The Mechanism of Action of the Fragile Histidine Triad, Fhit: Isolation of a Covalent Adenylyl Enzyme and Chemical Rescue of H96G-Fhit†

Kaisheng Huang, Abolfazl Arabshahi, Yaoming Wei,‡ and Perry A. Frey*

Department of Biochemistry, University of Wisconsin-Madison, 1710 University Avenue, Madison, Wisconsin 53726

Received February 2, 2004; Revised Manuscript Received March 24, 2004

ABSTRACT: The human fragile histidine triad protein Fhit catalyzes the Mg^{2+}-dependent hydrolysis of P^1-5′-*O*-adenosine-P^3-5′-*O*-adenosine triphosphate, Ap_3A, to AMP and ADP. The reaction is thought to follow a two-step mechanism, in which the complex of Ap_3A and Mg^{2+} reacts in the first step with His96 of the enzyme to form a covalent Fhit−AMP intermediate and release MgADP. In the second step, the intermediate Fhit−AMP undergoes hydrolysis to AMP and Fhit. The mechanism is inspired by the chain-fold similarities of Fhit to galactose-1-phosphate uridylyltransferase, which functions by an analogous mechanism, and the observation of overall retention in configuration at phosphorus in the action of Fhit (Abend, A., Garrison, P. N., Barnes, L. D., and Frey, P. A. (1999) *Biochemistry 38*, 3668−3676). Direct evidence in support of this mechanism is reported herein. Reaction of Fhit with [8,8′-^{3}H]-Ap_3A and denaturation of the enzyme in the steady state leads to protein-bound tritium corresponding to 11% of the active sites. Similar experiments with the poor substrate MgATP leads to 0.9% labeling. The mutated protein H96G-Fhit is completely inactive against $MgAp_3A$. However, it is chemically rescued by free histidine. H96G-Fhit also catalyzes the hydrolysis of adenosine-5′-phosphoimidazolide, AMP-Im, and of adenosine-5′-phospho-*N*-methylimidazolide, AMP-*N*-MeIm. The hydrolyses of AMP-Im and of AMP-*N*-MeIm by H96G-Fhit are thought to represent chemical rescue of the covalent Fhit−AMP intermediate. Wild-type Fhit is also found to catalyze the hydrolyses of AMP-Im and of AMP-*N*-MeIm nearly as efficiently as the hydrolysis of $MgAp_3A$. The results indicate that Mg^{2+} in the reaction of Ap_3A is required for the first step, the formation of the covalent intermediate Fhit−AMP, and not for the hydrolysis of the intermediate in the second step.

The fragile histidine triad (Fhit)[1] is encoded by the human gene *FHIT*, a candidate tumor suppressor gene (*1*). *FHIT* deletions and aberrant transcripts have been observed in tumors or tumor-derived cell lines (*2*−*7*). Fhit is a dinucleoside polyphosphate hydrolase with highest activity against Ap_3A (*8*, *9*). Fhit is a member of the HIT protein family, all of which contain a histidine triad motif, HxHxHxx, where the other residues are hydrophobic. The nucleotide binding Hint proteins are another branch of the HIT family (*10*), and more distant relatives are galactose-1-phosphate uridylyltransferase (GalT) and Ap_4A phosphorylases, in which glutamine replaces the third histidine (*8*). The sequence HPHGQ is in the active site of GalT (*11*). The chain folds in the structures of GalT, human Hint, and Fhit are strikingly similar (*10*, *12*−*16*). These nucleotidyltransferases and hydrolases constitute a superfamily known as GAFH for GalT, Ap_4A phosphorylase, Fhit, Hint (*3*). Fhit is a dimer of identical 16.9 kDa subunits, each of which contains a triad of histidine residues, His^{94}-Val^{95}-His^{96}-Val^{97}-His^{98}, that is conserved among species.

GalT catalyzes the reaction of Gal-1-P with UDPGlc to form UDPGal and Glc-1-P by a double displacement mechanism according to eqs 1 and 2 (*11*, *17*−*24*).

$$\text{UDPGlc} + \mathbf{E}\text{-His}^{166} \rightleftharpoons \mathbf{E}\text{-His}^{166}\text{−UMP} + \text{Glc-1-P} \quad (1)$$

$$\mathbf{E}\text{-His}^{166}\text{−UMP} + \text{Gal-1-P} \rightleftharpoons \mathbf{E}\text{-His}^{166} + \text{UDPGal} \quad (2)$$

The nucleophilic catalyst His^{166} is the central residue in a conserved triad of two histidines and one glutamine, His^{164}-Pro^{165}-His^{166}-Gly^{167}-Gln^{168}.

Fhit catalyzes the hydrolysis of Ap_3A to AMP and ADP by cleavage of the P_α−O bond and not the P_β−O bond (*25*). The hydrolysis of a P-chiral analogue of Ap_3A in $H_2{}^{18}O$ proceeds with overall retention of configuration at P, consistent with a double displacement mechanism of hydrolysis, as in the reaction of GalT (*25*). The apparent similarities of the active site triads in Fhit and GalT and the

† Supported by Grant No. GM30480 from the National Institute of General Medical Sciences.

* To whom correspondence should be addressed. Tel (608)262-0055; fax (608)265-2904; e-mail frey@biochem.wisc.edu.

‡ Current address: Metabasis Therapeutics, 9390 Towne Center Dr., San Diego, CA 92121.

[1] Abbreviations: Fhit, fragile histidine triad; *FHIT*, the gene encoding Fhit; GalT, galactose-1-P uridylyltransferase from *E. coli*; UDPGlc, uridine diphosphate glucose; UDPGal, uridine diphosphate galactose; Glc-1-P, α-D-glucose-1-phosphate; Gal-1-P, α-D-galactose-1-phosphate; Ap_3A, P^1-5′-*O*-adenosine-P^3-5′-*O*-adenosine triphosphate; AMP, adenosine-5′-phosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; AMP-Im, adenosine-5′-phosphoimidazolide; AMP-*N*-MeIm, adenosine-5′-phospho-*N*-methylimidazolide; UMP-Im, uridine-5′-phosphoimidazolide; NADH, reduced β-nicotinamide adenine dinucleotide; DMF, dimethylformamide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; IPTG, isopropyl thio-β-D-galactoside; PMSF, phenylmethylsulfonyl fluoride; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography.

10.1021/bi049762n CCC: $27.50
Published on Web 05/19/2004

similar chain folds of the two enzymes led to the proposition of similar mechanisms for the two reactions. According to this hypothesis, the action of Fhit also follows a double displacement mechanism, in which the ultimate adenylyl group acceptor is water, according to eqs 3 and 4.

$$Ap_3A + \mathbf{E}^{Fhit}\text{-}His^{96} \rightleftharpoons \mathbf{E}^{Fhit}\text{-}His^{96}{-}AMP + ADP \quad (3)$$

$$\mathbf{E}^{Fhit}\text{-}His^{96}{-}AMP + H_2O \rightleftharpoons \mathbf{E}^{Fhit}\text{-}His^{96} + AMP \quad (4)$$

Evidence supporting this mechanism includes the observation of a trace of protein-bound ^{32}P when Fhit is mixed with [α-^{32}P]-ATP, a poor substrate, and the observation that the reaction of Fhit proceeds with overall retention of configuration at phosphorus (P_α) consistent with double inversion in two steps (*15*, *25*).

While the mechanism of eqs 3 and 4 is reasonable on the basis of the presumption of similarity with that of GalT, it is supported by minimal evidence to date. Moreover, while mutation of His96 to asparagine inactivates the enzyme (*9*), proof of function does not follow. In this paper, we describe experiments quantitating the formation of a covalent AMP−enzyme with either Ap_3A or ATP. We further show how either imidazole or AMP-Im or AMP-*N*-MeIm can chemically rescue the specifically mutated and inactive H96G-Fhit. The latter two compounds serve as chemical rescuers of activity.

EXPERIMENTAL PROCEDURES

Materials. [2,8-^{3}H]-ATP and [2,8-^{3}H]-ADP were from American Radiolabeled Chemicals, Inc., and [8,8′-^{3}H]-Ap_3A was from Moravek Biochemicals. Imidazole, *N*-methylimidazole, triethylamine, diethyl ether, lithium perchlorate, triphenylphosphine, $MgCl_2$, molecular sieves, and DMF were purchased from Aldrich. 2,2′-Dipyridyl disulfide, AMP, ADP, ATP, potassium phosphoenolpyruvate, Ap_3A, NADH, myokinase, pyruvate kinase, HEPES, MOPS, CHES, and lactic dehydrogenase were purchased from Sigma. Acetone, methanol, KCl, NaCl, NaOH, and HCl were obtained from Fisher Scientific. Ready gel and gelcode blue stain reagent were from BioRad and Pierce, respectively.

Synthesis of AMP-Im. The method published for the synthesis of UMP-Im with slight modifications produced AMP-Im (*26*). The dried AMP-Im was judged to be of sufficient purity (>98% by HPLC and NMR) for use as isolated. ^{1}H NMR (200 MHz, Bruker) in D_2O, referenced to 3-(trimethylsilyl)-1-propane: δ (in ppm) 8.21 s, 8.14 s (each 1 H, H-2, 8), 7.87 s, 7.13 s, 6.93 s (each 1 H, H-2, 4, 5 on imidazole ring), 6.01 d (1 H, J = 4.88 Hz, H-1′), 4.74 t (1 H, J = 5.13, 4.88 Hz, H-2′), 4.43 t (1 H, J = 4.88, 4.64 Hz, H-3′), 4.28 m (1 H, H-4′), 4.10 m (2 H, H-5′). ^{31}P NMR in D_2O relative to 20% H_3PO_4, δ (in ppm) −8.59 (proton-decoupled singlet).

Synthesis of AMP-N-MeIm. A further modification of the above method produced AMP-*N*-MeIm as follows: Molecular sieves (5 g) and 20 mL of anhydrous DMF in a 50 mL round-bottom flask were cooled to −5 °C in an ice−salt bath with stirring. Then *N*-methylimidazole (2.5 g, 30 mmol), triphenyl phosphite (0.78 g, 3 mmol), and 2,2′-dipyridyl disulfide (0.67 g, 3 mmol) were added to the flask. After the temperature in the flask again decreased to −5 °C, dried AMP (1.02 g, 3 mmol) was added, and the reaction mixture was kept at −5 °C for 30 min. (HPLC analysis of aliquots indicated complete reaction after this time.) The reaction mixture was added dropwise with vigorous stirring to precooled anhydrous acetone containing $LiClO_4$ (1.5 g, 15 mmol) at 0 °C. The white precipitate was collected by filtration and washed several times with cooled anhydrous acetone. The product was dried and judged by HPLC to be of 90% purity. ^{1}H NMR (200 MHz, Bruker) in D_2O, referenced to 3-(trimethylsilyl)-1-propane: δ (in ppm) 8.23 s, 8.14 s (each 1 H, H-2, 8), 8.71 s, 7.29 s, 7.16 s (each 1 H, H-2, 4, 5 on imidazole ring), 5.98 d (1 H, J = 4.64 Hz, H-1′), 4.85 t (1 H, J = 4.64, 4.88 Hz, H-2′), 4.50 t (1 H, J = 4.88, 4.72 Hz, H-3′), 4.27 m (3 H, H-4′, 5′). ^{31}P NMR in D_2O relative to 20% H_3PO_4, δ (in ppm) −8.89 (proton-decoupled singlet).

Mutagenesis. Site-directed mutagenesis of *FHIT* was carried out using the QuickChange method (Stratagene). The mutagenesis primer used for H96G was the following: 5′-CAG ACT GTG AAG CAC GTT GGC GTC CAT GTT CTT CCC-3′. The nucleotide sequence of the mutated gene was verified by sequence analysis at the University of Wisconsin Biotechnology Center.

Purification of Fhit. Human Fhit and mutated H96G-Fhit were isolated from *Escherichia coli* strain SG100 cells transformed from pSGA02 carrying the wild-type or mutated gene according to the published method (*27*). An overnight 160 mL cell culture was used to inoculate 16 L of LB medium containing kanamycin and ampicillin. The culture was grown at 37 °C to A_{600} of 0.6. After addition of IPTG to 1 mM and growth at 37 °C for 6 h, the cells were harvested (~17 g) and suspended in 100 mL of buffer A (50 mM HEPES, pH 6.8, 10% v/v glycerol) containing 0.5 mM PMSF in the cold room. The cells were sonicated 5 min and centrifuged at 100 000*g* for 15 min to collect the supernatant fluid. After dilution to 150 mL, the extract was applied to a 400 mL column of DEAE-Sephacel as described (*27*). The fractions containing Ap_3A hydrolase activity from the DEAE-Sephacel column were combined and concentrated to 40 mL by ultrafiltration through a PM10 memberane, diluted to 100 mL, applied to a 200 mL column of Q-Sepharose, and eluted with a 1.0 L linear gradient of buffer A to 0.2 M NaCl in buffer A at a flow rate of 2 mL min^{-1}. Two peaks showing Fhit activity were collected and checked for purity by SDS−PAGE gel electrophoresis. The subunit concentration of Fhit was determined by use of ϵ_{280} = 8310 M^{-1} cm^{-1} calculated from the amino acid sequence of Fhit according to a published method (*28*).

Assay of Fhit Activity. A coupled assay monitoring AMP formation spectrophotometrically at 340 nm was developed. The coupling enzymes were myokinase (adenylate kinase), pyruvate kinase, and lactate dehydrogenase. Initial rates were measured in a Cary 50 spectrophotometer equipped with a thermostated cuvette holder. The rates observed as decreasing A_{340} due to consumption of NADH were twice the rate of AMP-Im hydrolysis and, when used to assay the wild-type enzyme, three times the rate of Ap_3A hydrolysis.

The detailed procedure was as follows: The assay was carried out at 26.5 °C in 1.00 mL solutions containing 0.1 M MOPS or CHES or 0.55 M imidazole-HCl buffer, as well as 300 μM NADH, 300 μM phosphoenolpyruvate, 100 μM ATP, 75 mM KCl, 2 mM $MgCl_2$, and 10−40 units of each

coupling enzyme at various pHs. Care was taken to ensure that the coupling enzymes did not limit the rate. The buffer, coupling enzymes, and substrates for the coupling enzymes were separately incubated in the cell holder at 26.5 °C for 20 min and then mixed just before adding either a substrate (AMP-Im, AMP-*N*-MeIm, or Ap_3A) or Fhit. The A_{340} was monitored for 2 min to obtain the background rate (if any) and allow the temperature to stabilize at 26.5 °C; then the Fhit was added to initiate the enzymatic reaction. The initial rate of decreasing A_{340} (NADH) was measured. The rates were calculated using the extinction coefficient 6220 M^{-1} cm^{-1} for NADH and computer fitted to the Michaelis−Menten equation to obtain parameters and standard errors.

Isolation of Covalent AMP−Fhit. The reaction mixtures for adenylylation by Ap_3A consisted of 20 μM Fhit, 8 mM [8,8′-^{3}H]-Ap_3A (2.7 μCi μmol^{-1}), 10 mM $MgCl_2$, and 50 mM HEPES buffer at pH 7.5. The reactions were started by addition of Fhit and stopped by addition of 25 μL of 3 M NaOH per 0.1 mL of reaction mixture. The reactions were quenched after 40, 90, or 120 s. [8-^{3}H]-AMP and unreacted [8-^{3}H]-Ap_3A were removed by ultrafiltration using an Amicon Ultrafree-MC centrifugal filter unit equipped with a 5000 MW cutoff membrane. The filter cup was washed at least three times by ultrafiltration with 0.3 mL of HEPES buffer at pH 8.0. Radiochemical assays of the washes verified that free radioactive molecules had been removed from the samples. The filter cups containing the labeled Fhit were placed in liquid scintillation vials and analyzed in a Beckman LS6500 liquid scintillation spectrometer.

The reaction mixtures for adenylylation by ATP consisted of 15 μM Fhit, 0.25 mM [2,8-^{3}H]-ATP (52 μCi μmol^{-1}), 0.5 mM $MgCl_2$, and 50 mM HEPES buffer at pH 7.5. The reactions were quenched after 20, 40, or 60 min, low molecular weight tritiated molecules were removed, and radiochemical analysis was conducted as described above in the adenylylation by Ap_3A.

Product Inhibition by MgADP. Product inhibition of the initial rate of Ap_3A hydrolysis by wild-type Fhit was evaluated by use of an HPLC assay method for AMP formation as follows. The reaction mixtures (1.00 mL) consisted of Ap_3A at 20, 30, 40, 80, 160, or 320 μM and 0.1 M MOPS buffer at pH 7.0, in addition to MgADP. Initial rates were measured in the absence of MgADP and in the presence of 1 or 5 mM MgADP. The reactions were quenched by adjustment of pH to 2 with 5 M $HClO_4$, and 80 μL of each quenched solution was injected into a Beckman HPLC system equipped with an anion exchange column. The column was eluted with a gradient formed from 100% buffer A (50 mM NaH_2PO_4, pH 4.0, adjusted with acetic acid) to 65% buffer B (1 M NaCl in buffer A) within 20 min. The flow rate was 2 mL min^{-1}, and detection was at 260 nm. Initial rates of AMP production were computer-fitted to the equation for noncompetitive inhibition, using Cleland's program.

Ap_3A−[^{3}H]-ADP Exchange. To an Ependorff vial (1 mL) were added 100 μL of 4 mM Ap_3A, 10 μL of 200 mM $MgCl_2$, 125 μL of 10 mM [^{3}H]-ADP (0.5 μCi μmol^{-1}), and 755 μL of MOPS buffer (pH 7.0). The solution was equilibrated at room temperature for 10 min, and then 10 μL of 10 μM wild-type Fhit was added, and the reaction was mixed, held at room temperature for 10 min, and finally quenched by heating the mixture in boiling water for 3 min.

Table 1: Fhit-Catalyzed Exchange of [^{3}H]-ADP into Ap_3A

reaction	tritium (dpm) in Ap_3A
Fhit (0.1 μM), Ap_3A, Mg^{2+}, [^{3}H]ADP[a]	2.1×10^4
0.05 μM Fhit	1.2×10^4
−Fhit	0
−Fhit + H96G-Fhit	0
−Ap_3A + H96G-Fhit	0
−[^{3}H]ADP + [^{3}H]AMP	0

[a] The reaction mixture and procedure is described in the Experimental Procedures section.

A sample (80 μL) of the reaction mixture was subjected to HPLC as described above for the initial rate assays of product inhibition by MgADP. The Ap_3A peak was collected and subjected to radiochemical analysis for tritium in a LS 6500 multipurpose liquid scintillation counter. Control experiments were carried out by the same method except for key omissions and substitutions.

RESULTS

Isolation of a Covalent Fhit−AMP Complex. The best substrate described for Fhit is Ap_3A; however, Fhit also catalyzes the hydrolysis of ATP to AMP and PP_I at a much lower rate. Because Fhit catalyzes hydrolysis, the putative Fhit−AMP intermediate undergoes hydrolysis rapidly and cannot be isolated easily. In experiments with radiolabeled ATP incubated with Fhit, small amounts of label could be detected on electrophoresis gels at the location of the protein band (*15*). We undertook to quantify the amount of covalent Fhit−AMP in the steady state of the reaction of Ap_3A or ATP. We incubated Fhit with [2,8-^{3}H]-ATP or [8,8′-^{3}H]-Ap_3A, Mg^{2+}, and buffer and then denatured the protein in the steady state by addition of base. We then separated the protein from excess radiolabeled substrate by ultrafiltration, washed the protein free of small, tritiated molecules by repeated ultrafiltration with unlabeled buffer, and counted the protein for radioactivity. The radioactivity measurements showed that a maximum of 0.9% of the active sites were labeled, presumably as Fhit−[^{3}H]-AMP, when the substrate was [2,8-^{3}H]-ATP, whereas 11% of the sites were labeled in the steady state with [8,8′-^{3}H]-Ap_3A.

Exchange of [^{3}H]ADP into Ap_3A. If the mechanism of eqs 3 and 4 is correct, Fhit should catalyze the reverse of eq 3. This could be observed as the exchange of radiolabeled ADP into Ap_3A. The normal course of the reaction is hydrolysis of Ap_3A to AMP and ADP, and reversibility would be required to detect such an exchange. That eq 3 is reversible is indicated by the fact that product inhibition experiments with ADP show noncompetitive inhibition with respect to Ap_3A and an inhibition constant of 1.2 mM (Experimental Procedures section). Therefore, if the mechanism is correct, Fhit should catalyze the exchange of [^{3}H]-ADP into Ap_3A when the concentration of the diphosphate is higher than millimolar, and this should be detectable by the appearance of tritium in residual Ap_3A upon quenching the reaction in midstream.

As shown by the data in Table 1, Fhit indeed catalyzes the exchange of modest amounts of [^{3}H]-ADP into residual Ap_3A. The radioactivity detected in Ap_3A is a small percentage of the total tritium in the reaction, as expected because of the hydrolysis of Ap_3A under the conditions of

Table 2: Kinetic Parameters for Catalysis by H96G-Fhit

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
AMP-Im[a]	0.91 ± 0.03	26 ± 1	$(3.5 \pm 0.1) \times 10^4$
AMP-*N*-MeIm[b]	0.62 ± 0.06	180 ± 30	$(0.19 \pm 0.01) \times 10^4$
Ap_3A/0.55 M Im[c]	0.0014[d]	230 ± 27[e]	9.2 ± 0.3[d]

[a] At the optimal pH of 5.8. [b] At the optimal pH of 6.6. [c] At pH 8.9. [d] Apparent value. [e] Apparent value for Ap_3A.

Table 3: Kinetic Parameters for Catalysis by Wild-Type Fhit

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
AMP-Im[a]	4.4 ± 0.07	6.2 ± 0.3	$(6.4 \pm 0.2) \times 10^5$
AMP-*N*-MeIm[b]	4.2 ± 0.1	5.3 ± 0.3	$(7.2 \pm 0.3) \times 10^5$
Ap_3A[b]	3.5 ± 0.1	2.9 ± 0.2	$(11.6 \pm 0.7) \times 10^5$

[a] At the optimal pH of 7.0. [b] At the optimal pH of 7.3.

the exchange reaction. However, the extent of exchange is proportional to the concentration of Fhit. Moreover, the exchange is not detected when either Fhit or Mg^{2+} are excluded. Nor is exchange detected when H96G-Fhit is substituted for wild-type Fhit. Other control experiments in Table 1 further support the validity of the exchange data.

Chemical Rescue of H96G-Fhit. Mutation of Fhit to H96G-Fhit abolishes activity toward Ap_3A, as does mutation to other amino acids, and this supports the assignment of His96 as the nucleophilic catalyst in the proposed mechanism (eqs 3 and 4). The mutation of His96 to Gly96 deletes the imidazole ring and creates the possibility of chemical rescue by free imidazole, in analogy with precedent in work on GalT (*23*). In preliminary experiments, imidazole was found to reconstitute the hydrolysis of Ap_3A by H96G-Fhit at a slow rate.

Another approach to chemical rescue of H96G-Fhit is the hydrolysis of AMP-Im. The complex of H96G-Fhit with AMP-Im should be analogous to the covalent intermediate $\mathbf{E}^{Fhit}$-His^{96}−AMP in eqs 3 and 4 in that the imidazole ring of AMP-Im should occupy the vacated subsite for the imidazole ring of His^{96}, and the AMP moiety should occupy the same binding site as in the covalent intermediate. Then H96G-Fhit should catalyze the hydrolysis of AMP-Im, as in the hydrolysis of $\mathbf{E}^{Fhit}$-His^{96}−AMP according to eq 4. H96G-Fhit is in fact a good catalyst of the hydrolysis of AMP-Im. The kinetic parameters k_{cat} and k_{cat}/K_m are set forth in Table 2. AMP-*N*-MeIm incorporates the positively charged *N*-methylimidazolium ring in place of imidazole, and it is nearly as reactive as AMP-Im despite the steric requirements of the methyl group. The results support the mechanism of eqs 3 and 4.

Also included in Table 2 are the apparent kinetic parameters for the chemical rescue of Ap_3A hydrolysis by H96G-Fhit. The values of k_{cat} and k_{cat}/K_m for the hydrolysis of AMP-Im by H96G-Fhit are 650- and 3800-fold larger than the apparent values for the hydrolysis of Ap_3A at 0.55 M imidazole. Incomplete data indicate that the value of K_m for imidazole is likely to be about 0.5 M, so 0.55 M imidazole represents about 55% of saturation.

AMP-Im as a Substrate for Fhit. H96G-Fhit is inactive in the hydrolysis of Ap_3A; however, wild-type Fhit catalyzes the hydrolysis of AMP-Im. Thus, the wild-type enzyme accepts AMP-Im and AMP-*N*-MeIm as if they were ordinary substrates. The kinetic parameters in Table 3 show that AMP-Im is nearly as good a substrate as Ap_3A. However, the Fhit-catalyzed hydrolysis of AMP-Im is Mg^{2+}-independent, whereas the hydrolysis of Ap_3A requires Mg^{2+}. This suggests that the role of Mg^{2+} in the hydrolysis of Ap_3A is solely complexation with the leaving group as MgADP, and Mg^{2+} is not required for the second step of the mechanism, the hydrolysis of the Fhit−AMP intermediate in eq 4.

DISCUSSION

The present results support the mechanism of eqs 3 and 4 for the action of Fhit in catalyzing the hydrolysis of Ap_3A. Observation of protein-bound tritium in a denatured covalent Fhit−[^{3}H]-AMP supports the double displacement mechanism in the Fhit-catalyzed hydrolysis of ATP and Ap_3A. The radioactivity in denatured Fhit should be tritium associated with the covalent Fhit−[^{3}H]-AMP in the steady state just prior to denaturation. The exceedingly low level of labeling by [2,8-^{3}H]-ATP (0.9% of sites) relative to [8,8′-^{3}H]-Ap_3A (11% of sites) is to be expected if the two molecules react by the same basic mechanism of eqs 3 and 4 at extremely different rates. Reaction 3 would lead to the same covalent intermediate with both substrates, albeit at different rates, and the rate constants in reaction 4 will be the same for both substrates. Then the slower reaction of ATP arises from the low values for rate constants in reaction 3 with ATP. The value of k_{cat}/K_m for ATP is about 1/100000th that for Ap_3A (*15*). Because reaction 4 is much faster than reaction 3 only a trace of Fhit−AMP can be present in the steady state, approximately 0.9% of the sites. In the reaction of Ap_3A, reaction 3 is much faster than in the case of ATP and almost as fast as reaction 4. Therefore, a much higher concentration of Fhit−AMP is present in the steady state, 11% of the active sites.

The chemical rescue experiments are based on the principle that mutation of His96 to glycine will vacate the subsite for the imidazole ring of His96 in the tertiary structure. Thus, the locus of the imidazole ring of His96 in Fhit would be vacated in H96G-Fhit. Then free imidazole may well bind to the vacated site in H96G-Fhit and react in the same way as the imidazole ring of His96 in wild-type Fhit. This phenomenon should manifest itself in two signals. One is that free imidazole should rescue the first step of the mechanism in the cleavage of Ap_3A. In this process, the imidazole bound to H96G-Fhit should mimic the imidazole ring of His96 in Fhit in the cleavage of Ap_3A according to eq 3, as illustrated in Figure 1A. The second is that AMP-Im should chemically rescue the second step of the mechanism, that is, eq 4. In this process, the complex of H96G-Fhit with Im-AMP should mimic the covalent intermediate $\mathbf{E}^{Fhit}$-His^{96}−AMP in eq 4, as illustrated in Figure 1B, and undergo hydrolysis. Both conditions are met, as shown by the data in Table 2. Furthermore, AMP-*N*-MeIm also chemically rescues the second step, eq 4, albeit somewhat less efficiently than AMP-Im. Chemical rescue by imidazole is much less efficient than rescue by either AMP-Im or AMP-*N*-MeIm. Nevertheless, the three chemical rescue results support the mechanism of eqs 3 and 4 for the action of Fhit.

H96G-Fhit does not catalyze the hydrolysis of Ap_3A. We do not present structural evidence for the preservation of the global structure of Fhit in H96G-Fhit. However, the activity of H96G-Fhit in catalyzing the hydrolysis of AMP-Im is about $^1/_4$ of the activity of wild-type Fhit in the hydrolysis

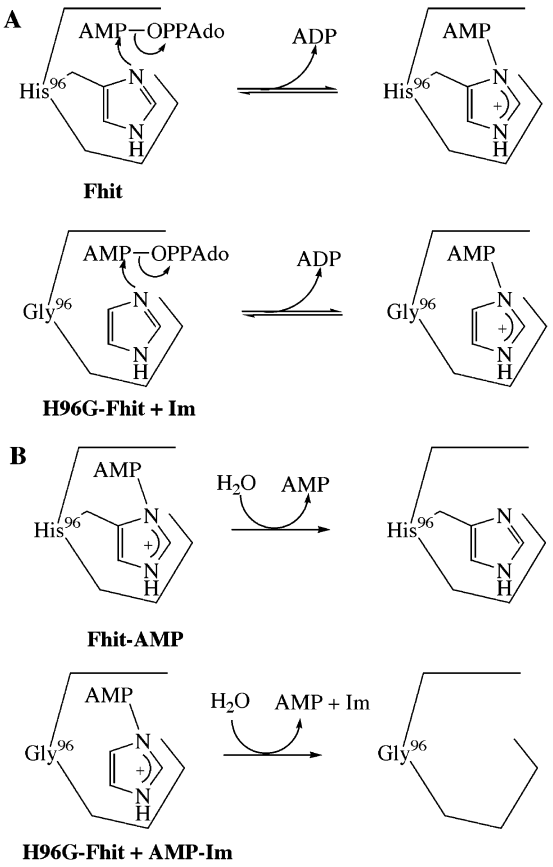

FIGURE 1: Chemical rescues of H96G-Fhit by imidazole and AMP-Im. In part A, the upper reaction illustrates the nucleophilic attack by the imidazole ring of His96 on Ap_3A to form the covalent intermediate Fhit−AMP in the first step of the action of Fhit according to eq 3. The lower reaction illustrates how imidazole binds in the space vacated by the mutation of His96 to glycine in H96G-Fhit and undergoes the analogous reaction with Ap_3A in the chemical rescue of the mutated enzyme. In part B, the upper reaction illustrates the hydrolysis of the covalent intermediate Fhit−AMP according to eq 4. The lower reaction illustrates how AMP-Im undergoes the analogous reaction in the active site of H96G-Fhit in the chemical rescue of the mutated enzyme.

of Ap_3A (Tables 2 and 3). The high activity of H96G-Fhit in chemical rescue implies that the structural integrity of Fhit is preserved in H96G-Fhit.

In an unanticipated outcome, we observe that wild-type Fhit also catalyzes the hydrolysis of AMP-Im and of AMP-*N*-MeIm at rates of 5−10 times those observed with H96G-Fhit. The kinetic parameters show that AMP-Im and AMP-*N*-MeIm are nearly as efficiently hydrolyzed as Ap_3A (Table 3). This represents a newly discovered catalytic activity of Fhit. The mechanism seems likely to follow the same course of eqs 3 and 4 as for Ap_3A, in which AMP-Im reacts with Fhit in place of Ap_3A in eq 3 to form E^{Fhit}-His^{96}−AMP, which then undergoes hydrolysis to AMP and Fhit according to eq 4. A difference is that the Fhit-catalyzed hydrolysis of Ap_3A requires Mg^{2+}, whereas the hydrolyses of AMP-Im and of AMP-*N*-MeIm do not require a divalent metal ion. Presumably $MgAp_3A$ is the true substrate in the hydrolysis of Ap_3A. The phosphoramidase activity of Fhit is significant in view of the phosphoramidase activity of Hint in the hydrolysis of adenosine-5′-phosphoramidate (*29*).

In the foregoing, we postulate that wild-type Fhit and H96G-Fhit catalyze the hydrolysis of AMP-Im by different mechanisms. In the action of wild-type Fhit, AMP-Im reacts as a conventional substrate with the imidazole ring occupying the position of the leaving group for substrates of Fhit. In the action of H96G-Fhit, the imidazole ring of AMP-Im occupies the site vacated by the mutation of His96 to glycine. This interpretation is supported by the following facts: First, Fhit is known to display very little specificity for the leaving group or for the presence of a nucleotide in the leaving group position (*25*). Second, consider the values of K_m in Tables 2 and 3 for the hydrolysis of AMP-Im and AMP-*N*-MeIm by Fhit and H96G-Fhit. The value of K_m for the hydrolysis of AMP-*N*-MeIm by H96G-Fhit is 7 times that for AMP-Im. This relationship can be expected on the basis of the steric requirements for the methylimidazole group relative to the imidazole group in occupying the limited space available in the site vacated by the mutation of His96 to glycine. In contrast, the values of K_m in the hydrolysis of these molecules by Fhit are very similar with that for AMP-*N*-MeIm actually being slightly smaller than that for AMP-Im. This can be understood on the basis that the leaving group site is sterically less restrictive in Fhit than the imidazole binding site in H96G-Fhit. We find great kinetic flexibility in the action of Fhit on molecules with different leaving groups.

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BI049762N