Enterococcus faecalis mevalonate kinase

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Abstract

Gram-positive pathogens synthesize isopentenyl diphosphate, the five-carbon precursor of isoprenoids, via the mevalonate pathway. The enzymes of this pathway are essential for the survival of these organisms, and thus may represent possible targets for drug design. To extend our investigation of the mevalonate pathway in *Enterococcus faecalis*, we PCR-amplified and cloned into pET-28b the *mvaK1* gene thought to encode mevalonate kinase, the fourth enzyme of the pathway. Following transformation of the construct EFK1-pET28b into *Escherichia coli* BL21(DE3) cells, the expressed C-terminally hexahistidine-tagged protein was purified on a nickel affinity support to apparent homogeneity. The purified protein catalyzed the divalent ion-dependent phosphorylation of mevalonate to mevalonate 5-phosphate. The specific activity of the purified kinase was 24 $\mu\text{mole}/\text{min}/\text{mg}$ protein. Based on sedimentation velocity data, *E. faecalis* mevalonate kinase exists in solution primarily as a monomer with a mass of 32.2 kD. Optimal activity occurred at pH 10 and at 37°C. ΔH_a was 22 kcal/mole. Kinetic analysis suggested that the reaction proceeds via a sequential mechanism. K_m values were 0.33 mM (mevalonate), 1.1 mM (ATP), and 3.3 mM (Mg^{2+}). Unlike mammalian mevalonate kinases, *E. faecalis* mevalonate kinase utilized all tested nucleoside triphosphates as phosphoryl donors. ADP, but not AMP, inhibited the reaction with a K_i of 2.7 mM.

Keywords: *Enterococcus faecalis*; isoprenoid biosynthesis; isopentenyl diphosphate; mevalonate 5-phosphate; mevalonate pathway

The lipids known as isoprenoids participate in processes as diverse as cell-wall biosynthesis (Reusch 1984), electron transport (Meganathan 1996), photosynthetic light harvesting (Johnson and Schroeder 1996), lipid membrane structure and intracellular signaling (Cane 1999). The basic building block for isoprenoid biosynthesis in all forms of life is isopentenyl diphosphate (IPP). Synthesis of IPP can occur either by the mevalonate pathway (Fig. 1) or by the glyceraldehyde-3-phosphate-pyruvate, or nonmevalonate, pathway (Lange et al. 2000). Although many Gram-negative bacteria utilize the nonmevalonate pathway, eukaryotes, archaea, Gram-positive cocci, and the spirochete *Borrelia burgdorferi* employ exclusively the mevalonate pathway (Rohmer 1999; Kim et al. 2000; Wilding et al. 2000a,b).

The use of knock-out mutants of *Streptococcus pneumoniae* and *Staphylococcus aureus* established the essentiality of the genes that encode the mevalonate pathway enzymes in Gram-positive cocci. Mutants that lacked the *mvaA* and *mvaS* genes that encode HMG-CoA reductase and HMG-CoA synthase, respectively, required an external source of mevalonate for survival, and the virulence of *S. pneumoniae* knockouts in mice was severely attenuated (Wilding et al. 2000a). These discoveries are particularly important in light of the recent development of multidrug-resistant strains of pathogenic bacteria (Walsh and Howe 2002; Paulsen et al. 2003). Inhibitors directed against the enzymes of this pathway might serve as antibiotics against these pathogens.

Mevalonate kinase, the fourth enzyme of the mevalonate pathway, catalyzes a nucleophilic attack by a C5 anion of mevalonate on the γ -phosphate of ATP forming mevalonate-5-phosphate:

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Figure 1. Intermediates and enzymes of the mevalonate pathway for isopentenyl diphosphate biosynthesis.

Mevalonate kinases have been purified and characterized from animal, plant, and archaeal sources (Tchen 1958; Potter and Miziorko 1997; Huang et al. 1999). A deficiency of mevalonate kinase in human subjects results in mevalonic aciduria and hyperimmunoglobulemia D (Houten et al. 2001).

Crystal structures of mevalonate kinase include a 2.4 Å structure of an ATP binary complex of the rat enzyme (Fu et al. 2002) and a 2.4 Å native structure of the thermostable enzyme from *Methanococcus jannaschii*. The overall fold of *M. jannaschii* mevalonate kinase resembles that of homoserine kinase from the same organism (Zhou et al. 2000; Yang et al. 2002). Site-directed mutagenesis has implicated several residues as important for catalysis. Glu 193_H (subscripts on residue numbers refer to the enzymes from *Enterococcus faecalis* [E], human [H], and rat [R]) of the human enzyme and Lys 13_R of the rat enzyme appear to be important for binding MgATP, and Ala 334_H appears to participate in binding mevalonate (Hinson et al. 1997; Potter and Miziorko 1997; Potter et al. 1997). Asp 204_H is thought to be the catalytic base that deprotonates the C-5 hydroxyl of mevalonate, generating the nucleophile that

attacks the γ -phosphate of ATP (Potter and Miziorko 1997).

We previously cloned, expressed, and characterized the *E. faecalis* enzymes that catalyze the first three reactions of the mevalonate pathway, acetoacetyl-CoA thiolase (Hedl et al. 2002), HMG-CoA synthase (Sutherland et al. 2002), and HMG-CoA reductase (Hedl et al. 2002). We report here the cloning, expression, purification, and characterization of *E. faecalis* mevalonate kinase, the fourth enzyme of the mevalonate pathway, and the first characterized eubacterial mevalonate kinase.

Results

Expression and purification of the *E. faecalis* mvaK1 gene product

Plasmid EFK1-pET28b was expressed in *Escherichia coli* BL21(DE3) cells at 16°C and the resulting C-terminally His-tagged polypeptide was purified on an NiNTA column. Protein yields averaged 18–20 mg per liter of dry cells. Migration on SDS-PAGE was consistent with the predicted molecular weight of 33,842 Da (Fig. 2). The specific activity of the purified enzyme was 24 μ mole/min/mg protein.

Multimeric state

The multimeric state of *E. faecalis* mevalonate kinase was investigated by sedimentation velocity analytical ultracentrifugation (Fig. 3). Two components with sedimentation coefficients of 2.56 and 3.78, consistent with molecular masses of 32.2 kD and 71.1 kD, were observed. The mass ratio of the 32.2-kD peak to that of the 71.1-kD peak was

Figure 2. SDS-PAGE of the expressed EFK1-pET28b construct purified by nickel affinity chromatography. Recombinant, C-terminally His-tagged *E. faecalis* mevalonate kinase. Numbers indicate molecular sizes in kilodaltons.

Figure 3. Sedimentation velocity ultracentrifugation of *E. faecalis* mevalonate kinase. (A) Sedimentation boundaries were measured by using Rayleigh interference optics plotted against radial position. The data are shown for 25-min intervals and represent one-fifth of the data used in the analysis. The jagged lines represent the observed fringes, and the smooth lines represent the calculated best-fit distribution calculated by using Sedfit 8.5 and the Lamm equation model (www.analyticalultracentrifugation.com; Schuck et al. 2002). (B) Best-fit $c(s)$ sedimentation coefficient distribution, allowing for systematic time-invariant noise. The uncorrected values for the sedimentation coefficients for the minor and major peaks are 2.56 S and 3.78 S, respectively.

Figure 4. Effect of temperature and of hydrogen ion concentration. (Left) Temperature. Assays of reaction 1, the phosphorylation of mevalonate to mevalonate-5-phosphate, were conducted at the indicated temperatures under otherwise standard conditions. (Inset) Selected data shown as an Arrhenius plot. (Right) Hydrogen ion concentration. Assays were conducted in 50 mM sodium acetate, 50 mM glycine, 50 mM Tris, 50 mM MES, and 200 mM KCl at the indicated pH, but under otherwise standard conditions. Fraction of maximal activity ranges from 0 (no activity) to 1 (maximal activity observed). Assays employed substrate concentrations that approximated $V_{\max}$, 11.4 $\mu\text{mole/min/mg}$ protein.

10 : 1. The monomer mass calculated from the amino acid sequence is 33.8 kD.

Effect of temperature and pH on activity

Optimal activity for catalysis of reaction 1 by *E. faecalis* mevalonate kinase occurred at 37°C (Fig. 4, left). An Arrhenius plot of selected data yielded a ΔH_a of 22 kcal/mole (Fig. 4, left, inset). Optimal activity occurred at pH 10.0 (Fig. 4, right).

Kinetic parameters

K_m values derived from Lineweaver–Burke plots were 0.33 mM for mevalonate, 1.1 mM for ATP, and 3.3 mM for Mg^{2+} (Fig. 5). Table 1 summarizes these data and compares them to those of other characterized mevalonate kinases.

Mechanism

The rate of reaction 1 was measured at varying concentrations of mevalonate and ATP. A double reciprocal plot of $1/[\text{ATP}]$ versus $1/\text{fraction of maximal activity}$ at several mevalonate concentrations yielded lines that intersected on the y-axis, consistent with a sequential mechanism (Fig. 6).

Specificity of the divalent cation and of the phosphoryl donor

Although Mg^{2+} represents the probable physiologic cation for *E. faecalis* mevalonate kinase, catalysis proceeded in the presence of Mn^{2+} , Zn^{2+} , or Ca^{2+} . Similarly, although ATP is

Figure 5. Dependence of the initial velocity of reaction 1 on the concentration of substrates. Assays were conducted at the indicated concentrations of ATP, mevalonate or Mg^{2+} , but under otherwise standard conditions. (Left) ATP. (Middle) Mevalonate. (Right) Magnesium ion.

the likely phosphoryl donor in vivo, GTP, CTP, TTP, UTP, and ITP all served as alternate phosphoryl donors. K_m values for UTP (2.8 mM), TTP (1.9 mM), ITP (1.4 mM), ATP (1.1 mM), CTP (0.8 mM), and GTP (0.3 mM) were similar for all 5 nucleoside triphosphates.

Effect of adenosine di- and monophosphate

ADP and AMP structurally resemble ATP, the substrate for the reaction. Investigation of inhibition by ADP and AMP revealed that ADP acts an inhibitor of *E. faecalis* mevalonate kinase competitive with ATP with a K_i of 2.7 mM (Fig. 7). By contrast, concentrations of AMP as high as 100 mM did not significantly inhibit activity.

Discussion

E. faecalis mevalonate kinase represents the first characterized prokaryotic form of this enzyme. It exhibits approximately 20% sequence homology with the DNA-based inferred primary structures of the putative mevalonate kinases

of several other Gram-positive pathogens (*Enterococcus faecium*, *Staphylococcus epidermis*, *Staphylococcus haemolyticus*, *S. aureus*, *S. pneumoniae*, and *Streptococcus pyogenes*; Wilding et al. 2000a). Shared structural identity is considerably lower for the characterized mevalonate kinases from eukaryotes and archaea. As might be anticipated, however, previously identified residues implicated in ligand recognition or catalysis remain conserved across kingdoms. Lys 12_E, Glu 137_E, and Asp 148_E of *E. faecalis* mevalonate kinase are the apparent cognates of Lys 13_R, Glu 193_H, and Asp 204_H of the rat and human enzymes (Fig. 8).

The kinetic properties of the purified recombinant C-terminally His-tagged *E. faecalis* mevalonate kinase resembled those for other characterized mevalonate kinases

Figure 6. Mevalonate kinase proceeds via a sequential mechanism. Assays employed the indicated concentrations of ATP and either 1.0 mM (circles), 0.5 mM (squares), or 0.33 mM (diamonds) mevalonate under otherwise standard conditions. Lines were fit to the data using the graphing program of Microsoft Excel.

Table 1. Kinetic parameters for mevalonate kinase from *E. faecalis* and other sources

Source	$V_{\max}$ ($\mu\text{mole}/\text{min}/\text{mg}$)	K_m^{ATP} (mM)	$K_m^{\text{mevalonate}}$ (mM)	pH optimum
<i>E. faecalis</i>	11.4	1.1	0.33	10
Rat liver ^a	29.4	1.75	0.271	N.R. ^c
Pig liver ^b	17.5	0.30	0.019	N.R. ^c
Human liver ^c	37	0.024	0.074	N.R. ^c
<i>M. jannaschii</i> ^d	390	0.092	0.068	8.0–8.5

^a Tanaka et al. 1990.

^b Beytia et al. 1970.

^c Popjak 1969.

^d Huang et al. 1999.

^e N.R.: not reported, but assayed at pH 7.0.

Figure 7. Inhibition by ADP of the phosphorylation of mevalonate to mevalonate-5-phosphate. Assays were conducted in the presence of 0 mM (open circle), 10 mM (filled circle), or 20 mM (square) ADP at the indicated concentrations of ATP under otherwise standard conditions.

(Table 1). As anticipated for an enzyme from a mesophile, the temperature optimum was 37°C. Although the number of data points is limited by the assay employed, the pattern of intersecting lines for a plot of $1/[ATP]$ versus $1/\text{specific activity}$ at several concentrations of mevalonate is, as was shown for the hog liver enzyme (Beytia et al. 1970), consistent with a sequential catalytic mechanism.

E. faecalis mevalonate kinase shares with both the hog enzyme (Beytia et al. 1970) and the yeast enzyme (Tchen 1958) a requirement for a divalent cation. Although it seems likely that the essential cation in vivo probably is Mg^{2+} , preliminary investigations suggest that equal concentrations of Mn^{2+} , Zn^{2+} , or Ca^{2+} could each serve this function in vitro. Similar considerations apply to the phosphoryl donor. Although ATP, GTP, CTP, UTP, TTP, and ITP each served as the phosphoryl donor in vitro with similar K_m and V_{max} values, we consider ATP the most likely in vivo donor of the phosphoryl group. Broad specificity for the phosphoryl donor also characterizes yeast mevalonate kinase (Tchen 1958). By contrast, pig liver mevalonate kinase uses only ATP or ITP (Beytia et al. 1970). No data are available for

the nucleotide specificity of the sole archaeal mevalonate kinase, possibly because the coupled spectrophotometric assay used to study this enzyme, which detects formation of ADP, does not lend itself to the investigation of phosphoryl donor specificity or of inhibition by ADP. ADP inhibits *E. faecalis* mevalonate kinase with a K_i of 2.7 mM, a value close to the 1.1 mM K_m for ATP. By contrast, although similar in structure to ATP and ADP, AMP failed to inhibit, implying a requirement for a di- or tri-phosphate moiety for binding to the enzyme. Other nucleoside monophosphates were not investigated as potential inhibitors. These observations suggest that *E. faecalis* mevalonate kinase might be feedback-inhibited by ADP and that carbon flux through this enzyme might respond in vivo to ATP/ADP ratios.

Because a single monomer contains a complete active site, catalysis would not seem to require dimerization (Fu et al. 2002; Yang et al. 2002). The multimeric state of mevalonate kinase can, however, vary between monomer and dimer. Based on gel filtration data, rat and pig liver mevalonate kinases are homodimers (Beytia et al. 1970; Tanaka et al. 1990). The crystal structure of the rat holoenzyme revealed a dimer with a dimerization domain of two α -helices, one from each monomer. This domain comprises about 7.6% of the protein surface (Fu et al. 2002). By contrast, *M. jannaschii* kinase formed dimers in solution (Huang et al. 1999), but monomers in crystals (Yang et al. 2002). Our investigation of the mevalonate kinase of *E. faecalis* revealed that it exists primarily as a monomer in solution, with some dimer apparent (Fig. 3). Differences between the apparent multimeric state of the *E. faecalis* kinase and other characterized mevalonate kinases may reflect factors such as protein concentration, the experimental method employed, and the lack of primary structure conservation between the region required for dimerization of the rat kinase and the corresponding sequence of the *E. faecalis* enzyme. Differences in multimeric states between bacterial and eukaryotic enzymes are also apparent in phosphomevalonate kinase, a member of a superfamily that includes mevalonate kinase. Although all known eukaryotic phosphomevalonate kinases exist as dimers, the *S. pneumoniae* enzyme is a monomer both in solution and in the crystalline states (Romanowski et al. 2002).

Human	10	A P G K V I L H G E H A V V H G K V A	193	E R M I H G N P S G V D
Rat	10	A P G K V I L H G E H A V V H G K V A	193	E R V I H G N P S G V D
<i>E. faecalis</i>	9	T P S K I I L M G E H A V V Y G E P A	137	E K I A H G N P S G I D
<i>M. jannaschii</i>	4	A T G K V I L F G E H A V V Y G Y R A	243	E K E I Q G K A S I T D
		*		*

Figure 8. Selected sequences of characterized mevalonate kinases. Amino acid sequences from mevalonate kinases were aligned using CLUSTALW (www.expasy.ch). Conserved residues are in bold type. Asterisks indicate the active site lysine, aspartate, and glutamate. The active site lysine and glutamate are thought to stabilize $MgATP$, and the aspartate to serve as the base that deprotonates the C-5 hydroxyl of mevalonate, generating the nucleophile that attacks the γ -phosphate of ATP (Potter and Mizioro 1997; Potter et al. 1997).

Materials and methods

Reagents

Purchased materials included Vent DNA polymerase (New England Biolabs), isopropyl- β -D-thiogalactopyranoside (IPTG), T4 DNA ligase and restriction enzymes (New England Biolabs), shrimp alkaline phosphatase (Promega), 55 mCi/mmol 2-[14 C]-(*R,S*)-mevalonate (Amersham), prestained low-range protein standards and Bradford reagent (Bio-Rad). NiNTA agarose (Qiagen) was used for Ni-affinity chromatography. Plasmid DNA preparations employed a QIAprep Spin Miniprep Kit (Qiagen) and agarose gel extractions a QIAGEN Gel Extraction Kit. Synthetic oligonucleotides were prepared by IDT, Inc. of Coralville, Iowa. Unless otherwise specified, all other reagents were from Sigma.

Plasmids, bacterial strains, and culture media

Expression vector pET28b(+) was from Novagen. Bacterial strains used included *E. coli* strains DH5 α and BL21(DE3) (Invitrogen). Genomic DNA from *E. faecalis* strain 41 (Glaxo SmithKline culture collection) was used for amplification of the *mvaK1* open reading frame (PubMed locus number NP_814642) thought to encode mevalonate kinase. Luria-Bertani (LB) medium and agar (Sambrook et al. 1989) supplemented with 50 μ g of kanamycin per milliliter served for the growth of *E. coli* strains.

Construction of the expression plasmid

The *mvaK1* gene that encodes a 314-residue polypeptide was PCR-amplified from *E. faecalis* genomic DNA using a forward primer (5'-CGT TCT TCA TAT GCA AGA AGG ACT TTT GTC C-3') and a reverse primer (5'-GTA TGC GCT CAA GCT TTT CCT GAA AGC -3') to introduce HindIII and BamHI restriction sites (underlined). The resulting 0.9-kb fragment was gel purified and ligated into Hind III and BamHI-digested pET28b(+) that had been dephosphorylated and gel purified (Sambrook et al. 1989). The resultant plasmid was termed EFK1-pET28b. Plasmid DNA was isolated from transformed *E. coli* DH5 α cells and the insert was sequenced by the Iowa State University DNA Sequencing Facility to confirm the presence of unaltered *mvaK1*.

Expression and purification of the gene product

E. coli BL21(DE3) cells transformed with EFK1-pET28b were grown initially in 10 mL LB_{kan} at 37°C for 15 h. The cultures were transferred to 100 mL LB_{kan}, grown for an additional 6 h at 37°C, and transferred to 1 L of LB_{kan}. Following addition of 0.5 mM IPTG at a cell density of 90 Klett units, growth was continued at 16°C for 48 h until the cells reached a density of about 300 Klett units. Cells were harvested by centrifugation, washed with 0.9% saline, and suspended in 500 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 10 (Buffer A). Lysis in a French pressure cell gave the cell lysate. The supernatant liquid obtained by centrifugation of the cell lysate (30,000 rpm, 60 min, 4°C) was applied to a NiNTA column. The column was washed with Buffer A, then eluted successively with 50 and 100 mM imidazole in Buffer A. Fractions with high activity were combined and stored at -70°C. Protein concentrations were determined by the method of Bradford (1976).

Assay of mevalonate kinase activity

Standard assay conditions for phosphorylation of mevalonate were modified from Popjak (1969). Briefly, the assays included 5 mM ATP, 1.0 mM (*R,S*)-mevalonate, 2.5 μ Ci 2-[14 C]-(*R,S*)-mevalonate, 1.0 mM dithiothreitol (DTT), 10 mM MgCl₂, 210 mM KCl, and 70 mM CAPS (pH 10), in a final volume of 130 μ L. Reactions were initiated by the addition of 2-[14 C]-(*R,S*)-mevalonate in 1 mM nonlabeled (*R,S*)-mevalonate. After a 10-min incubation at 37°C, the reaction was stopped by heating for 10 min at 94°C, then centrifuged (13,000 rpm, 15 min). Portions of the supernatant liquid were then applied either to a Kodak cellulose TLC sheet or to a Whatman 3 mm sheet and chromatographed in *n*-butanol:water:formic acid (77:13:10) in a small volume TLC chamber. One-centimeter sections were then removed and counted in 4 mL EcoLume scintillation liquid (ICN). Mevalonate phosphate migrated to an *R_f* of 0.2 to 0.3, and mevalonolactone to an *R_f* of 0.9–1.0. One enzyme unit (eu) represents the turnover, in 1 min, of 1 μ mole of mevalonate. Reported results represent mean values for at least duplicate determinations.

Sedimentation velocity analytical ultracentrifugation

E. faecalis mevalonate kinase, 1.0 mg/mL or 0.4 mg/mL in 210 mM KCl, 70 mM CAPS (pH 10), was added to a Beckman analytical ultracentrifuge cell with sapphire windows and a charcoal-filled epoxy centerpiece. The cell was placed in a Beckman Model XL-1 centrifuge and allowed to come to thermal equilibrium at 20°C for 1 h. The sample was then spun at 50,000 rpm for 6 h. Rayleigh interference scans were taken at 3-min intervals.

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