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Engineering Human Fhit, a Diadenosine Triphosphate Hydrolase, into an Efficient Dinucleoside Polyphosphate Synthase

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The putative human tumor suppressor gene *FHIT* encodes Fhit, the fragile histidine triad protein. Fhit is thought to participate in a signal transduction pathway involving dinucleoside polyphosphates.[1] Fhit catalyzes the Mg^{2+}-dependent hydrolysis of P^1-5′-O-adenosine-P^3-5′-O-adenosine triphosphate (Ap_3A) to AMP and MgADP.[2] Mutation of His96 to glycine disables Fhit as a catalyst for the hydrolysis of phosphoanhydrides such as Ap_3A.[3] However, the mutated enzyme H96G-Fhit efficiently catalyzes the *synthesis* of phosphoanhydride bonds in reactions of nucleoside-5′-phosphimidazolides with nucleoside di- and triphosphates. H96G-Fhit can be employed in the synthesis of a wide range of dinucleoside tri- and tetraphosphates. We here describe the use of H96G-Fhit to catalyze the synthesis of Ap_3A, Ap_3C, Ap_3G, Ap_3T, Ap_3U, Cp_3U, Tp_3U, dAp_3U, Ap_4A, Ap_4U, and the fluorescent Ap_4*etheno*-C.

The hydrolytic action of wild-type Fhit on Ap_3A proceeds by a double replacement mechanism through a covalent Fhit-AMP intermediate according to eqs 1 and 2.[3−5]

$$\mathbf{E}^{\text{Fhit}}\text{-His}^{96} + \text{MgAp}_3\text{A} \rightleftharpoons \mathbf{E}^{\text{Fhit}}\text{-His}^{96}\text{-AMP} + \text{MgADP} \quad (1)$$

$$\mathbf{E}^{\text{Fhit}}\text{-His}^{96}\text{-AMP} + \text{H}_2\text{O} \rightarrow \mathbf{E}^{\text{Fhit}}\text{-His}^{96} + \text{AMP} \quad (2)$$

Mutation of His96 into glycine eliminates the nucleophilic imidazole ring of His96, blocks the formation of the intermediate $\mathbf{E}^{\text{Fhit}}$-His^{96}-AMP in eqs 1 and 2, and inactivates the enzyme for Ap_3A hydrolysis.[3] However, H96G-Fhit efficiently catalyzes the Mg^{2+}-independent hydrolysis of AMP−Im to AMP and imidazole in a process that mimics reaction 2 in the action of wild-type Fhit.[3]

We tested the hypothesis that high concentrations of MgADP would react with the noncovalent Michaelis complex H96G-Fhit·AMP-Im and produce Ap_3A plus imidazole, according to eq 3, analogous to the reversal of eq 1. The Ap_3A formed would be stable

$$\mathbf{E}^{\text{H96G−Fhit}}\cdot\text{AMP-Im} + \text{MgADP} \rightarrow \mathbf{E}^{\text{H96G−Fhit}} + \text{Ap}_3\text{A} + \text{Im} \quad (3)$$

because of the inactivity of H96G-Fhit in the hydrolysis of Ap_3A at low concentrations of imidazole.[6] We found this to be the case and employed this engineered enzyme to produce Ap_3A from AMP-Im. We further found that H96G-Fhit catalyzed the formation of Ap_3U from AMP-Im and UDP.

The action of H96G-Fhit in producing Ap_3U proved to be pH-dependent. Small-scale reactions conducted at pH 7.75, 7.0, 6.5, 6.0, 5.5, and 5.0 gave yields of 25%, 26%, 37%, 57%, 73%, and 64%, respectively.[7] Large-scale synthesis was therefore carried out at pH 5.5.[7] We also found wild-type Fhit to catalyze the production of dinucleoside triphosphates from AMP-Im and nucleoside diphosphates, but at much lower yields, owing to the Fhit-catalyzed hydrolysis of dinucleoside triphosphates (data not shown). The advantage of H96G-Fhit for this synthesis is the absence of hydrolytic activity.

Table 1. Yields and Extinction Coefficients for Dinucleoside Polyphosphates Produced by Action of H96G-Fhit

dinucleoside polyphosphate	yield[a] (%)	A_{260}[a] (cm^{-1} mM^{-1})	wild-type Fhit hydrolysis products[b]
Ap_3A	76	25.2	AMP + ADP
Ap_3C	75	20.8	AMP + CDP (68%); CMP + ADP (32%)
Ap_3G	63	25.8	AMP + GDP (78%); GMP + ADP (22%)
Ap_3T	67	22.4	AMP + TDP
Ap_3U	73	23.6	AMP + UDP (80%); UMP + ADP (20%)
Cp_3U	71	15.9	CMP + UDP (71%); UMP + CDP (29%)
Tp_3U	69	18.5	UMP + TDP
dAp_3U	71	23.8	dAMP + UDP (22%); UMP + dADP (78%)
Ap_4A	75	26.6	AMP + ATP
Ap_4U	73	22.7	AMP + UTP (68%); UMP + ATP (32%)
Ap_4*etheno*-C	64	20.9	AMP + *etheno*-CTP (77%); *etheno*-CMP + ATP (23%)

[a] Extinction coefficients and yields at pH 5.5 with nucleoside di- or triphosphates at 80 mM. [b] At pH 7.0.

Although Ap_3A is the best substrate for wild-type Fhit, the enzyme does not display significant specificity for the leaving group.[5] In accord with this, H96G-Fhit efficiently catalyzes the reactions of nucleoside diphosphates (MgNDP) other than MgADP with AMP-Im to produce the corresponding dinucleoside triphosphates (Ap_3N). Yields are given in Table 1, and the physicochemical properties of the products are given in the Supporting Information. As an example, consider the reaction of MgUDP with AMP-Im catalyzed by H96G-Fhit. The initial rates of formation of AMP and Ap_3U are plotted in Figure 1 as a function of the concentration of MgUDP. The figure shows that AMP production due to hydrolysis of AMP-Im is essentially blocked and Ap_3U production is increased at high MgUDP concentrations.

At the high concentrations of AMP-Im and MgADP employed for synthesis, H96G-Fhit also accepts other nucleoside-5′-imidazolides, including UMP-Im and CMP-Im. Thus, essentially any dinucleoside triphosphate can be efficiently synthesized by the procedure described employing the engineered enzyme H96G-Fhit (Table 1).

The low specificity of H96G-Fhit for both the nucleoside-5′-phosphoimidazolide and MgNDP is also displayed by wild-type Fhit. This is shown in the right column of Table 1, which records the product yields in the Fhit-catalyzed hydrolysis of the dinucleoside triphosphates synthesized by use of H96G-Fhit. It is clear that wild-type Fhit will accept most nucleosides in either the leaving group or hydrolytic positions, except for thymidine, which must be in the leaving group position.

The low specificity of H96G-Fhit can be extended to the synthesis of dinucleoside tetraphosphates. At lower concentrations of $MgCl_2$ the engineered enzyme accepts nucleoside diphosphates

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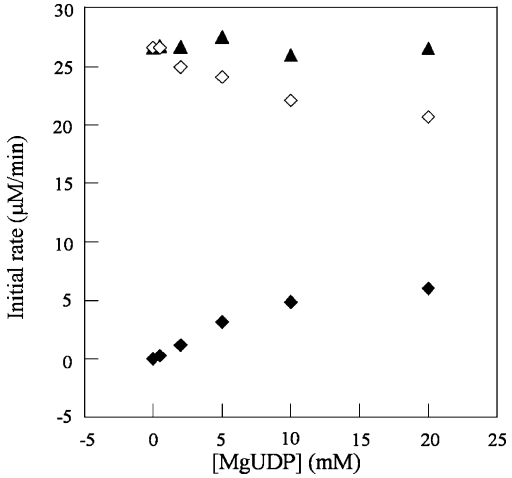

Figure 1. Effect of MgUDP on the H96G-Fhit-catalyzed reactions of AMP-Im. Initial rates of product formation as functions of increasing MgUDP concentrations are plotted for the reaction of AMP-Im catalyzed by H96G-Fhit. The reaction mixtures contained 100 μM MOPS buffer (pH 7.5), 2 μM H96G-Fhit, 300 μM AMP-Im (saturating), and MgADP varied from 0.5 to 20 mM. The reactions were quenched after 4 min by adjustment to pH 12 with 5 M NaOH. Aliquots of the reaction mixtures (80 μL) were analyzed in a Beckman HPLC system equipped with an anion exchange column, with elution and detection as described.[7] The initial rates of Ap_3A formation, AMP produced, and AMP-Im disappearance were calculated. Symbols: filled triangles, initial rate for AMP-Im disappearance; open diamonds, initial rate for AMP production; filled diamonds, initial rate for Ap_3U formation.

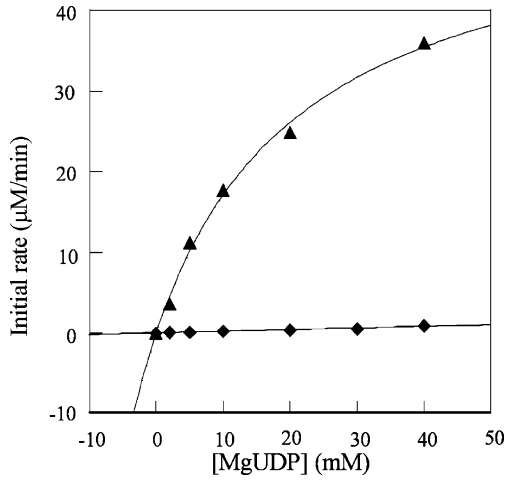

Figure 2. Nonenzymatic and H9G-Fhit-catalyzed rates of production of Ap_3U. The rates of enzymatic and nonenzymatic production of Ap_3U from AMP-Im and MgUDP were measured in a 500 μL reaction mixture containing 200 mM MES buffer at pH 5.5, saturating AMP-Im (400 μM), 6 μM H96G-Fhit, and MgUDP at 2, 5, 10, 20, and 40 mM. After 4 min at room temperature, the reactions were quenched by adjustment to pH 12 with 5 M NaOH. An aliquot (40 μL) of each reaction mixture was analyzed by HPLC and the initial rate of Ap_3U formation calculated. The symbols are as follows: triangles, initial rate for the H96G-Fhit-catalyzed reaction (the solid line is calculated from the kinetic parameters $k_{cat} = 9.2 \pm 0.7$ min^{-1} and K_m for MgUDP $= 20 \pm 3$ mM); diamonds, initial rates for the nonenzymatic reaction under the same conditions (the solid line represents the fit of the second-order rate equation to the data, with $k = 52 \pm 3$ M^{-1} min^{-1}).

in reactions with AMP-Im, and ATP does not react well. However, at twice the concentration of $MgCl_2$ employed in the synthesis of dinucleoside triphosphates, H96G-Fhit accepts either ATP or UTP in reaction with AMP-Im to produce Ap_4A or Ap_4U in good yields (Table 1).

Also included in Table 1 are the extinction coefficients for the dinucleoside polyphosphates synthesized. Because these molecules are hypochromic, the extinction coefficients had to be measured on the basis of the amounts of products formed by the action of wild-type Fhit on the molecules synthesized using the engineered H96G-Fhit.[8]

AMP-Im is an activated form of AMP that can be used in the nonenzymatic synthesis of the phosphoanhydride linkage, as well as the phosphodiester linkage.[9−13] Thus, nonenzymatic reactions of nucleoside-5-imidazolides with nucleoside di- and triphosphates should produce dinucleoside tri- and tetraphosphates. The nonenzymatic reactions are less specific and far slower than the process with the engineered enzyme. The advantages of the engineered enzyme can be appreciated by comparing the kinetic course of the enzymatic and nonenzymatic production of Ap_3U under otherwise identical conditions. Data are shown in Figure 2. The engineered enzymatic rate is much faster than the nonenzymatic rate at all concentrations of MgUDP and approaches saturation with a K_m value of 20 mM for MgUDP.

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Supporting Information Available: NMR spectral assignments and MS characterizations of Ap_3A, Ap_3C, Ap_3G, Ap_3T, Ap_3U, Cp_3U, Tp_3U, dAp_3U, Ap_4A, Ap_4U, and Ap_4*etheno*-C. This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) The chemical rescue of H96G-Fhit by imidazole requires high concentrations, e.g. 0.5 M, and is very slow.[3]
(7) H96G-Fhit and AMP-Im were prepared as described.[3] To determine the pH optimum for Ap_3U synthesis, the reaction mixtures consisted of 100 mM UDP, 100 mM $MgCl_2$, 600 μM AMP-Im, 60 μM H96G-Fhit-H96G, and 0.2 M MES or HEPES buffer. After reaction for 2 min at room temperature, 40 μL samples were analyzed by HPLC, using an anion exchange column eluted with a gradient changing from 100% buffer A (50 mM NaH_2PO_4 at pH 4.0) to 65% buffer B (1 M NaCl in buffer A) within 20 min at a flow rate of 2 mL min^{-1}. The concentration of Ap_3U was calculated by using the extinction coefficient $\epsilon_{260} = 23.6$ mM^{-1} cm^{-1}.[8] This method was employed to obtain the transformation yields in Table 1. Large-scale synthesis was conducted as follows. To 5 mL of 200 mM MES buffer (pH 5.5) containing 80 mM UDP, 160 mM $MgCl_2$, and 0.5 mM H96G-Fhit was added solid AMP-Im to 80 mM. The reaction proceeded to completion at room temperature in 60 min. The reaction mixture was diluted 5-fold with double-distilled water and applied to a pre-packed anion exchange column (200 mL of Q-Sepharose fast flow). The column was eluted with a 2.0 L linear gradient of buffer A (10 mM $CH_3COONa-CH_3COOH$, pH 4.0) to 0.5 M NaCl in buffer A at a flow rate of 2 mL min^{-1}. The fractions containing Ap_3U were pooled, adjusted to pH 7.0, and freeze-dried. The dried product was desalted by passage through a column of Biogel P2. The yield of Ap_3U based on AMP-Im is 54% with 12% Ap_2A as a byproduct.
(8) Extinction coefficients of dinucleoside tri- and tetraphosphates at 260 nm were determined as follows for the example of Ap_3U. A sample of Ap_3U (200 μL) with a known value of absorbance at 260 nm and in 200 mM MES buffer (pH 7.0) was completely hydrolyzed by addition of 0.01 μM wild-type Fhit and 1.0 mM $MgCl_2$ to give AMP, UDP, UMP, and ADP. A 40 μL sample of the product mixture was analyzed by HPLC for its content of AMP + ADP, determined spectrophotometrically using the known extinction coefficient for these molecules (15.0 mM^{-1} cm^{-1}). The sum of AMP and ADP was taken as the total amount of Ap_3U, and the extinction coefficient for Ap3U was calculated from the measured absorbance of the original solution. For samples that did not contain adenosine, the same procedure was followed using the extinction coefficient for uridine or thymidine nucleotides (10 mM^{-1} cm^{-1}).
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