$-BuLi, $n$-Butyllithium; CSD, Cambridge Structural Database; EDTA, Ethylenediaminetetraacetic acid; FAA, Fumarylacetoacetate; FAH, Fumarylacetoacetate hydrolase; HMPOBA, 4-(hydroxymethylphosphinoyl)-3-oxo-butanoic acid; IPTG, Isopropyl $\beta$-D-thiogalactopyranoside; LB, Luria broth; NMR, Nuclear magnetic resonance; PCR, Polymerase chain reaction; PMSF, Phenylmethylsulfonyl fluoride; THF, Tetrahydrofuran; TMS, Tetramethylsilane; TMSI, Iodotrimethylsilane;
Summary

Fumarylacetoacetate hydrolase (FAH) catalyzes the hydrolytic cleavage of a carbon-carbon bond in fumarylacetoacetate to yield fumarate and acetoacetate as the final step of Phe and Tyr degradation. This unusual reaction is an essential human metabolic function, with loss of FAH activity causing the fatal metabolic disease hereditary tyrosinemia type I (HT1). An enzymatic mechanism involving a catalytic metal ion, a Glu/His catalytic dyad and a charged oxyanion hole was previously proposed based on recently determined FAH crystal structures. Here we report the development and characterization of a FAH inhibitor, 4-(hydroxymethylphosphinoyl)-3-oxo-butyric acid (HMPOBA), that competes with the physiological substrate with a Ki of 85 µM. The crystal structure of FAH complexed with HMPOBA refined at 1.3 Å resolution reveals the molecular basis for the competitive inhibition, supports the proposed formation of a tetrahedral alkoxy transition state intermediate during the FAH catalyzed reaction and reveals a Mg$^{2+}$ bound in the enzyme's active site. The analysis of FAH structures corresponding to different catalytic states reveals significant active site side chain motions that may also be related to catalytic function. Thus, these results advance the understanding of an essential catabolic reaction associated with a fatal metabolic disease and provide insight into the structure-based development of FAH inhibitors.

Introduction

The aromatic amino acids Phe and Tyr are catabolized by a pathway of six enzyme catalyzed reactions. The inherited metabolic diseases phenylketonuria, alkaptonuria and tyrosinemia types I, II and III are associated with mutations of these enzymes. Mutations in fumarylacetoacetate hydrolase (FAH; EC 3.7.1.2) cause hereditary tyrosinemia type I (HT1), the most severe disease associated with Phe and Tyr catabolism (1). HT1 is an autosomal recessive disease with an acute
form that causes death in infancy due to liver failure and a chronic form that causes an early age death due to hepatocellular carcinoma, liver cirrhosis and neurologic crises (2, 3). The lethal nature of HT1 is most likely due to the accumulation of the toxic intermediate fumarylacetoacetate in the absence of FAH activity (4-6). This reactive metabolite may cause direct DNA (7) and tissue damage, depletion of cellular glutathione levels (8, 9) and induction of apoptosis (10). HT1 is currently treated by combined liver/kidney transplantation (11) and pharmacologic inhibition of hydroxyphenylpyruvate dioxygenase (12), which catalyzes an earlier step in Phe/Tyr catabolism.

A mouse model of HT1 has been developed (13, 14) and can be treated effectively using adenoviral and retroviral based gene therapy vectors to restore FAH activity (14-16). These studies have demonstrated that hepatocytes expressing FAH exhibit a strong selective advantage over FAH deficient cells in vivo. Therefore, it has been suggested that metabolic overloading of the Phe/Tyr catabolic pathway and/or inhibitors of FAH may be of use to improving the efficiency and permanence of liver gene therapy strategies, in general (15). The overexpression of FAH carried by a gene therapy vector harboring another corrective gene or the expression of inhibitor resistant variants of FAH could be used as a selectable marker in the treatment of liver diseases other than HT1.

FAH catalyzes the final reaction of Phe/Tyr catabolism, the formation of fumarate and acetoacetate from fumarylacetoacetate hydrolysis (17, Scheme 1). FAH is a homodimer of 46 kDa subunits that catalyzes the hydrolytic cleavage of carbon-carbon bonds in a variety of diketo acid substrates (18). In contrast to common biochemical hydrolysis reactions involving amide and ester bonds, hydrolytic cleavage of relatively stable carbon-carbon bonds is a fairly unusual reaction. Only ten enzymes having this primary function have been described by the Enzyme
Commission (EC 3.7.1.1-3.7.1.10). However, FAH is structurally distinct from other carbon-carbon bond hydrolases known to be members of the α/β-hydrolase fold family (19, 20). In addition to the degradation of aromatic amino acids, carbon-carbon bond hydrolysis reactions are also required for the degradation of aromatic hydrocarbons by soil bacteria (21). Therefore, further understanding of this type of reaction is of potential use in bioengineering efforts aimed at the bioremediation of toxic hydrocarbon wastes, such as polychlorinated biphenyls (PCBs).

Crystal structures of FAH and FAH complexed with its physiological products, fumarate and acetoacetate, were recently determined (22). The FAH structure consists of a 120 residue N-terminal domain of unknown function and a 300 residue C-terminal domain defined by a novel β-sandwich roll structure that forms an active site in close proximity to the dimer interface (Figs. 1A, 1B). The FAH active site occurs in a solvent filled cavity that is complementary in shape and charge to the fumarylacetoacetate substrate (22). The base of the active site is formed by a metal ion coordinated by four side chain carboxyl groups at the edge of the β-roll, while the sides are formed by helices and turns located above the β-roll. The product acetoacetate binds to FAH by providing two oxygen ligands to a Ca^{2+} ion present in the metal ion binding site. Fumarate binding involves an Arg and two Tyr side chains near the entrance to the active site.

These structural observations, along with the finding that an D233V substitution causing HT1 (23) and a E201G mutation causing a phenotype like HT1 in mice (D. Johnson personal communication), led to a proposed mechanism involving the active site His133 imidazole ring as a general base catalyst in the direct activation of a nucleophilic water molecule (22). The sidechains of Arg237, Gln240 and Lys253 are proposed to stabilize the formation of a tetrahedral alkoxy transition state intermediate, and the metal ion is proposed to function in binding substrate and stabilizing an acetoacetate carbanion leaving group.
Molecules containing phosphorus have proven to be effective inhibitors of enzyme catalyzed hydrolytic and condensation reactions. Substrate analogs containing phosphonate, phosphonoamidate and phosphinate groups have been used as effective noncovalent inhibitors of aspartylproteases (24) and metalloproteases (25), while phosphonate and phosphinate bisubstrate inhibitors have been used to inhibit farnesyltransferase (26). Of particular interest to the enzymatic mechanism of FAH are studies indicating that the tetrahedral geometry associated with the phosphorus groups in these types of inhibitors closely approximates both geometric and electronic aspects of the transition state (24, 27, 28). Thus, the effectiveness of these compounds as inhibitors is presumably associated with similarities to the high-energy reaction intermediates stabilized during catalysis.

The synthesis of an inhibitory phosphinate substrate analog of FAH and its characterization using kinetic and crystallographic methods are reported here. As an effective inhibitor of a carbon-carbon hydrolase, HMPOBA represents the first example of a phosphorus-based analog targeting this class of enzymes. These results provide a context for future structure-based design of FAH inhibitors and provide additional insight into the mechanism by which FAH catalyzes the hydrolysis of carbon-carbon bonds.

EXPERIMENTAL PROCEDURES

Inhibitor Synthesis and Characterization-Products of the synthesis (Scheme 2) were confirmed using GC/MS data acquired on a Hewlett Packard 5995, and by recording $^1$H, $^{13}$C and $^{31}$P NMR data using a Bruker AC-300 (300.13 MHz) spectrophotometer. Solvents were dried by passing over activated alumina, and all reactions were performed under a positive pressure of dry N$_2$. 

- 5 -
**Chloromethyl methylphosphonate (product 1):** Oxalyl chloride (6.54 ml, 75.0 mmol) was added dropwise to a stirred solution of dimethyl methylphosphonate (6.2 g, 50.0 mmol) in CH₂Cl₂ (3 ml) with cooling at 0°C. This reaction was left to warm to room temperature overnight. The volatiles were removed *in vacuo* to yield a yellow oil which was suspended in THF (80 ml) and used directly. MS *m/z* 128.

**4-(Methoxyphosphinoyl)-3-oxo-butanoic Acid, 1,1-dimethylethyl ester (product 2):** *t*-Butyl acetoacetate (17 ml, 102.5 mmol) was added dropwise to a stirred slurry of NaH (2.46 g, 102.5 mmol) in THF (120 ml) with cooling at 0 °C. After 10 min the reaction was cooled in a dry ice/acetone bath, whereupon *n*-BuLi (1.6 M in hexanes, 64.1 ml, 102.5 mmol) was added dropwise. The red-orange reaction was stirred for 40 min. The phosphonochloridate, I, from above was cooled in a dry ice/acetone bath, whereupon the red-orange dianion solution was transferred via cannula (dropwise over 15 min) while stirring. After 2 hr, the reaction was quenched with HOAc (17.5 ml), water (200 ml) was added, and THF was removed *in vacuo*. This mixture was extracted with CH₂Cl₂ (3 x 200 ml), the organic extract washed with 250 ml H₂O/EDTA (0.01%) and dried *in vacuo*. Silica gel chromatography (EtOAc/Hexane, 1:1) of the rust colored oil afforded 2 (70% yield). Only data for the keto form are reported. ¹H NMR (CDCl₃) δ 1.47 (9H, s), 1.58 (3H, d, J₃-CH_P = 14.6 Hz), 3.28 (2H, d, J₃-CH_P = 17.9 Hz), 3.58 (2H, s), 3.78 (3H, d, J₃-H_CP = 11.3 Hz). ¹³C NMR (CDCl₃) δ 14.06 (1C, d, J₃-CP = 97.9 Hz), 27.90 (3C, s), 45.46 (1C, d, J₃-CP = 77.96 Hz), 51.33 (1C, d, J₃-COP = 6.79 Hz), 51.60 (1C, s), 82.34 (1C, s), 165.85 (1C, s) 98.12 (1C, d, J₃-CCP = 5.43 Hz). ³¹P NMR (CDCl₃) δ 48.5.

**4-(Hydroxymethylphosphinoyl)-3-oxo-butanoic Acid, dipotassium salt (HMPOBA, product 3):** The dry phosphinic ester (0.61g, 2.45 mmol) was dissolved in CH₂Cl₂ (7 ml) and dry cyclopentene (24.5 mmol, 2.2 ml). TMSI (7.83 mmol, 1.11 ml) was added dropwise to the
stirred solution at 0° C and the reaction was left for 2 hours. The volatiles were removed in vacuo, and toluene (5 ml) was added and evaporated. The residue was dissolved in THF (2 ml) and transferred via cannula to a stirred solution of KHCO₃ (4.73 mmol, 0.47 g) in water (10 ml). CO₂ was removed under vacuum, and the pH was adjusted to 7 with KOH. Following evaporation in vacuo, residual volatiles were removed by successive evaporation with CH₃CN (2 x 5 ml) and CCl₄ (5 ml). The residue was dried in vacuo overnight to yield 0.86 g of a pale yellow solid (137 %). The solid (0.422 g) was dissolved in H₂O (5 ml), and the phosphorus concentration (0.2 M) was assayed (29). The excess mass was shown by neutron activation analysis to result from I⁻ (presumably as KI). ¹H NMR (H₂O/D₂O, 9:1) δ 3.59 (2H, s), 3.14 (2H, d, J_HCP = 17.4 Hz), 1.35 (3H, d, J_HCP = 14.2 Hz). ¹³C NMR (H₂O/D₂O, 9:1) δ 18.91 (1C, d, J_CP = 97.9 Hz), 51.59 (1C, d, J_CP = 71.6 Hz), 56.60 (1C, s), 177.00 (1C, s), 207.74 (1C, d, J_CCP = 32.27 Hz). ³¹P NMR (H₂O/D₂O, 9:1) δ 33.34.

Kinetic Assays-Kinetic assays utilized human FAH expressed with an N-terminal poly His tag using the vector pQE-30 (Qiagen, Santa Clarita, CA). Bacteria containing the pQE-FAH plasmid were grown in LB media and induced overnight with 0.5 mM IPTG. The bacteria were harvested by centrifugation and lysed by sonication. Lysates were centrifuged, and recombinant FAH was purified from the supernatant using metal chelate chromatography by eluting with a solution containing 50 mM sodium phosphate pH 6.0, 300 mM NaCl, 10% glycerol, 1 mM PMSF and 100 mM EDTA. FAH activity was assayed spectrophotometrically using acetopyruvate (AP) (Sigma) and fumarylacetoacetate (FAA) prepared enzymatically from homogentisic acid (Sigma) as described (17, 18, 30). Assays were initiated by adding recombinant His-tagged FAH to reaction mixtures that covaried AP (0.625, 0.833, 1.25, 2.5 and 5.0 mM) with HMPOBA (0.0, 250, 500 and 750 µM) or covaried FAA (3, 8 and 15 µM) with
HMPOBA (0, 125, 250, 500 or 750 µM). The disappearance of AP and FAA was followed at 292 nm and 330 nm for 2 to 3 min at 37°C, respectively. Extinction coefficients of 9,510 and 13,500 cm⁻¹M⁻¹ were used for AP and FAA, respectively. Initial rates were fit to the competitive inhibition model by non-linear regression analysis using SigmaPlot (Jandell Scientific).

**Expression, Crystallization and Structure Determination**-Mouse FAH was expressed as a glutathione S-transferase fusion protein (22) using pGEX4T-1 (Pharmacia) and was purified using glutathione Sepharose (Pharmacia). Mouse and human FAH share 88% sequence identity, with strict conservation of the active site residues. The fusion protein was digested using Bovine thrombin (Sigma) in 20 mM Tris, 10 mM CaCl₂, pH 7.4. The free FAH was separated from GST using a MonoQ column (Pharmacia). FAH was at least 90% pure as assessed by Coomassie Blue stained SDS polyacrylamide electrophoresis gels and was concentrated using a Centricon ultrafiltration device (Amicon). FAH concentrations were determined using an extinction coefficient of 1.31 ml·cm⁻¹·mg⁻¹ at 280 nm. FAH was crystallized by the hanging drop vapor diffusion method at room temperature by combining 10 µL of a 3 mg/mL FAH solution with 5 µL of a precipitant solution containing 17-18% PEG 8000, 0.03-0.15 M nickel acetate, 0.00-0.24 M sodium acetate, 0.1 M sodium cacodylate pH 6.5 (22). Acetate was maintained at a constant 0.3 M concentration. FAH crystallizes under these conditions in the space group P2₁ with unit cell dimensions of 64.1 x 109.5 x 67.5 Å³ and β = 102.4°.

Crystals of FAH were soaked with 0.1 M HMPOBA in precipitant solution for 16 hours at room temperature prior to data collection. Data were collected on beamline X12C at the Brookhaven National Laboratory National Synchrotron Light Source at 100 K using a cryoprotectant solution containing 30 % PEG 400, 0.3 M sodium acetate, 0.1 M sodium cacodylate pH 6.5. Data were integrated, scaled and merged using DENZO and SCALEPACK.
Details of the data collection and processing are given in Table 1. The structure of FAH complexed with HMPOBA was determined by refining coordinates of the mouse FAH structure against data collected from a single soaked crystal. The positions of the ligand in the FAH active site was apparent in electron density maps calculated using 2Fo-Fc and Fo-Fc coefficients. Iterative cycles of model building using the program O (32) were followed by restrained refinement using the programs REFMAC (33) and XPLORv3.8 (34) and automated water building using the program ARPP (33). A model of FAH complexed with HMPOBA consisting of 835 amino acid residues, 1 Ni$^{2+}$, 2 Mg$^{2+}$, 2 Ca$^{2+}$, 2 HMPOBA molecules, 2 acetate molecules and 833 water molecules has been refined to a crystallographic R-factor of 0.181 (R-free=0.199) at 1.30 Å resolution. Details of the model refinement are given in Table 1. Unrestrained refinement and refinement including model H atoms did not improve the R-free residual. Figures were created using O (32) and MOLSCRIPT (35).

**Supplementary NMR data.** NMR data were obtained using a Bruker AC-300 (300.13 MHz) spectrophotometer. $^1$H (300.13 MHz) and $^{13}$C (75.45 MHz) resonances were recorded in parts per million downfield from TMS. $^{31}$P (121.49 MHz) spectra were recorded in parts per million downfield from 85% phosphoric acid for CDCl$_3$ samples, and downfield from 1% phosphoric acid for D$_2$O samples.

**4-(Methoxyphosphinoyl)-3-oxo-butanoic Acid, 1,1-dimethylethyl ester (product 2):**

$^1$H NMR (CDCl$_3$) keto: $\delta$ 1.47 (9H, s, -OC(CH$_3$)$_3$), 1.58 (3H, d, J$_{HCOP}$ = 14.6 Hz, CH$_3$P), 3.28 (2H, d, J$_{HCOP}$ = 17.9 Hz, -P(O)CH$_2$C(O)-), 3.58 (2H, s, -CH$_2$C(O)O-), 3.78 (3H, d, J$_{HCOP}$ = 11.3 Hz, -OCH$_3$). enol: $\delta$ 1.49 (9H, s, -OC(CH$_3$)$_3$), 1.60 (3H, d, J$_{HCOP}$ = 14.6Hz, CH$_3$P-), 2.74 (2H, d, J$_{HCOP}$ = 17Hz, -P(O)CH$_2$C(O)-), 3.58 (2H, s, -CH$_2$C(O)O-), 3.78 (2H, d, J$_{HCOP}$ = 11.3 Hz, CH$_3$P), 12.41 (1H, s, -OH). $^{13}$C NMR (CDCl$_3$) keto: $\delta$ 14.06 (1C, d, J$_{CP}$ = 97.9 Hz, CH$_3$P), 27.90
(3C, s, -OC(CH3)3), 45.46 (1C, d, J_Cp = 77.96 Hz, -P(O)CH2C(O)-), 51.33 (1C, d, J_COP = 6.79 Hz, CH3OP), 51.60 (1C, s, -C(O)CH2C(O)-), 82.34 (1C, s, OC(CH3)3), 165.85 (1C, s, -CH2C(O)O-), 98.12 (1C, d, J_CCP = 5.43 Hz). 31P NMR (CDCl3): δ 48.5.

4-(Hydroxymethylphosphinoyl)-3-oxo-butanoic Acid, dipotassium salt (HMPOBA, product 3): 1H NMR (H2O/D2O, 9:1) δ 3.59 (2H, s, C4H2), 3.14 (2H, d, J_HCP = 17.4 Hz, C9H2), 1.35 (3H, d, J_HCP = 14.2 Hz, C15H2). 13C NMR (H2O/D2O, 9:1) δ 18.91 (1C, d, J_Cp = 97.9 Hz, C15), 51.59 (1C, d, J_Cp = 71.6 Hz, C9), 56.60 (1C, s, C4), 177.00 (1C, s, C2), 207.74 (1C, d, J_CCP = 32.27 Hz, C5). 31P NMR (H2O/D2O, 9:1) δ 33.34.

RESULTS

Inhibitor Synthesis and Characterization- HMPOBA was synthesized according to Scheme 2 with a 70% yield via a three step synthesis from dimethyl methylphosphonate. The phosphonochloridate (product 1) was made by reaction of dimethyl methylphosphonate with oxalyl chloride. Dianions of β-keto esters have previously been shown to react with acyl chlorides to form terminally acylated products (36). Similarly, reaction of this phosphonochloridate with the dianion of t-Butyl acetoacetate resulted in phosphinylation of the terminal carbon to yield the phosphinic ester (product 2). Two equivalents of dianion were used in the reaction, for a proton is made acidic upon phosphinylation. Deprotection of the phosphinic ester with TMSI followed by basic workup circumvented decarboxylation which occurs in acid, and the di-potassium salt of HMPOBA was produced (product 3).

Kinetic Assays- Hydrolysis of acetopyruvate and the physiological substrate was followed spectrophotometrically by loss of absorbance at 292 and 330 nm, respectively. Initial velocities varied as a function of substrate concentration in the absence of inhibitor according to simple Michaelis-Menten kinetics. Substrate inhibition of FAH by AP (17) was not observed at
concentrations 15-fold higher than the $K_m$. Steady state kinetic assays performed as a function of HMPOBA concentration were fit according to the competitive inhibition pattern. Global fitting of the data measured at three FAA and five inhibitor concentrations yielded a $K_i$ of $30.3 \pm 7.7 \mu M$ and a $K_m$ of $0.4 \pm 0.1 \mu M$ (Data not shown). Global fitting of data measured at five AP and four inhibitor concentrations yielded a $K_i$ of $84.8 \pm 4.2 \mu M$ and a $K_m$ of $1.3 \pm 0.1 \text{mM}$ (Figure 2). The $K_i$ obtained from AP assays is likely to be more accurate, given difficulties associated with making measurements below the $K_m$ of FAA.

Structure of the FAH/HMPOBA Complex—The crystal structure of mouse FAH complexed with HMPOBA was determined by soaking an enzyme crystal with the inhibitor. The inhibitor was readily identified in electron density maps calculated at near atomic resolution (1.3 Å) using crystallographic phases from a previously determined structure of mouse FAH (22) and the structure factors measured from the soaked crystal. Crystallographic data and refinement statistics are given in Table 1. Refinement of this model against the 1.3 Å data has enabled atomic positions in the model to be defined by electron density that discriminates between C, N and O atoms (Fig. 3) with an overall coordinate error estimated at less than 0.05 Å.

The HMPOBA binds the FAH active site through hydrogen bond and/or charge interactions between the pro-S phosphinoyl oxygen atom and the Gln240 NE2, Arg237 NH1 and Lys253 NZ side chain atoms, which are separated by respective distances of 2.9, 2.9 and 2.8 Å from the inhibitor oxygen atom (Fig. 4). The pro-R phosphinoyl oxygen atom interacts with His133 at a distance of 2.7 Å and a water molecule at a distance of 2.8 Å bound between the Arg237 NH2 and Thr350 OG1 atoms (Fig. 4). HMPOBA provides two ligands to the FAH active site Ca$^{2+}$ present in the catalytic metal ion binding site through the carbonyl oxygen atom (O8) and one carboxyl oxygen atom (O1). The HMPOBA carboxyl oxygen atoms are also located at 2.9 and
3.0 Å from the main chain N atom of Thr350. While the enzyme ligand interactions appear to be dominated by the electrostatic interactions described above, several complementary Van der Waals contacts are also made between the inhibitor and FAH. The methylene carbon bonded to the phosphorus atom is located at respective distances of 3.9 and 4.0 Å from the CD1 and CD2 atoms of Leu247, which originates from the opposite FAH subunit as part of the dimer interface (Fig. 1B). The inhibitor carbonyl carbon atom is located 3.8 Å from the Tyr159 CE2 atom. The inhibitor methylene carbon atom positioned between the carbonyl and carboxyl groups is located 3.7 Å from the Tyr128 CD1 atom and 3.2 Å from the Phe127 O atom. The HMPOBA methyl carbon atom is located 3.8 Å from the Tyr128 CE1 atom.

Active Site Metal Ions-In addition to the Ca$^{2+}$ occupying the catalytic metal ion binding site, a second divalent metal ion was identified in the active site during the refinement of the FAH/HMPOBA complex when a water molecule bound to the Asp233 side chain was found to have an abnormally low temperature factor and an associated positive peak in Fo-Fc difference maps. The isotropic temperature factors of equivalent water molecules in each active site refined to 3.0 and 3.3 Å$^2$, while the surrounding oxygen atoms had temperature factors ranging from 8.3 to 9.6 Å$^2$. Furthermore, the three carbonyl, one carboxyl and one hydroxyl oxygen atoms surrounding each of the water molecules provided an excess of hydrogen bond acceptors. Reassignment of this peak to Mg$^{2+}$ in both active sites eliminated the positive feature from the Fo-Fc difference map and resulted in refined temperature factors for the Mg$^{2+}$ ions of 8.7 Å$^2$. Examination of several other FAH structures refined between 1.55 and 1.90 Å (22) indicate similar anomalies with the water bound to the Asp233 side chain, and in every case reassignment to Mg$^{2+}$ is consistent with the refined temperature factors and difference maps. Thus, FAH
contains a binuclear metal binding site centered about the Asp233 sidechain (Fig. 4), with one carboxyl oxygen binding the catalytic metal ion and the other binding Mg²⁺.

The hexagonal coordination geometry for the catalytic metal ion site is best described as a distorted square bipyramid, whereas the pentavalent Mg²⁺ coordination is best described as a distorted square pyramid. Details of the coordination geometries are given in Table 2. The largest distortions of the octahedral Ca²⁺ coordination involve ligands from the bound inhibitor. These distortions are likely to be due to covalent bonding restraints on the positions of the O1 and O8 atoms within the β-keto acid portion of HMPOBA. The O1 and O8 atoms form a six membered ring with the carbonyl, methylene and carboxyl C atoms and the Ca²⁺ that is strained by both a 73° O1-Ca²⁺-O8 angle and a nearly planar configuration of all but the methylene C atom.

Alternate Active Site Side Chain Conformations-FAH crystal structures representing free enzyme, the enzyme/HMPOBA complex and the enzyme/product complex reveal distinct side chain conformations for several catalytic residues within the active site. The CA positions of free FAH, represented by the structure of the selenomethionine substituted enzyme refined at 1.9 Å, were superimposed with those of the FAH/HMPOBA complex with an overall RMS deviation of 0.21 Å, while those of the FAH/product complex refined at 1.9 Å were superimposed with the FAH/HMPOBA structure with an RMS deviation of 0.33 Å. The active site of the free FAH structure and the FAH/HMPOBA complex contain an acetate bound to Tyr128 and Arg142 (Figs. 3, 4), and a second acetate binds the Ca²⁺ ion in the free FAH structure (22). The largest side chain motions in the active site are associated with Tyr128, Glu199, Ser235, Gln240 and Lys253. The position of the Glu199 OE2 atom in the HMPOBA complex differs by 2.2 Å relative to both the free and product complex structures (Fig. 5). This Glu199 motion is largely
the result of respective 20º and 50º rotations about the side chain Chi2 and Chi3 angles in the HMPOBA complex relative to the free and product complex structures. The position of the Ca²⁺ ligand, Glu199 OE1, does not change significantly between the structures. A 100º Chi3 rotation changes the position of the Lys253 NZ atom by 1.6 Å in the HMPOBA complex relative to the free structure. The Gln240 side chain NE2 atom also moves about 1.6 Å and 1.3 Å towards the active site entrance in the product complex relative to the respective free and HMPOBA complex structures. The Tyr128 OH atom moves by 1.2 Å in the FAH/HMPOBA and FAH/product complexes relative to that of the free enzyme, but the position of this atom differs by less than 0.3 Å between the structures of the two complexes. The Arg142 NH2 and Tyr244 OH atoms near the entrance to active site also move between 0.7 and 0.9 Å in the complexes relative to the free enzyme. Moderate shifts (0.6-0.8 Å) in the positions of the Val137, Arg237, Pro246 and Leu247 side chain atoms are also observed. In contrast, the main chain CA atoms of the residues mentioned above deviate by less than 0.4 Å, with the exception of Arg142, which has CA positional differences of 0.6 and 0.5 Å in the product and HMPOBA complexes relative to the free structure, and Ser235, having a CA positional difference of 0.5 Å between the HMPOBA and product complex.

DISCUSSION

Mechanistic Implications of Phosphorus Based Hydrolase Inhibitors—Our studies of the phosphinoyl compound HMPOBA demonstrate the potential for this class of molecules to effectively inhibit a hydrolase that acts on carbon-carbon bonds. Phosphorus containing compounds have previously been characterized as noncovalent inhibitors and transition state analogs of other hydrolases, including aspartyl- and metalloproteases (24, 25). A variety of crystallographic, kinetic and structure/function data suggest that inhibitors containing tetrahedral
phosphorus groups can share characteristics of tetrahedral transition state intermediates (24, 27, 28). While the Ki for HMPOBA is higher than expected for a true transition state analog, it is reasonable to conclude that our results approximate many of the geometric and electronic features expected for an intermediate of FAH catalyzed reactions.

The FAH/HMPOBA complex shares many features in common with the previously proposed mechanism of FAH (22). According to this mechanism, substrate binding to the catalytic metal ion positions a carbonyl carbon atom for nucleophilic attack by hydroxide to give rise to a tetrahedral alkoxy intermediate (Fig. 6). Consistent with this mechanism, the HMPOBA acetoacetyl group interacts directly with Ca$^{2+}$ present in the catalytic metal binding site, and the tetrahedral phosphinoyl group occupies the position predicted for the alkoxy intermediate (22). The HMPOBA pro-S oxygen atom is likely to be ionized and accepting three hydrogen bonds from the Arg237, Gln240 and Lys253 side chains (Fig. 4). These side chains are proposed to function as an oxyanion hole, stabilizing the negative charge acquired at this position during catalysis (Fig. 6). The pro-R oxygen atom occupies the position of a water molecule hydrogen bonded to the His133 and Glu199 side chains (Fig. 5) in the free FAH structure (22). This water molecule is proposed to function as the active site nucleophile (22) as part of a catalytic triad involving the His133 and Glu365 side chains (Fig. 4). Glu199 may function along with His133 to direct the nucleophilic attack by orienting the lone pairs of electrons on the water molecule in the direction of the carbonyl carbon atom. Therefore, the FAH/HMPOBA structure shares several features of the proposed catalytic intermediate, and provides further support for the involvement of a metal ion and the His133, Arg237, Gln240 and Lys253 side chains in catalysis.

Several features of the FAH/HMPOBA structure are likely to account for a relatively high Ki. The most significant factor is likely to be the lack of a carboxyethylene group in HMPOBA
(Schemes 1, 2), which could interact with the charged or polar groups of Tyr128, Arg142 and Tyr244 (Figs. 3-6). The importance of these interactions is suggested by the binding of acetate (Figs. 3, 4) or fumarate at this location in all FAH structures reported to date (22). Potential van der Waals interactions between the fumaryl group and nonpolar atoms present in the side chains of Tyr128, Val137, Phe141, Tyr244 and Pro246 are also absent from the HMPOBA complex. Slight differences in the tetrahedral phosphorus center relative to the predicted alkoxy intermediate (Fig. 6) may also exist. The pro-R oxygen atom is likely to be charge neutral and accepting two hydrogen bonds from the protonated imidazole of His133 and a water molecule (Fig. 4). This arrangement differs slightly from the proposed tetrahedral intermediate, where the protonated His133 NE2 atom is adjacent to a hydroxyl proton, rather than a ketone oxygen (Fig. 6). Differences in the atomic radii of tetrahedral C and P could also have slight steric effects. Additional studies are underway to assess these possibilities and to improve inhibitor binding by adding groups to the HMPOBA framework to exploit electrostatic and hydrophobic interactions near the entrance to the active site.

Active Site Metal Ions-The structure of the FAH/HMPOBA complex is consistent with both catalytic and structural requirements for divalent metal cations. In addition to substrate binding, the catalytic metal ion is expected to stabilize the enolate form (Fig. 6) of the acetoacetate carbanion leaving group (22). Charge neutralization and prevention of carboxylate side chain repulsion in this region of the FAH structure are other likely roles for the catalytic metal ion. Ca\(^{2+}\) occupancy of the catalytic metal binding site is consistent with several different FAH structures refined at 1.3 to 1.9 Å resolution. The catalytic metal binding site must have significantly higher affinity for Ca\(^{2+}\) than the crystallization additive Ni\(^{2+}\); however, exogenous
Ca$^{2+}$ is added during the FAH purification (Materials and Methods). Studies to identify the physiologically relevant catalytic metal ion are underway.

The Ca$^{2+}$ coordination geometry (Table 2) is consistent with values obtained from small molecule analogs of Ca$^{2+}$/carboxylate complexes; however, the observed Mg$^{2+}$ coordination is unusual. The HMPOBA O1 and O8 atoms, the Asp233 OD2 and the Glu201 OE2 atoms are planar ligands of Ca$^{2+}$. The Asp126 OD2 and Glu199 OE1 atoms are axial ligands. The average carboxylate oxygen distance to the Ca$^{2+}$ of 2.31±0.04 Å (n=10) compares favorably with a mean value of 2.37 Å and range of 2.27-2.49 Å recently reported in a survey of the Cambridge Structural Database (CSD, ref. 41). This finding further supports the accuracy of the 1.3 Å refinement, which placed no restraints on the metal ion coordination geometries. The significantly longer distance between the HMPOBA carbonyl oxygen (Table 2) and Ca$^{2+}$ may be due to the lack of a formal charge on O8. While metal-carbonyl oxygen complexes were not part of the CSD survey (41), the distances between Mg$^{2+}$ and the main chain carbonyl oxygen atoms (Table 2) are about 0.3 Å longer than found in representative CSD structures (M. Harding, personal communication). The Asp233 OD1, Trp234 O, Gly256 O and Thr257 OG atoms are planar ligands of Mg$^{2+}$. The Lys253 O is the axial ligand. The distances between Mg$^{2+}$ and the Asp233 OD1 and the Thr257 OG atoms are respectively 0.3 and 0.6 Å longer than the mean distance reported for relevant Mg$^{2+}$ complexes (41).

The newly identified Mg$^{2+}$ appears to have a role in stabilizing the structure of the β-roll that forms the central scaffold of the FAH active site and a possible role in intersubunit communication. In contrast to Ca$^{2+}$, Mg$^{2+}$ is not added exogenously and is not accessible to solvent within the active site. The Mg$^{2+}$ is located within the β-roll structure near the dimer interface, linking main chain atoms at the end of a helix between Phe250 and Phe255 with two β-
strands between Thr257 and Ile259 and between Phe226 and Ala236 (Fig. 1). Mg$^{2+}$ may also stabilize a loop between Trp234 and Lys253 that positions Pro246 and Leu247 within the active site of the opposite subunit. While half-site reactivity or catalytic cooperativity has not been reported, the FAH structure seems suited to such functions. Interactions between the Leu247 side chain of one subunit and HMPOBA bound in the opposite subunit were described above. Pro246 also makes an intersubunit interaction with Tyr244 of the fumaryl binding site and interacts with fumarate in the FAH/product complex (22). Therefore, changes within the fumaryl and/or acetoacetyl binding sites could be transmitted through the 234/253 loop to the active site in the opposite subunit. The catalytic residues Arg237, Gln240 and Lys253 and the D233V, W234G and P249T mutations causing HT1 (19) also occur in this region of the structure. Therefore, Mg$^{2+}$ appears to be important for joining secondary structures within the β-roll, as well as having a potential roles in communication between active sites and positioning the side chains of the oxyanion hole.

Mg$^{2+}$ could have additional catalytic effects. Mg$^{2+}$ could maintain a favorable overall charge within the active site by balancing the formal negative charges associated with the dicarboxylic acid substrate and the four carboxylate ligands to the catalytic metal ion. Assuming Arg142, Arg237 and Lys273 are protonated, the net formal charge of the substrate bound active site in the presence of two divalent cations is +1. Finally, Mg$^{2+}$ could affect properties of the catalytic metal ion, such as Lewis acidity, through the shared Asp233 carboxylate ligand. The importance of this residue in normal FAH function is indicated by the HT1 associated D233V mutation (23), which is anticipated to affect both the catalytic metal ion and Mg$^{2+}$ binding sites.

Side Chain Mobility—A comparison of FAH structures bound to different ligands indicates conformational mobility within the FAH active site. Four of the five active site residues having
the largest positional differences also have central roles in the proposed mechanism. This observation suggests that these conformational differences may be significant to substrate binding or product release, formation of the leaving group and proton transfer to the leaving group. The observed positional deviations are significantly larger than the estimated coordinate errors of the refined structures and involve side chains that are well defined by electron density. Indeed, these side chains have temperature factors that are less than 24.0 Å², indicating they occupy distinct, stable conformations in different structures, rather than being mobile within a given structure.

Entry to the FAH active site appears restricted in the FAH structures, indicating that a conformational change is required for substrate binding. A small opening to the active site is defined by a triangular arrangement of the Arg142 NH1, Pro246 CB and Tyr244 OH atoms separated by distances of 5.3 to 5.5 Å. Considering the Van der Waals radii of the atoms, this opening is not large enough to accept substrates, products or inhibitors. However, ligand binding to FAH within the crystal lattice suggests the entrance to the active site does open. The entrance to the active site is likely to occur near the end of a helix formed between Ser130 and Gly143. This region has some of the highest temperature factors found in the FAH structures and shows some of the largest RMS deviations between main chain positions. Furthermore, a G369V mutation causing HT1 is located adjacent to this region (23). A transient opening of Phe141 and Arg142 away from the active site would expose an elongated entrance. Conversely occupancy of the fumarate binding site would cause the active site to close by creating the hydrogen bond network between Tyr128, Arg142 and Tyr144. It is also interesting to note that while fumarate binding might be expected to affect the conformation of residues near the entrance to the active site, HMPOBA binding also resulted in significant conformational changes in Tyr128, Arg142
and Tyr244 relative to the free structure. Therefore, ligand occupancy of the acetoacetate binding site can apparently induce conformational changes near the entrance to the active site.

Conformational changes affecting the oxyanion hole may accompany the transformation of the tetrahedral alkoxy intermediate to the planar fumarate carboxyl group (Fig. 6). Substantial differences in the Arg237, Gln240 and Lys253 side chains positions are observed between the different FAH structures. The separation of products following the breakdown of the tetrahedral intermediate may be indicated in comparing the HMPOBA and product structures, which reveals a 2.3 Å difference between the positions of the HMPOBA phosphorus and the fumarate carboxyl carbon atoms. Thus, fumarate formation may pull Gln240 in the direction of the entrance to the active site.

Side chain mobility may also be related to proton transfer to the carbanion leaving group. This step may involve the protonated forms of either the fumarate carboxyl group or the Lys253 amine group. His133 is unlikely to serve as the general acid, as the NE2 atom is located over 5.1 Å away from the HMPOBA equivalent of the acetoacetate methyl group, the C9 atom. The superimposed structures reveal that a fumarate carboxyl oxygen is located 2.9 Å from the HMPOBA C9 atom. This distance would allow for direct or water-mediated proton transfer between the protonated carboxyl and the leaving carbanion. Another possibility involves the Lys253 rotation away from an interaction with the Ser235 OG in the free structure to interact with HMPOBA as part of the oxyanion hole. This motion may be coupled with a change in the conformation of Glu199, which pivots about the catalytic metal ion to accept a hydrogen bond from Lys253 in the HMPOBA structure (Fig. 5), and suggests Lys253 could function as the proton donor. Indeed, a preferred rotamer conformation resulting from a 90º rotation about the side chain Chi2 angle brings the Lys253 NZ atom within 3.0 Å of the HMPOBA C9 atom.
Summary—Studies of more exact transition-state analogs, currently under development in our laboratories, are expected to address many of these issues. In particular, a crystal structure of FAH complexed with an analog which incorporates a competent mimic of the fumaryl portion of FAA should allow us to probe the role of the 140/147 loop in the closing/opening portion of the active site and to ascertain the related functions of Phe141 and Arg142 in covering the respective binding sites of acetoacetate and fumarate. Additionally, it is expected that such an analog will serve as a more potent inhibitor of FAH, with profound therapeutic properties for hepatic repopulation, far superior to that observed with HMPOBA.

ACKNOWLEDGMENTS

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Figure Legends

Figure 1. CA trace. A CA trace of the FAH dimer is shown in stereo A) viewed normal and B) viewed parallel to the dyad axis between subunits. The position of the active site is indicated by the inclusion of spheres representing the HMPOBA model, the Mg$^{2+}$ and Ca$^{2+}$. The Mg$^{2+}$, Ca$^{2+}$ and the atoms C, N, O and P are respectively colored grey, green, orange, blue, red and pink. Individual subunits are colored green and blue.

Figure 2. HMPOBA inhibition of FAH. A double reciprocal plot shows the steady-state kinetic data obtained at different inhibitor concentrations (0, ●; 250, ○; 500, ■; and 750 µM, □). Data were fit according to the competitive inhibition model with solid lines indicating the pattern derived from global non-linear fitting of the data, as described in the Materials and Methods section. Results of triplicate measurements made at each substrate and inhibitor concentration are shown.

Figure 3. Electron density. A section of an omit map in the vicinity of the active site is shown. The Fo-Fc map is contoured in dark blue at a level of 4 RMS. The 2Fo-Fc map is contoured in light blue at a level of 1.75 RMS. The Ca$^{2+}$ and Mg$^{2+}$ are colored yellow; C, N, O and P atoms are respectively colored orange, blue, red and green. Dumbbell shaped Fo-Fc contours around the Ca$^{2+}$ and Mg$^{2+}$ are suggestive of anisotropic thermal parameters.

Figure 4. Stereo diagram of the active site. A stereo diagram of the active site is shown with Ca$^{2+}$, Mg$^{2+}$ and P colored light gray, and C, N, and O respectively colored dark gray, white and black. The HMPOBA compound is centrally located in the figure, with key hydrogen bonds indicated by dashed lines. Arg237, Thr350 and the HMPOBA bound water are in the foreground of the figure. To maintain an unobstructed view, the side chains for Arg137 and Asp126...
(background) have not been labeled. An acetate molecule present in the active site is labeled Act.

Figure 5. Side chain mobility in the FAH active site. Active site side chains having the largest positional differences between free FAH (dark gray), FAH/HMPOBA (light gray) and FAH/product (medium gray) structures are shown in stereo. His133 is included as a residue with a position that deviates little between these structures.

Figure 6. Breakdown of the alkoxy transition state intermediate. Formation of fumarate and the enolate form of the acetoacetate carbanion leaving group are represented schematically. The flow of electrons is indicated by arrows. Interactions stabilizing the transition state are indicated by dashed lines.
Table 1) Crystallographic Data and Refinement

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<tr>
<th>Observations</th>
<th>Completeness</th>
<th>Rmerge</th>
<th>I/sigI</th>
<th>B-factor (Å²)</th>
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<tr>
<td>Unique/Total</td>
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<td></td>
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<tr>
<td>227,518/672,278</td>
<td>96.6(81.8)</td>
<td>0.046(0.366)</td>
<td>15.6(1.4)</td>
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R Rfree Coordinate Ramachandran Plot RMSD from Ideal

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<th>Angles</th>
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<td>0.181(0.234)</td>
<td>0.199(0.249)</td>
<td>0.047 Å</td>
<td>92.5%</td>
<td>7.5%</td>
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The data were integrated and reduced using DENZO and SCALEPACK (31). Rsym=Σ | I- <I> | / Σ I, where I is the integrated intensity of a given reflection. Numbers in parenthesis represent the values obtained in the highest resolution bin (1.36-1.30 Å). The refinement residual, R= Σ | Fobs - Fcalc | / Σ Fobs. The overall B-factor was obtained from Wilson scaling statistics. The overall coordinate error is estimated according the the Cruickshanks method implemented in REFMAC (33).
<table>
<thead>
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<th>Metal-Ligand Distances (Å)</th>
<th>Ligand-Metal-Ligand Angles (degrees)</th>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-HMPOBA O1</td>
<td>2.33±0.01  Asp126 OD2-Ca&lt;sup&gt;2+&lt;/sup&gt;-HMPOBA O1 97.82±0.25</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td></td>
<td>Gly256 O-Mg&lt;sup&gt;2+&lt;/sup&gt;-Thr257 OG1 74.64±0.05</td>
</tr>
</tbody>
</table>

The HMPOBA O1 and O8 atoms respectively occur in carboxyl and carbonyl groups. Aside from protein geometric restraints, metal coordination geometry was not restrained during refinement. Values are averages between the two active sites with ranges indicated by the error values.
Fig 2)
Fig 3)
Fig 4)
Fig 5)
Fig 6).
Scheme 1)

\[
\begin{align*}
\text{O-O} & \quad \text{+H}_2\text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{+} & \quad \text{H}_2\text{O} \\
\end{align*}
\]

Scheme 2)

1) NaH
2) n-BuLi

1) TMSI
2) KHCO_3, KOH

- 36 -
Mechanistic inferences from the crystal structure of fumarylacetoacetate hydrolase with a bound phosphorus-based inhibitor
Raynard L Bateman, P. Bhanumoorthy, John F. Witte, Ronald W. McClard, Markus Grompe and David E. Timm

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