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Mutation and inhibition studies of mevalonate 5-diphosphate decarboxylase

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Abstract—Mevalonate 5-diphosphate decarboxylase plays an important role in regulating cholesterol biosynthesis, which was studied through incubation with various synthetic substrate analogs and characterization of mutated enzymes. The results are potentially useful for further developing inhibitors that block the mevalonate pathway which is a drug target for treating cardiovascular disease and cancer.

In animal cells, the cholesterol biosynthetic pathway contains a unique series of three sequential ATP-dependent enzymes that convert mevalonate to isopentenyl diphosphate (IPP): mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and mevalonate 5-diphosphate decarboxylase (MDD) (Fig. 1).[1] These three enzymes catalyze consecutive steps downstream from the HMG-CoA reductase in the mevalonate pathway, and are responsive for cholesterol intake in animals. The pathway has been exploited in the design of drugs treating cardiovascular disease[2] and cancer.[3] In addition, inhibition of insect juvenile hormone biosynthesis by fluorinated mevalonate analogs has been reported as a potential method for pest control.[4] Several investigators have suggested the involvement of MDD as important regulatory steps in the biosynthesis of cholesterol.[5]

The X-ray structure of MDD from yeast has been solved without bound ligand,[6] and the enzyme belongs to GHMP (Galactokinase, Homoserine kinase, Mevalonate kinase, and Phosphomevalonate kinase) superfamily of small-molecule kinases. Kinases are a ubiquitous group of enzymes central to many biochemical processes such as metabolism, gene regulation, and signal transduction.[7] A variety of substrate analogs of MDD targeting mevalonate 5-diphosphate binding site have been studied,[8] and some have been found to be enzyme inhibitors. In the present study, we report our further investigation of rat MDD through characterization of mutated enzymes. A variety of substrate analogs were synthesized and incubated with rat MDD, and structure–activity relationships were obtained for the enzymatic reaction. The study increased our understanding of MDD and may be useful for drug discovery for the purpose of treating cardiovascular disease and cancer, or for pest control.

The rat liver MDD with N-terminal hexa His-tag was obtained and assayed as previously described.[8c] Tris buffer was used instead of phosphate buffer for storing the enzyme and for the enzyme assays. A sequence alignment analysis of MDDs from different sources indicates that acidic residue D306 and basic residues K23, K27, R154, R162, K208 are highly conserved, and may have important roles in substrate binding or catalysis. Mutant expression plasmids of rat MDD K23A, K27A, R154A, R162A, K208A, D306A, D306E, D306N were subsequently constructed using site-directed mutagenesis.

Abbreviations: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GHMP, galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase; GPP, geranyl diphosphate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate; MDD, mevalonate 5-diphosphate decarboxylase, also known as mevalonate 5-pyrophosphate decarboxylase or MPD; MVAPP, mevalonate 5-diphosphate; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; PP, pyrophosphate or diphosphate; TsCl, toluene sulfonyl chloride.

Keywords: Mevalonate 5-diphosphate decarboxylase; Substrate analog; Mutation; Cholesterol; Cardiovascular disease; Cancer.

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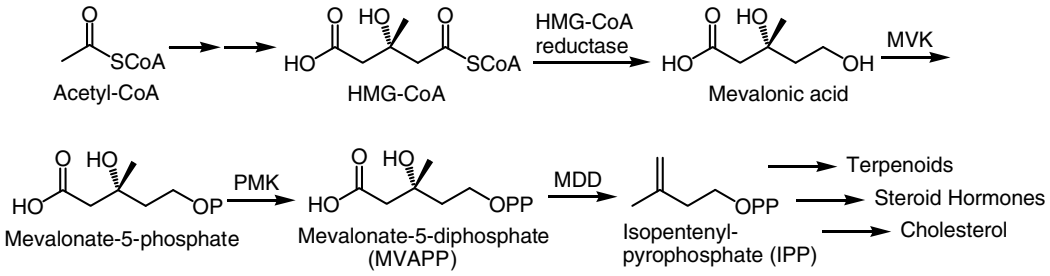

Figure 1. Mevalonate pathway in animal cells.

The mutated enzymes were overexpressed and purified with a nickel metal affinity column to apparent homogeneity based on SDS–PAGE. Spectrophotometric activity assays demonstrated that mutants K23A, R162A, K208A, D306A, D306E, and D306N have no detectable activity. The low activities of mutants K27A and R154A were detected and their kinetic parameters were obtained as shown in Table 1. For K27A, the V_{max} value is decreased to 0.14 ± 0.01 μmol/min/mg, and K_M values increased to 0.75 ± 0.16 mM for mevalonate 5-diphosphate and 1.87 ± 0.15 mM for ATP. For R154A, the V_{max} value is 0.34 ± 0.02 μmol/min/mg, and K_M values are 0.92 ± 0.29 mM and 1.40 ± 0.20 mM for mevalonate 5-diphosphate and ATP, respectively.

The MDD-catalyzed reaction has been proved to be a stepwise process, which includes phosphorylation at 3-OH and decarboxylation.[8a] For mevalonate kinase, another member of GHMP superfamily, the highly conserved acidic residue D204 is determined to play a crucial catalytic role for phosphorylation at 5-OH of the substrate.[9] It is reasonable to assume that a conserved aspartate residue in MDD may also work as a general base to abstract proton from 3-hydroxy of the substrate. A sequence alignment demonstrates that D306 is located in a highly conserved motif (^{306}DAGPN^{310}), and the three mutants D306A, D306E, and D306N were all found to have no detectable activity. This result strongly suggests that D306 has a catalytic role in the reaction. During the progress of this work, Krepkiy and Miziorko reported their work with assignment of a crucial catalytic role to Asp302 of yeast MDD,[10] which is consistent with our result.

Based on our assumption that positively charged residues may directly interact with negatively charged substrates (mevalonate 5-diphosphate and ATP), the highly conserved basic residues K23, K27, R154, R162, and K208 were chosen for mutation. The residue K23 is located at a highly conserved motif (^{23}KYWGK^{27}). From the crystal structure of yeast MDD, the corresponding Lys18 is very close to Asp302 (2.75 Å), forming an invariant acid/base pair.[6] The activity of the mutated enzyme K23A is below detectable levels, indicating that the residue is critical for catalysis or substrate binding. Krepkiy and Miziorko reported a 30-fold increase in activity and 16-fold inflation of K_M for ATP for yeast MDD K18M mutant, indicating that Lys18 influences the active site but is not crucial for reaction chemistry.[10] Our results show that the mutation of the residue to alanine has a larger effect on the activity of the protein. Comparing with the case of mevalonate kinase, residue K23 in rat MDD may function like K13 in rat MVK to interact with γ-phosphoryl group of ATP and 3-OH of mevalonate 5-diphosphate by salt bridge or hydrogen bond.[9b]

Table 1. Kinetic parameters for MDD wild-type and mutated enzymes

MDD	V_{max} (μmol/min/mg)	$K_{M\ (MVAPP)}$ (mM)	$K_{M\ (ATP)}$ (mM)
Wild type	6.60 ± 0.31	0.036 ± 0.003	0.53 ± 0.09
K27A	0.14 ± 0.01	0.75 ± 0.16	1.87 ± 0.15
R154A	0.34 ± 0.02	0.92 ± 0.29	1.40 ± 0.20

A 47-fold decrease in activity was found for the K27 mutant. A 21-fold inflation of K_M for mevalonate 5-diphosphate and 3.5-fold inflation of K_M for ATP were found for the mutant. Though the residue K27 is also located in the same invariant motif of MDD with K23, the assay result indicates that K27 is less significant for the reaction. The changes of K_M suggest that K27 affects the binding of mevalonate 5-diphosphate more than ATP. The residues R154 and R162 are located in another highly conserved motif of MDD (^{154}RRGSGS ACRS^{163}). The activities of their corresponding mutated enzymes are quite different. The activity of R154A mutant was decreased by 19-fold, while no activity was found for the R162A mutant, suggesting the residue R162 may play an important role for the reaction by providing a positive center. The Ser153 of yeast MDD in the same conserved motif was proposed to bind with Mg-ATP at the MDD active site.[11] Finally, the activity of K208A mutant is undetectable by spectrophotometric measurement, indicating that the residue is also crucial for the activity of the enzyme. More work is necessary to investigate its catalytic or structural role.

The above results indicate that mutations of highly conserved acidic residue D306 and basic residues K23, K27, R154, and R162 in rat MDD to alanine caused significant changes in enzymatic activities. D306 may play a crucial role for the catalytic reaction as a general base to abstract proton from 3-OH of mevalonate 5-diphosphate. The mutated basic residues may function critically in binding with negatively charged substrates, or play a role in the second step of the catalytic reaction: elimination of phosphate and carbon dioxide.

Table 2. IC_{50} values of substrate analogs for rat MDD

Compound	IC_{50} for rat MDD (μM)
4	7.5
8	20
17	60
18	80
19	120
20	140
25	50

In order to further understand rat MDD catalyzed reaction, we synthesized a variety of substrate analogs, which were incubated with the enzyme. 2-Fluoromevalonate 5-diphosphate has been found to be an irreversible inhibitor of rat MDD,[8c] and we further synthesized 2,2-difluoromevalonate 5-diphosphate (**4**)[12] and 3-ethyl-2-fluoro-3,5-dihydroxy-pentanate 5-diphosphate (**8**)[13] as shown in Figures 2 and 3 for inhibition studies. 4-Hydroxy-2-butanone reacted with 4-toluene sulfonyl chloride in the presence of pyridine to give 4-tosyloxy-2-butanone (**1**), which was then reacted with fluorinated ethyl 2-bromoacetate through Reformatsky reaction yielding compound **2**. Compound **3** was obtained from compound **2** through a reaction with pyrophosphate, and its subsequent hydrolysis gave 2,2-difluoromevalonate 5-diphosphate (**4**). Compound **8** was synthesized following same synthetic strategy as that for compound **4**. Both compounds were found to be competitive inhibitors only, since the inhibitions were not time-dependent. Both inhibitions were competitive with mevalonate 5-diphosphate, and the IC_{50} values of 7.5 and 20 μM were determined for compounds **4** and **8**, respectively, as shown in Table 2. These results indicated that an extra fluorine at carbon 2 position and the replacement of methyl group at carbon 3 with an ethyl group will both affect the nucleophilic attack at carbon 2 by an enzyme nucleophile, which was proposed for inhibition of MDD by 2-fluoromevalonate 5-diphosphate.

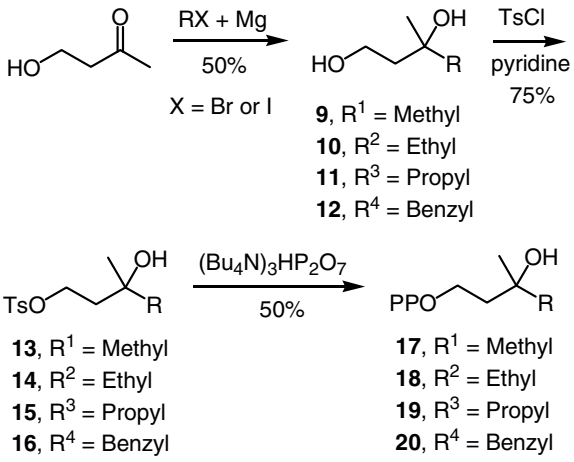

Figure 4. Organic syntheses of substrate analogs (**17**–**20**).

Based on our results from the site directed mutagenesis study, several basic residues play important role in enzymatic reaction, which can probably interact with negatively charged substrate. Therefore, it is interesting to know the kinetic properties of the substrate analogs with carboxylate group replaced with various other functional groups. Since the charged group usually makes the molecule difficult to penetrate the cell membrane, it would be better if it can be replaced with a neutral group. A variety of substrate analogs (**17**–**20**, **25**) were synthesized[14] for this purpose as shown in Figures 4 and 5. Compounds **17**–**20** were synthesized using a

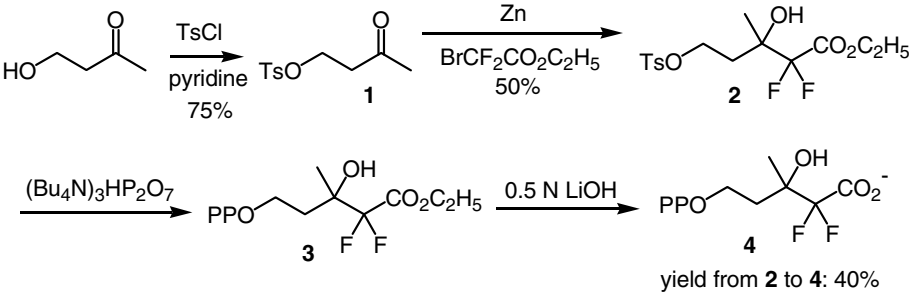

Figure 2. Organic synthesis of 2,2-difluoromevaloate 5-diphosphate (**4**).

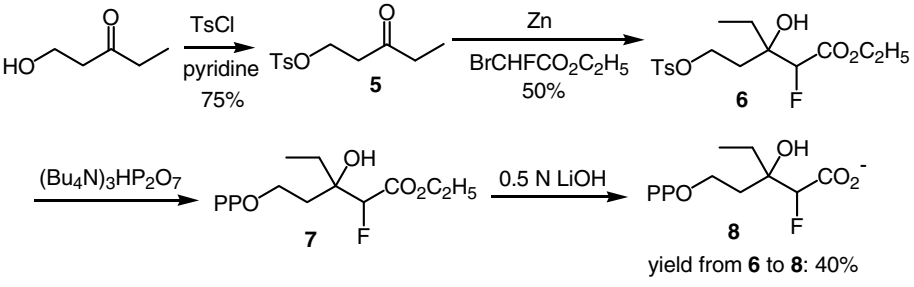

Figure 3. Organic synthesis of 3-ethyl-2-fluoro-3,5-dihydroxy-pentanate 5-diphosphate (**8**).

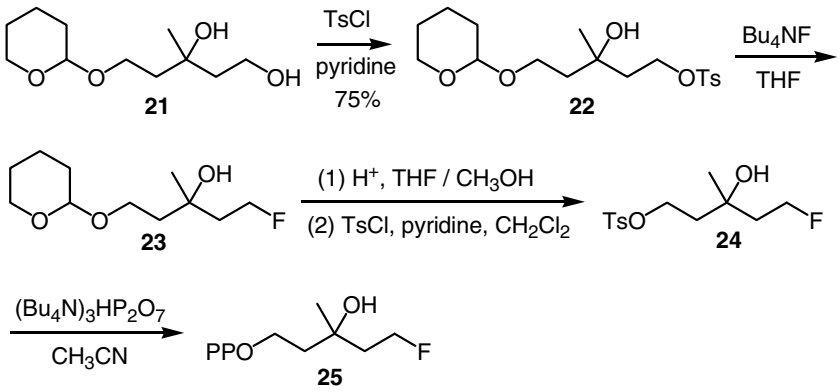

Figure 5. Organic synthesis of substrate analog (**25**).

similar strategy to that for the synthesis of compound **4**. Difference is that we used the Grignard reaction here instead of the Reformatsky reaction, since the reagents do not have carboxylate ester functional group. Two equivalents of Grignard reagent were used, with the first equivalent reagent consumed by reacting with hydroxyl group, which saved one protection and one deprotection steps. The synthesis of compound **25** also followed a similar strategy, which includes two stepwise S_{N2} reactions on two sides of the molecule with some protection and deprotection steps. These compounds were found to be mild inhibitors of rat MDD as shown in Table 2, and all are competitive with respect to mevalonate 5-diphosphate, suggesting that carboxyl group is relatively important for the binding and reaction of the substrate.

In summary, MDD is an essential enzyme in mevalonate pathway regulating cholesterol biosynthesis. The enzyme was studied through incubation with various synthetic substrate analogs and characterization of several mutated enzymes. The results increased our understanding of MDD and are potentially useful for developing inhibitors that target the mevalonate pathway for treatment of cardiovascular disease and cancer.

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12. The spectra data of 2,2-difluoromevaloate 5-diphosphate (**4**) are shown as following: ^{1}H NMR (300 MHz, D_2O, TMS) δ 1.34 (s, 3H, CH_3), 1.92–2.11 (m, 2H), 4.07–4.13 (m, 2H, $POCH_2$); ^{31}P NMR (121 MHz, D_2O) δ −1.03 (m).
13. The spectra data of 3-ethyl-2-fluoro-3,5-dihydroxy-pentanate 5-diphosphate (**8**) are shown as following: ^{1}H NMR (300 MHz, D_2O, TMS) δ 0.95 (t, J = 7.5 Hz, 3H), 1.67 (m, 2H), 2.02 (m, 2H), 4.10 (q, 2H), 4.78 (m, 1H, partially mixed with solvent signals); ^{31}P NMR (121 MHz, D_2O). δ −7.71 (d, J = 18.3 Hz), −3.54 (d, J = 19.0 Hz).
14. (a) The spectra data of 3-hydroxy-3-methyl-butanyl pyrophosphate (**17**) are shown as following: ^{1}H NMR (300 MHz, D_2O, TMS) δ 1.28 (s, 6H, $2CH_3$), 1.92 (t, J = 7.2 Hz, 2H, CH_2C), 4.04–4.11 (m, 2 H, OCH_2); ^{31}P NMR (121 MHz, D_2O) δ −6.30 (d, J = 17.0 Hz), −5.90 (d, 17.5 Hz). (b) The spectra data of 3-hydroxy-3-methyl-pentanyl pyrophosphate (**18**) are shown as following: ^{1}H NMR (300 MHz, D_2O) δ 0.83 (t, 7.2 Hz, 3H, CH_2CH_3), 1.14 (s, 3H, CH_3), 1.48 (q, J = 7.5 Hz, 2H, CH_2CH_3), 1.82 (t, J = 6.8 Hz, 2H, CH_2C), 3.98 (q, J = 7.2 Hz, 2H, OCH_2); ^{13}C NMR (75 MHz, D_2O) δ 8.1, 25.8, 34.2, 40.6, 63.1, 73.3; ^{31}P NMR (121 MHz, D_2O) δ −6.10 (d,

J = 20.2 Hz), −3.65 (d, J = 19.6 Hz). (c) The spectra data of 3-hydroxy-3-methyl-hexyl pyrophosphate (**19**) are shown as following: ^{1}H NMR (300 MHz, D_2O) δ 0.88 (t, J = 6.9 Hz, 3H, CH_2CH_3), 1.19 (s, 3H, CH_3), 1.28–1.48 (m, 4H, $CH_2CH_2CH_3$), 1.86 (t, J = 7.2 Hz, 2H, CH_2C), 3.99–4.06 (q, 2H, OCH_2); ^{31}P NMR (121 MHz, D_2O) δ −6.12 (d, J = 19.5 Hz), −3.82 (d, J = 19.5 Hz). (d) The spectra data of 3-hydroxy-3-phenyl-butanyl pyrophosphate (**20**) are shown as following: ^{1}H NMR (300 MHz, D_2O) δ 1.22 (s, 3H, CH_3), 1.93 (t, J = 7.2 Hz, 2H, CH_2C), 2.88 (s, 2H, CH_2Ph), 4.11–4.18 (m, 2H, OCH_2), 7.15–7.26 (m, 5 H, ArH); ^{31}P NMR (121 MHz, D_2O) δ −5.97 (d, J = 18.9 Hz), −4.68 (d, J = 19.6 Hz); ^{13}C NMR (75 MHz, D_2O) δ 26.1, 41.6, 48.2, 63.4, 73.1, 126.0, 128.9, 131.6, 138.4. (e) The spectra data of 5-fluoro-3-hydroxy-3-methyl-pentanyl diphosphate (**25**) are shown as following: ^{1}H NMR (300 MHz, D_2O) δ 1.20 (s, 3H, CH_3), 1.85 (t, J = 6.6 Hz, 2H, CH_2), 1.95 (t, J = 6.0 Hz, 2H, CH_2), 3.99 (q, 2H, OCH_2), 4.62 (dt, J_{HF} = 46.2 Hz, J_2 = 5.9 Hz, 2H, CH_2F); ^{31}P NMR (121 MHz, D_2O) δ −6.67 (d, J = 22.0 Hz), −6.45 (d, J = 23.2 Hz).