Biochemical and genetic aspects of mevalonate kinase and its deficiency
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Chapter 4

Identification and characterization of three novel missense mutations in mevalonate kinase cDNA causing mevalonic aciduria, a disorder of isoprene biosynthesis


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Three novel missense mutations in MVK causing mevalonic aciduria

Mevalonic aciduria is a rare autosomal recessive metabolic disorder, characterized by psychomotor retardation, failure to thrive, hepatosplenomegaly, anemia and recurrent febrile crises. The disorder is caused by a deficient activity of mevalonate kinase due to mutations in the encoding gene. Thus far, only two disease-causing mutations have been identified. We now report four different missense mutations including three novel ones, which were identified by sequence analysis of mevalonate kinase cDNA from three mevalonic aciduria patients. All mutations affect conserved amino acids. Heterologous expression of the corresponding mutant mevalonate kinases as fusion proteins with glutathione S-transferase in Escherichia coli cells showed a profound effect of each of the mutations on enzyme activity. In addition, immunoblot analysis of fibroblast lysates from patients using specific antibodies against mevalonate kinase identified virtually no protein. These results demonstrate that the mutations not only affect the activity but also the stability of the mutant proteins.

Introduction
The isoprene pathway supplies cells with intermediates for the biosynthesis of a variety of compounds with important functions in cellular processes. These compounds include ubiquinone-10 and heme A, both necessary for electron transport, dolichol, essential for protein glycosylation, isopentenyl tRNA, involved in protein translation, and farnesyland geranylgeranyl groups used for the isoprenylation of proteins which function in cell signaling and cell differentiation. In addition to these non-sterol isoprenoids, the pathway produces cholesterol, a structural component of membranes and precursor for bile acids and steroid hormones.

At present, three different inherited disorders have been recognized, which are caused by defects in this pathway. Two of these disorders, Smith-Lemli-Opitz (SLO) syndrome and desmosterolosis, only affect the synthesis of cholesterol and its derivatives and are caused by a deficient activity of 7-dehydrocholesterol reductase and desmosterol reductase, respectively [1]. The biochemical defect in SLO syndrome was confirmed recently by the identification of mutations in the gene coding for 7-dehydrocholesterol reductase [2-4], but the gene mutated in desmosterolosis patients remains to be identified.

The third disorder of the pathway is mevalonic aciduria, which is caused by a deficient activity of mevalonate kinase (MK). MK is the first enzyme to follow 3-hydroxy-3-methylglutaryl-CoA reductase in the isoprene pathway and converts mevalonate into 5-phosphomevalonate. As a consequence of the MK deficiency, high levels of mevalonic acid are present in plasma and urine from mevalonic aciduria patients [5, 6].

So far, ~15 patients with mevalonic aciduria have been reported. Although most patients suffered from an often fatal, multi-systemic disease, their clinical presentation varied considerably. Severely affected patients had profound developmental delay, dysmorphic features, cataracts, hepatosplenomegaly, lymphadenopathy, anemia, diarrhea and malabsorption, and died early in infancy, while milder affected patients only showed psychomotor retardation, hypotonia, myopathy and ataxia. All patients were characterized by recurrent febrile attacks associated with lymphadenopathy, an increase in the size of the liver and spleen, arthralgia, edema and morbilliform rash [6]. Since the enzyme reaction catalyzed by MK occurs in the first, unbranched part of the pathway, an effect on the synthesis of both sterol and non-sterol isoprenoids is predicted. Accordingly, the ubiquinone-10 concentration
in plasma from some mevalonic aciduria patients appeared to be decreased [7]. The plasma cholesterol level, however, was usually normal or, at most, slightly decreased [6].

Since the cloning of human MK cDNA [8], only two disease-causing missense mutations have been reported. The resulting two amino acid changes, N301T [8] and A334T [9], have a profound effect on enzyme activity as shown by heterologous expression of the mutant proteins in COS-7 cells and in Escherichia coli. Here we report four different missense mutations causing mevalonic aciduria, three of which are novel. These were identified after sequence analysis of MK cDNAs from three patients clinically diagnosed with mevalonic aciduria. Subsequent studies demonstrated that all four mutations affect both enzyme activity and protein stability of the resulting mutant MKs.

Results

Biochemical characterization of three mevalonic aciduria patients

All three patients used for this study were diagnosed with mevalonic aciduria based on clinical signs (Table 1) and on the basis of increased concentrations of mevalonic acid in plasma and urine. Judged from their clinical presentation and the fact that they died at an early age, patients 1 and 3 were severely affected. This is also reflected by the almost complete absence of MK activity in fibroblasts of both patients (Table 2). Since patient 2 showed a milder clinical presentation and is currently 20 years old, he was predicted to have some residual MK activity. Measurements in fibroblasts of this patient, however, also revealed virtually no MK activity (Table 2).

<table>
<thead>
<tr>
<th>Clinical findings</th>
<th>Patient 1 (F)</th>
<th>Patient 2 (M)</th>
<th>Patient 3 (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychomotor retardation</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Recurrent febrile crises</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Anemia</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypotonia and myopathy</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ataxia and cerebellar atrophy</td>
<td>-</td>
<td>++/+++</td>
<td>-</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Dysmorphic features</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cataracts</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Outcome*</td>
<td>4.5 months†</td>
<td>20 years</td>
<td>5 months†</td>
</tr>
</tbody>
</table>

*Present age or † age at death.
+++ , severe; ++ , moderate; + , present; -- , absent.

Mutation analysis of MK cDNA

To identify the mutations that cause the mevalonic aciduria, MK mRNAs of the three patients were amplified by RT-PCR in two overlapping fragments. Sequence analysis of the amplified cDNA fragments showed that patient 1 was homozygous for a T>C transition at nucleotide 803, which changes the isoleucine at position 268 into a threonine (I268T). Patient 2 was compound heterozygous for an A>C transversion at nucleotide 59 and a G>A transition at nucleotide 1000, which change the histidine at position 20 into a proline (H20P) and the alanine at position 334 into a threonine (A334T), respectively. Compound heterozygosity was
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confirmed by separate cloning of the two mutant cDNAs. Patient 3 was homozygous for a G>A transition at nucleotide 928, which changes the valine at position 310 into a methionine (V310M).

Table 2. MK and glutamate dehydrogenase (GDH) activities in cultured skin fibroblast lysates from the three mevalonic aciduria patients and a control subject.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Amino acid change(s)</th>
<th>(MK)a</th>
<th>(GDH)b</th>
<th>Ratio x 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>171</td>
<td>350</td>
<td>0.489</td>
</tr>
<tr>
<td>Patient 1</td>
<td>I268T</td>
<td>0.3</td>
<td>149</td>
<td>0.002</td>
</tr>
<tr>
<td>Patient 2</td>
<td>H20P, A334T</td>
<td>0.5</td>
<td>174</td>
<td>0.003</td>
</tr>
<tr>
<td>Patient 3</td>
<td>V310M</td>
<td>0.4</td>
<td>203</td>
<td>0.002</td>
</tr>
</tbody>
</table>

a Expressed as pmol/min/mg protein.
b Expressed as nmol/min/mg protein.

Alignment of various eukaryotic (Fig. 1A) and prokaryotic (not shown) MK protein sequences revealed that all mutations affect conserved amino acids. The valine at position 310 and the alanine at position 334 are conserved in nearly all MK sequences, whereas the isoleucine at position 268 is conserved in mammalian MKs. The histidine at position 20 is part of a spatially conserved motif, which is present in all MK sequences but also in several galactokinases [8] (Fig. 1B). The alignment in Figure 1A also includes the amino acid sequence of mouse MK, the corresponding cDNA of which was identified and cloned after screening of the expressed sequence tag (EST) database with the human MK sequence.

Heterologous expression of mutant MKs
To examine whether the mutations affect the enzyme activity, we expressed the mutant MK proteins in E. coli. Since initial attempts to express full-length 6xHis-tagged wild-type MK protein in E. coli did not yield an active enzyme (not shown), we expressed MK as a fusion protein with glutathione S-transferase (GST). Previously, it was shown that this approach hardly influences the kinetic properties of MK when compared with the purified liver enzyme [9]. Apparently, the fusion of MK with GST stabilizes the enzyme and therefore provides a useful method to test the effect of mutations on the function of the enzyme. An additional advantage of using GST as a fusion partner for MK is that the MK activity can be normalized with the activity of GST to correct for differences in expression, which allows direct comparison of the effects of the various mutations on the enzyme activity of MK. Measurements of the enzyme activity of wild-type and mutant MK-GST proteins in total E. coli lysates revealed a profound effect of all four mutations on MK activity (Fig. 2). No activity could be detected for the H20P mutant allele. The A334T mutation resulted in a very low residual activity, which confirms the earlier observation of its effect on the affinity of the corresponding MK for its substrate mevalonate [9]. Both the I268T and V310M mutations resulted in mutant MKs with strongly reduced enzyme activities. To exclude that the reduced enzyme activities were the result of an inability to produce the various mutant MK-GST fusion proteins in E. coli, we analyzed their expression by immunoblotting. All mutant MKs were found to be expressed in comparable amounts (data not shown).
**Figure 1.** Amino acid sequence conservation of the mutated amino acids. Conserved amino acids are boxed and arrowheads indicate the amino acids mutated in the patients. A. Alignment of the amino acid sequences of human (Hs), rat (Rn) and mouse (Mm) MK (GenBank accession nos M88468, P17256 and AF137598, respectively). B. Alignment of the N-termini of MK and galactokinase (GAL) amino acid sequences of human, rat, mouse, Arabidopsis thaliana (At), Saccharomyces cerevisiae (Sc), Schizosaccharomyces pombe (Sp), Methanobacterium thermoautotrophicum (Mt), Methanococcus jannaschii (Mj), Archaeoglobus fulgidus (Af) and E. coli (Ec).

**Immunoblot analysis of MK in fibroblasts from patients**

To determine whether the identified mutations also affect the stability of the mutant MK proteins, skin fibroblast lysates of all three patients were subjected to immunoblot analysis using affinity-purified antibodies directed against human MK (Fig. 3). In the lysates of patient 1 and 3 no MK protein could be detected, indicating that the I268T and V310M mutations found in these patients strongly affect the stability of the protein. Although hardly visible on the photograph, very low levels of MK protein could be detected repeatedly in lysates of patient 2. Since it has been shown previously that a patient who was homozygous for the A334T mutation had near normal protein levels [9], the strongly reduced levels of MK protein in lysates of this patient suggest that the H20P mutation also affects the stability of the enzyme.
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**Figure 2.** Relative enzyme activities of wild-type and mutant MK-GST fusion proteins expressed in *E. coli*. MK and GST were both assayed in *E. coli* lysates and the MK/GST ratio was calculated. The ratio for the wild-type allele (0.44±0.07) is used as the 100% value. The error bars indicate 1 SD.

**Figure 3.** Immunoblot analysis of MK in fibroblast lysates. Equal amounts of protein (100 μg) of mevalonic aciduria patients 1, 2 and 3, four control subjects and, as a reference, 40 μg of human liver protein from a control subject were separated by 10% SDS-PAGE and analyzed on immunoblot with MK-specific antibodies. The sizes of the immunoreactive protein species are equal to the predicted molecular weight of 42 kDa for MK.

**Discussion**

In this paper we report four different mutations in MK cDNA causing mevalonic aciduria. Although patient 2 was already reported to be heterozygous for the A334T mutation, the second allele remained obscure [9]. We now discovered that this patient is in fact a compound heterozygote for the A334T mutation and a novel missense mutation, H20P. The two other patients appeared to be homozygous at the cDNA level for two novel missense mutations, I268T and V310M. Whether these patients are homozygous for these mutations at the genome level awaits the elucidation of the chromosomal structure of the MK gene. Since the parents of patient 3 are related, it is expected that this patient is homozygous.
The enzyme activity measurements revealed almost no MK activity in fibroblasts from all three patients and thus no apparent correlation between residual enzyme activity and the severity of the disease. The identified mutations in the two severely affected patients 1 and 3, I268T and V310M, respectively, resulted in strongly reduced activity upon expression in E. coli, although some residual activity could still be measured. In immunoblots of fibroblast lysates of these patients, however, no MK protein was identified, indicating that these mutations also, or maybe primarily, affect the stability of the protein.

The A334T and H20P mutations identified in the relatively mildly affected patient 2 result in a strongly reduced and a complete absence of enzyme activity, respectively, when expressed in E. coli. In addition, the levels of MK protein in fibroblast lysates from this patient were strongly reduced as revealed by immunoblotting. Since it was shown previously that the A334T mutation results in a stable protein with a 30-fold elevated $K_m$ for the substrate mevalonate [9], these results suggest that the H20P allele also codes for an unstable protein. The relatively mild phenotype of patients with the A334T allele might be explained by the fact that the encoded mutant protein still has substantial residual activity when plasma mevalonate levels are elevated [9].

The H20P mutation occurs in a spatially conserved region found in all MKs and galactokinases (Fig. 1). Two putative functions for this region have been proposed [10]. One function is that this region is involved in targeting of MK to peroxisomes. The histidine at position 20 is part of an N-terminal amino acid stretch that shows a perfect match with the consensus sequence of peroxisomal targeting signal type 2 (PTS2), which is involved in targeting of a certain class of peroxisomal matrix proteins [11]. Several observations are in favor of a function for this domain in peroxisomal targeting. For instance, it has been reported that MK is localized in rat liver peroxisomes [12, 13]. Furthermore, MK deficiency has been reported in patients diagnosed with the peroxisomal biogenesis disorders Zellweger syndrome and rhizomelic chondrodysplasia punctata type 1 [12, 14, 15]. In Zellweger syndrome, there is a generalized loss of peroxisomal functions, while in rhizomelic chondrodysplasia punctata type 1, there is only a partial loss of peroxisomal functions due to an impaired import of proteins with a PTS2 [16-18]. However, although the H20P mutation disrupts the PTS2 consensus sequence, the actual role of this sequence in peroxisomal targeting of MK still remains to be demonstrated.

The second function of this conserved N-terminal region may be in modulating the activity and stability of MK. It has been reported that amino acid changes introduced in the N-terminus of MK greatly affect the enzyme activity. For instance, when the lysine at position 13 is changed into a methionine, a strong effect on the $V_{\text{max}}$ and affinity of the enzyme for ATP is observed [10]. In addition, changing the glutamic acid at position 19 into an alanine or a glutamine, or the histidine at position 20 into an alanine, resulted in unstable and completely insoluble proteins upon expression in E. coli, suggesting structural importance for these amino acids [19]. This is in accordance with our expression studies in E. coli, which showed that the H20P mutation resulted in an inactive enzyme. Furthermore, the strongly reduced protein levels in an immunoblot of fibroblasts of patient 2 suggest instability in vivo of the MK protein encoded by this allele. Although these data suggest that the N-terminal region is important for enzyme activity and/or stability, they do not necessarily exclude a second function in peroxisomal targeting.
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Materials and Methods

Mevalonic aciduria patients
Patient 1 [20] was a girl born to unrelated parents who presented with minor dysmorphic features (micrognathia), hepatosplenomegaly, gastrointestinal problems and failure to thrive. Hematological parameters were suggestive of a congenital infection, but this was excluded. The girl had a massive mevalonic aciduria, which showed a steady increase during her life. She died suddenly in a state of shock at the age of 4.5 months.

Patient 2 is a boy born to unrelated Dutch parents. At the age of 6 years, he was clinically evaluated for his cerebellar ataxia, hypotonia and strongly elevated creatine kinase activity. NMR and computed tomography of the brain revealed cerebellar hypoplasia together with an enlarged IVth ventricle. Following the detection of mevalonic acid in the urine [21], low concentrations of bile acids in the blood and an almost complete absence of the fat-soluble vitamins A, E and D were noticed. Serum concentration of cholesterol was normal [22]. From his 7th year, the patient has been on an oral therapy providing him with bile acid (Chenofalk 2x100 mg daily) and vitamin A, E and D. At present, the patient is 20 years of age and in a good clinical and mental condition (height p90 and weight p20).

Patient 3 was a girl born to first cousin-related parents of Pakistani origin. She presented with recurrent fever, joint pains and swelling, hepatosplenomegaly and a moderate failure to thrive. She died during one of the febrile episodes having made little developmental progress.

Table 1 summarizes the clinical findings in these patients.

Mutation analysis
First strand cDNA was synthesized from RNA isolated from cultured human skin fibroblasts as described elsewhere [23]. The first strand cDNA was used as template in a PCR with two different sets of primers tagged with either a −21M13 or an M13rev extension and designed to amplify the MK cDNA in two overlapping fragments. The first fragment was amplified using primer set MK−21−3 (5'-tgg aac gcg gct gat GCG GCA GGA TTC CCA GGA G-3') and MK622−403 (5'-cag gaa aca gct atg acc TGA CAG CAT TGT CCA CTC CG-3'). The second fragment was amplified using primer set MK521−540 (5'-tgg aac gcg gct gat TCA ACA GGT GGA CCA AAG AG-3') and MK21238−1219 (5'-cag gaa aca gct atg acc ATC CAG AAA GGG GCA TCT GG-3'). The PCR fragments were sequenced in both directions by means of −21M13 and M13rev fluorescent primers, on an applied biosystems 377A automated DNA sequencer, according to the manufacturer's protocol (Perkin-Elmer, Foster City, CA).

Cloning of wild-type and mutant MK cDNAs
The open reading frame (ORF) of the wild-type MK cDNA was amplified by PCR using primer set MK10−29 (5'-cg ata gga tcc GAA GTC CTA GTG GTG TCT GC-3') and MK1195−1177 (5'-cga tag tga CCT CTC AGA GGC CAT CCA G-3'). The primers introduce a 5' BamHI site and a 3' KpnI site (underlined). The PCR product was ligated into the pGEM-T vector (Promega Corp., Madison, WI) and sequenced to exclude PCR-introduced mutations. The wild-type ORF subsequently was released as a BamHI-NotI fragment from pGEM-T and ligated in-frame with the GST ORF into the BamHI and NotI sites of pGEX-4T-1 (Pharmacia Biotech, Uppsala, Sweden). cDNAs containing the mutations H20P, V310M and I268T were cloned from patients into pGEM-T as described for the wild-type MK cDNA. The H20P mutation was introduced into the pGEX-4T-1 plasmid by replacing the BamHI-HindIII

Table 1 summarizes the clinical findings in these patients.
fragment of the wild-type ORF. To introduce the I268T mutation, a Smal-Sacl fragment of the wild-type ORF in pGEX-4T-1 was replaced. V310M was introduced in the wild-type ORF in pGEX-4T-1 by replacing a Saci-KpnI fragment.

Cloning of the mouse MK cDNA
In order to identify the mouse MK cDNA, the EST database of the National Center for Biotechnology Information (NCBI) was screened for mouse sequences homologous to human MK. Numerous homologous mouse EST sequences were identified which together spanned the entire putative ORF. The ORF was subsequently amplified by PCR using primer set mmMK-20-1 (5'-GGG CAG AAG TCT CAG AAG CC-3') and mmMK1225-1209 (5'-cg ata gaa ttG TGG TGT GTC GGG TGG T-3') and primer set mmMK4-24 (5'-cg ata gaa tcc TTG TCA GAA GCT CTG CTG GTG-3') and mmMK1225-1209. Both PCR products were ligated into pGEM-T and sequenced. The second primer set introduced a 5' BamHI and a 3' EcoRI site, which were used for subcloning of the ORF into the pGEX-4T-1 vector to confirm its identity by means of expression as a GST fusion protein in E. coli.

Enzyme assays
MK was measured radiochemically making use of 14C-labeled mevalonate [24]. Glutamate dehydrogenase (GDH) activity served as a control. GST activity was assayed spectrophotometrically at 335 nm by measuring the formation of the conjugate of glutathione and 1-chloro-2,4-dinitrobenzene [25].

MK expression in E. coli
pGEX-4T-1 plasmids containing the various MK ORFs were transformed into CaCl2 competent DH5α or commercially available Invα (Invitrogen, Carlsbad, CA) E. coli cells. Cells were grown from a 100-fold diluted fresh overnight culture for 4 hours in LB medium, induced with 2 mM isopropyl-β-thiogalactopyranoside (Promega) and subsequently grown for an additional 2 hours. Cells were lysed by sonication (twice for 15 s at 8 W output, with 1 min of cooling between the pulse periods). Lysates were adjusted to equal levels of GST activity before assaying MK activity.

Immunoblot analysis
A synthetic peptide corresponding to amino acid residues 76 to 90 of human MK (H2N-EQGDVTPTSEQVEK-COOH) was conjugated with keyhole limpet hemocyanin and used to produce antibodies in rabbits (Eurogentech, Seraing, Belgium). The crude antiserum was affinity-purified on a column containing MK-GST fusion protein coupled to cyanogen bromide-Sepharose as described [26]. The MK-GST fusion protein was affinity purified on glutathione-agarose columns according to the protocol of the supplier (Pharmacia Biotech). In immunoblots, the affinity-purified antibodies were used at a 1:100 dilution.

For immunoblot analysis, fibroblast cultures were harvested and lysed by sonication in 20 mM MOPS (pH 7.4) and 0.25% Triton X-100, supplemented with 25 μg/ml phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Equal amounts of total protein were separated by SDS-PAGE and transferred onto nitrocellulose by semi-dry immunoblotting [27]. Antigen-antibody complexes were visualized using anti-rabbit IgG-alkaline phosphatase conjugate (BioRad, Hercules, CA).
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References
