Chapter 2

Biochemical and genetic aspects of mevalonate kinase and its deficiency

Parts of this chapter have been published as a review in:
Mevalonate kinase (MK) is an essential enzyme in the mevalonate pathway, which produces numerous cellular isoprenoids. The enzyme has been characterized both at the biochemical and the molecular level in a variety of organisms. Despite the fact that mevalonate kinase is not the rate-limiting enzyme in isoprenoid biosynthesis, its activity is subject to feedback regulation by the branch-point intermediates geranyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate. Recently, the importance of mevalonate kinase was demonstrated by the identification of its deficiency as the biochemical and molecular cause of the inherited human disorders mevalonic aciduria and hyperimmunoglobulinaemia D and periodic fever syndrome. Although some progress has been made since the discovery of the genetic defect, the pathophysiology of these disorders is not yet understood, but eventually will give insight into the in vivo role of mevalonate kinase and isoprenoid biosynthesis in inflammation and fever. The subcellular localization of mevalonate kinase is still a matter of debate. The enzyme may be localized in the cytosol, or in peroxisomes, or in both cellular compartments. Here, we review the biochemical and molecular properties of MK, and discuss its biological significance, the regulation of its enzyme activity and finally its subcellular localization.

1. Introduction

Isoprenoids make up a large group of essential compounds involved in diverse cellular processes and include among others; 1) the side-chain of ubiquinone-10 and heme A, both necessary for mitochondrial electron transport in the respiratory chain; 2) isopentenyl tRNA, involved in protein translation; 3) dolichol, essential for N-linked protein glycosylation; and 4) farnesyl and geranylgeranyl groups used for prenylation of proteins involved in cell proliferation and differentiation. In addition to these non-sterol isoprenoid compounds, cells produce sterols such as cholesterol. Cholesterol is a structural component of cellular membranes, a precursor of bile acids and steroid hormones and required for embryonic development. Cellular isoprenoids can be synthesized by two different pathways, the glyceraldehyde 3-phosphate (GAP)-pyruvate pathway (nonmevalonate pathway) [1, 2] and the mevalonate pathway [3]. Metazoan organisms only use the mevalonate pathway.

The mevalonate pathway for the biosynthesis of isoprenoids starts with three acetyl-CoAs, which are converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in two enzyme steps. HMG-CoA is the substrate of the rate-limiting enzyme of the pathway, the highly regulated HMG-CoA reductase (HMGR, EC 1.1.1.34)[3]. The first enzyme to follow HMGR is mevalonate kinase (MK, ATP:(R)-mevalonate 5-phosphotransferase, EC 2.7.1.36), which catalyses the phosphorylation of mevalonate into 5-phosphomevalonate. The first indication for the involvement of MK in isoprenoid biosynthesis dates back to 1956 when it was reported that mevalonate, earlier identified as a growth factor for a Lactobaccillus strain [4], is a key intermediate in the biosynthesis of cholesterol [5]. Subsequently, it was shown that only one of the isomers (R-enantiomer) was biologically active [5] and that the conversion of mevalonate to squalene required adenosine triphosphate (ATP), Mg\(^{2+}\) or Mn\(^{2+}\) and reduced pyridine nucleotides [6, 7]. The ATP requirement suggested the formation of one or more phosphorylated intermediates from mevalonate, which was confirmed later when 5-phosphomevalonate was identified as the first product of the phosphorylation catalyzed by the enzyme accordingly called MK [8]. MK activity was characterized subsequently in bacteria, fungi, plants and mammals [9, 10]. Genes encoding MK (MVK) have been identified.
in not only several plants and animals, but also archaebacteria and eubacteria. Recently, the importance of MK in human metabolism was demonstrated by the identification of its deficiency as the biochemical and molecular cause of two inherited disorders, mevalonic aciduria (MA, MIM 251170) [11] and hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS, MIM 260920) [12, 13]. Here we review the biochemical and molecular aspects of MK, and discuss its biologic significance, the regulation of its enzyme activity, and its subcellular localization.

2. Biochemical characterization

2.1. Purification of MK

In 1958, the first partial purification of MK was reported from yeast autolysates [14], soon followed by the partial purification from pig liver [15]. Purification to homogeneity was reported later from pig and rat liver [16, 17]. The kinetic and binding properties determined from the purified enzymes are displayed in Table 1.

<table>
<thead>
<tr>
<th>Source of MK</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$ (RS)</th>
<th>$K_m$ ATP</th>
<th>$K_i$ GPP</th>
<th>$K_i$ FPP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol/min/mg protein</td>
<td>$\mu$M</td>
<td>$\mu$M</td>
<td>$\mu$M</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Pig liver [16]</td>
<td>17.5</td>
<td>18.6</td>
<td>302</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Rat liver [17]</td>
<td>29.4</td>
<td>271</td>
<td>1750</td>
<td>ND</td>
<td>2.5</td>
</tr>
<tr>
<td>Recombinant M. jannaschii [35]</td>
<td>387</td>
<td>68.5</td>
<td>92</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Recombinant human [22]</td>
<td>13.6</td>
<td>150</td>
<td>440</td>
<td>0.116</td>
<td>0.104</td>
</tr>
<tr>
<td>Recombinant human [21]</td>
<td>37</td>
<td>24</td>
<td>74</td>
<td>ND</td>
<td>0.01</td>
</tr>
<tr>
<td>Recombinant rat [41]</td>
<td>30</td>
<td>288</td>
<td>1240</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

The biochemical characterization of the (partially) purified enzymes allowed a detailed comparative study of their properties. In general, all purified enzymes are stable, although rat liver enzyme was reported to lose irreversibly all enzyme activity after freezing [17]. Furthermore, the pig and rat liver enzymes, unlike the yeast enzyme, are highly sensitive to acid and undergo irreversible inactivation under acidic conditions [14-17]. Both the pig and yeast enzyme are sensitive to compounds that react with sulphhydryl groups (monooiodoacetamide, p-chloromercuribenzoate and p-hydroxymercuribenzoate), but the pig liver enzyme is inactivated also readily by oxidation and requires sulphhydryl compounds for activation. The inactivation was reversed completely by addition of reduced glutathione, $\beta$-mercaptoethanol or dithiothreitol [14-16]. Furthermore, addition of either mevalonate or ATP prior to the incubation with sulphhydryl-specific reagents reversed the inactivation [16]. These data suggest that a sulphhydryl group in the active site is essential for MK activity.

In contrast to the lack of nucleotide specificity of the yeast enzyme, which nevertheless preferentially uses ATP [14], pig liver enzyme can only handle ATP or inosine triphosphate (ITP), but not GTP, CTP and UTP [15]. Furthermore, all characterized MKs need a divalent cation for activity, which together with ATP is the actual substrate of the enzyme. At low concentrations, $\text{Mn}^{2+}$ is by far the most active cation, but $\text{Mg}^{2+}$ becomes increasingly active at higher concentrations [14-16].
Purification to homogeneity of MK from pig and rat liver enabled the determination of its molecular weight [16, 17]. SDS-polyacrylamide gel electrophoresis of rat liver MK revealed a 40 kDa protein. Size determination by gel chromatography yielded a molecular weight of 86 kDa, consistent with a homodimeric structure [17]. The subunit size of pig liver MK was 52 kDa [18], which is in agreement with the reported native sizes of 98 kDa [16] and 104 kDa [18] as determined by gel filtration.

2.2. Inhibition of MK activity by isoprenoid intermediates

Dorsey and Porter [19] first noticed that the activity of pig liver MK was subject to feedback inhibition by low concentrations of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP). A similar inhibition was also reported for MK from rat and plant [17, 20]. The inhibition was competitive with respect to MgATP but uncompetitive with respect to mevalonate [19]. The $K_i$ value for the inhibition of rat liver MK activity was similar to that of the pig liver enzyme (table 1) [17, 19]. The inhibition by GPP and FPP was also determined using recombinant human MK (table 1) [21, 22], which revealed considerably lower $K_i$ values. In addition, Hinson et al. [22] found that geranylgeranyl pyrophosphate (GGPP) had an even higher relative inhibitory capacity than GPP and FPP ($K_i$ 59 nM vs. 116 nM and 104 nM, respectively). Whether or not inhibition by isoprenoid intermediates is relevant in vivo remains to be studied. However, when these $K_i$ values are compared with the $K_m$ values of the branch-point prenyltransferases, which use GPP, FPP and GGPP as a substrate, they are generally lower [22]. Only the affinity of the protein prenyltransferases for the isoprenoid substrate is higher [23-25]. Also in vitro experiments with rabbit reticulocyte lysates indicate that polyisoprenyl pyrophosphates inhibit their synthesis from mevalonate [26]. Thus, in addition to the fact that HMG R is the rate-limiting enzyme of cholesterol biosynthesis, the regulation of MK activity may be of functional importance too in the biosynthesis of isoprenoids.

2.3. Mechanism of the enzymatic reaction

Initial velocity kinetics analysis using purified pig liver MK with MgATP as variable substrate and mevalonate as fixed substrate and vice versa revealed a sequential reaction mechanism, i.e. both substrates bind to the enzyme prior to the release of the first product [16]. These experiments were verified using isotope exchange reactions. An exchange reaction with one of the products occurred only when both substrates were present, which is consistent with the proposed sequential reaction mechanism [16]. The study of the inhibition pattern by GPP and FPP revealed that substrates are added in an obligatory order. As mentioned above, both GPP and FPP are competitive inhibitors of MK when MgATP is the variable substrate, while uncompetitive inhibition occurs when mevalonate is varied. Thus, GPP and FPP bind to the enzyme-mevalonate complex to give a catalytically inactive complex, which suggests that the addition of substrates is ordered with mevalonate added first as shown in figure 1 [16, 19]. Studies on the inhibition of the reaction by the products revealed that 5-phosphomevalonate is a noncompetitive inhibitor of MK when MgATP or mevalonate is the variable substrate and a nearly uncompetitive inhibitor at saturating levels of MgATP. When ADP was used as inhibitor, competitive inhibition was observed if mevalonate was the varied substrate. These experiments suggested that the release of products is also sequential with 5-phosphomevalonate released first (figure 1) [16].
Figure 1. The proposed sequential reaction mechanism for MK enzyme activity. Mevalonate (A) is added first to the free enzyme (E) and forms an enzyme-substrate complex (EA), followed by the addition of MgATP (B). After reaction of the substrates, 5-phosphomevalonate (P) is released as the first product and ADP (Q) as the second product. (Figure adapted from Porter [9]).

3 Identification of the MVK gene and characterization of the encoded product

3.1. Molecular cloning of MK

The purification of MK from rat liver enabled the generation of specific antibodies against MK, which were used for immunoscreening of a rat liver cDNA library. This approach yielded several cDNA clones encoding MK. The full-length rat cDNA contained an open reading frame of 1185 bp and coded for a 395-amino acid polypeptide with a calculated molecular weight of 41,990 kDa [27]. The amino acid sequence showed significant similarity with the *Saccharomyces cerevisiae* RAR1 protein (regulation of autonomous replication). RAR1 was identified by screening for mutations that increase the mitotic stability of plasmids whose replication is dependent on weak origins of DNA replication [28]. Indeed, the RAR1 gene was soon found to be identical to *ERG12*, the gene affected in the *S. cerevisiae* ergosterol auxotrophic *erg12* strain, which exhibited no MK activity [29-31]. In yeast, *ERG12* is essential for spore germination and vegetative growth [30]. The cDNA encoding rat MK was used to clone the human *MVK* cDNA by hybridization to a B-lymphocyte cDNA library [32]. Furthermore, the yeast *erg12* strain was complemented functionally by *Arabidopsis thaliana* MK, followed by the cloning of this plant cDNA [33].

A search of the expressed sequence tag (EST) and protein databases reveals putative MKs from a large variety of organisms including mammals (mouse [34], *Bos taurus*), plants (*Glycine max*, *Medicago truncatula*), roundworms (*Caenorhabditis elegans*), insects (*Drosophila melanogaster*), slime molds (*Dictyostelium discoideum*) and fungi (*Schizosaccharomyces pombe*), confirming the general importance of MK and isoprenoid biosynthesis via the mevalonate pathway. Also archaeabacterial genomes contain putative MK ORFs based on homology with other MK sequences (*Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, *Pyrococcus horikoshii*, *Pyrococcus abyssi*, *Aeropyrum pernix*). In addition to cellular isoprenoids, archaeabacteria also depend on biosynthesis of isoprenoids for lipids in their cytoplasmic cell membrane. The putative *M. jannaschii* MK has been overexpressed recently, followed by purification and characterization of the protein which, indeed, turned out to have MK activity (table 1)[35]. This recombinant archaeabacterial enzyme is thermostable, has an optimal temperature for
activity between 70 and 75 degrees Celsius and, like other MKs, functions as a homodimer [35].

Recently, it was shown that many eubacteria use the GAP-pyruvate pathway instead of the mevalonate pathway for isoprenoid biosynthesis. However, genomic analysis revealed significant exceptions including gram-positive cocci, Streptomyces sp., and Borrelia burgdorferi, which possess genes predicted to encode all of the enzymes of the mevalonate pathway (including MK) but not the GAP-pyruvate pathway [36, 37].

3.2. Characterization of conserved regions of MK
Comparison of MK amino acid sequences revealed four conserved regions [33]. Figure 2 shows an alignment of the human (396 aa, 42 kDa), rat (395 aa, 42 kDa), A. thaliana (378 aa, 41 kDa) and S. cerevisiae MKs (443 aa, 49 kDa). The four conserved domains present in these proteins and indicated in the figure by solid bars are also present in all other MK amino acid sequences (not shown). These conserved domains can also be found in a large class of small metabolite kinases, the GHMP (Galacto, Homoserine, Mevalonate, Phosphomevalonate) kinase superfamily. Another member of this protein family is MPD. At this moment three crystal structures of protein members of the GHMP kinase superfamily have been determined. These include M. jannaschii MK, M. jannaschii homoserine kinase and S. cerevisiae MPD [38-40].

The role of these conserved regions in function, stability and reaction mechanism of MK has been studied by site-directed mutagenesis of human and rat recombinant MK [21, 41-43] and analysis of mutant proteins identified in patients with an MK deficiency [12, 34, 44, 45]. This was done by expression of these mutant proteins as recombinant fusion proteins in Escherichia coli and immunoblotting of fibroblast lysates from these patients with an MK-specific antibody (see also molecular basis of MK deficiency). Expression of MK as recombinant (fusion) protein does not significantly alter the kinetic properties of the enzyme when compared with the purified enzyme (table 1), which makes it a valid model for the biochemical characterization of mutant MK proteins. The catalytic reaction mechanism proposed after these studies is consistent with the later determined crystal structure of MK.

Three possible functions have been suggested for the first conserved region (amino acids 1 to 30 in the human sequence). The first function of this domain is being part of the catalytic site of MK. It has been suggested that it is important for stabilization of ATP-binding since pyridoxal phosphate, a lysine-specific reagent, is able to inactivate recombinant rat MK [41]. This inactivation does not occur in the presence of ATP, suggesting that pyridoxal phosphate reacts with a lysine in the active site of the enzyme [41]. This lysine was subsequently identified as the conserved lysine at position 13. The function of this residue in ATP-binding was further studied by changing it into a methionine using site-directed mutagenesis (K13M), followed by expression of the resulting recombinant mutant protein. The V_max of the purified enzyme was 56-fold decreased, which suggests that this lysine facilitates catalysis but does not play an indispensable role in the reaction catalysed by MK [41]. The K_d values of the wild-type and mutant enzyme for ATP revealed a >50-fold diminution in affinity for the mutant enzyme. This strongly suggests a role for Lys-13 in stabilization of ATP binding [41]. In the crystal structure of MK, however, Lys-13 is present in the catalytic site, making a role in stabilization of mevalonate binding via the C-3 hydroxyl group more likely [40].

The second possible function of this domain is stabilization of the protein’s tertiary or quaternary structure. When the conserved glutamic acid at position 19 was changed into an
alanine or a glutamine, or the conserved histidine at position 20 into an alanine, the expressed proteins were unstable and completely insoluble [21]. Also the H20P mutation, which was identified in both HIDS and MA patients (see molecular basis of mevalonate kinase deficiency), resulted in an inactive enzyme [34].

![Alignment](image)

**Figure 2.** CLUSTAL W multiple sequence alignment of human, rat, Arabidopsis thaliana and Saccharomyces cerevisiae MK. The GenBank accession number are A42919, A35629, S42088 and BVBYR1, respectively. The conserved regions are indicated by bars and the characterized amino acids by arrows.

Another function is that this region may be involved in targeting of MK to peroxisomes (see also subcellular localization of MK). This N-terminal amino acid stretch has a perfect match with the consensus sequence of a peroxisomal targeting sequence type 2 (PTS2, (R/K)(L/V/I)X₅(H/Q)(L/A)), which directs certain proteins via the PTS2 receptor protein (PEX7) to the protein import machinery of the peroxisome (for recent reviews on peroxisome biogenesis see [46, 47]). The observation that MK localizes to rat liver peroxisomes and its reported deficient activity in patients with diverse peroxisome protein import deficiencies (see 6, subcellular localization of mevalonate kinase) are in favor of a function in targeting for this domain. However, it is remarkable that the same domain including the PTS2 consensus sequence is part of the enzyme’s catalytic site and is found also in the MK amino acid sequence from archaeabacteria and eubacteria, which do not possess peroxisomes. The same domain including the PTS2 is present in the amino acid sequences of another member GHMP kinase superfamily, the non-peroxisomal galactokinases. Thus the
Biochemical and genetic aspects of mevalonate kinase and its deficiency

proposed role of this domain in targeting of the enzyme to the peroxisome needs to be reevaluated.

The second region (amino acids 135 to 158) is not only conserved in all MK amino acid sequences, but also in all other GHMP kinases [32, 48, 49]. The amino acid sequence of this region has the characteristics of an ATP binding site (GXGXXXAX_{13-26}K) [32, 48], however, the lysine is not invariably conserved [41]. This domain is also very hydrophobic, which makes it a good candidate for an isoprenoid-binding site [33]. The isoprenoids GPP, FPP and GGPP have been found to be competitive inhibitors of MK with respect to ATP, which is in line with the proposed role for this region in ATP binding. The crystal structures of MK, homoserine kinase and MPD confirm this hypothesis. This region resembles but is distinct from the classical phosphate-binding loops (P-loop) responsible for ATP-binding in other enzymes [38-40]. Further evidence for the proposed role of this domain as part of MK’s catalytic site comes from site-directed mutagenesis of the serine at position 146 into an alanine (S146A). This resulted in a protein with a 4000-fold decreased $V_{max}$ and a 40-fold increased $K_m$ for MgATP suggesting that it functions as a cation ligand and a hydrogen bond donor to a phosphoryl oxygen of the bound nucleotide [42].

Suggested functions for the third conserved region (amino acids 186 to 211) come from a study of carboxylate residues (aspartic and glutamic acids) in recombinant human MK. The importance of these residues for MK function is suggested by the complete inactivation of the enzyme upon modification of carboxylates by water-soluble carbodiimides [21]. The fact that only poor protection was observed after the substrates had been provided, points to the modification of multiple sites. Since only three glutamic acids (E19, E193 and E296) and one aspartic acid (D204) are conserved in all MK sequences, site-directed mutagenesis was performed to characterize these residues [21]. Changing the glutamic acid at position 193 into a glutamine resulted in a stable protein with a $V_{max}$ 52-fold lower than wild-type, whereas the $K_m$ values for ATP and mevalonate were both elevated, 20- and 40-fold respectively. These results are compatible with a role of the Glu-193 in the interaction of the enzyme with the cation of the MgATP substrate [21]. The most dramatic effect on the enzyme’s activity was observed when the aspartic acid at position 204 was changed into an alanine or an asparagine (D204A, D204N). These mutations resulted in stable enzymes without any enzyme activity [21]. The active sites of these enzymes were still intact, as concluded from the preserved $K_m$ and $K_d$ values. This suggests a crucial catalytic role for the Asp-204, most probably in deprotonation of the C-5 hydroxyl of mevalonate [21]. In accordance, Asp-155 (Asp-201 in the human sequence) in the M. jannaschii MK crystal structure is located close to the C-5 hydroxyl group of mevalonate and the γ-phosphorus of ATP [40]. An additional study of this region was performed by site-directed mutagenesis of the serine at position 201 into an alanine (S201A). This resulted in a protein with 100-fold increased $K_m$ for mevalonate, which could reflect a significant reduced binding efficiency [42].

The role of the fourth conserved region (amino acids 329 to 344) was elucidated after characterization of a mutation in this domain identified in an MA patient [44]. This mutation changed the alanine at position 334 into a threonine. Although this region has characteristics of an ATP-binding site (GXGXXGX_{15-21}AXK)[27, 32, 41], the mutation changed the affinity of the enzyme for its other substrate, mevalonate, when expressed as a recombinant fusion protein in E. coli [44]. This suggests a function of this region in the stabilization of mevalonate binding, which is confirmed by the crystal structure of MK [40].
Remarkably, all four conserved regions do not contain an invariably conserved cysteine, when all MK sequences currently known are considered. Although the cysteine at position 339 in region 4 is conserved in the majority of MK species, it is substituted for a serine in some eubacteria and in _M. thermoautotrophicum_. This is in disagreement with the previously suggested role for a sulfhydryl group in the active site of the enzyme (see also biochemical characterization) [14-16]. However, this cysteine might be of structural importance by stabilizing the P-loop since it seems to form a disulfide bond with another cysteine in the active site [40].

4  Mevalonate kinase deficiency

4.1. _Mevalonic aciduria and hyperimmunoglobulinaemia D and periodic fever syndrome_

Two disorders, MA and HIDS, are caused by a deficient activity of MK, which in principle may affect the biosynthesis of all isoprenoids. This is in contrast to other disorders caused by deficiencies in the isoprenoid pathway. MA was first described by Berger et al. in 1985 [50] and later recognized to be caused by a deficiency of MK enzyme activity [11]. In MA, MK enzyme activity is virtually absent when measured in cultured skin fibroblasts or lymphoblasts of patients (<0.5% of the control mean in our laboratory and 0 to 4% of the control mean in literature) [34, 51]. In HIDS, however, a residual MK activity is measured which varies between 1% and 7% in cultured skin fibroblasts and peripheral blood mononuclear cells (PBMCs) and 2% and 28% in cultured lymphoblasts [12, 52, 53]. As a result of this difference in MK function, excretion of mevalonate in urine varies significantly between the two syndromes. MA is characterized by a massive and constitutive excretion (1-56 mol/mol creatinine), which correlates with the severity of the clinical disease among patients as well as during the course of the disease within a subject [51]. In HIDS the excretion is moderate (0.005-0.040 mol/mol creatinine) and noted mainly during febrile crises [12]. Kelley reported that excretion in HIDS patients is at least 10-fold higher between fever episodes, and increases 100- to 500-fold above normal during febrile crises [54]. Normal excretion of mevalonate in urine is usually less than 0.001 mol/mol creatinine.

Clinically, both syndromes also can be distinguished from each other. Severely affected MA patients have profound developmental delay, dysmorphic features, cataracts, hepatosplenomegaly, lymphadenopathy, and anemia as well as diarrhea and malabsorption and often die in infancy. Less severely affected patients have psychomotor retardation, hypotonia, myopathy, and ataxia. The ataxia in these patients is probably due to a selective and progressive cerebellar atrophy [51]. All MA patients also suffer from recurrent episodes of fever, which may occur up to 25 times per year and last on average 4 to 5 days. These fever episodes are associated with lymphadenopathy, arthralgia, subcutaneous edema, gastrointestinal problems and skin rash [51]. Several severely affected patients died during such crises. During a crisis there were no obvious metabolic derangements noted, only a highly increased blood sedimentation rate [51]. MA may often present as cholestatic liver disease and a hematologic disorder mimicking congenital infection, myelodysplastic syndrome or even acute leukemia in childhood [55]. These hematologic abnormalities include a normocytic anemia with striking extramedullary hematopoiesis, hepatosplenomegaly, thrombocytopenia due to hypersplenism and leukocytosis with a left shift [55].

Compared to MA, HIDS is a relative benign condition, in which patients mainly suffer from strikingly similar fever episodes, which occur every 2 to 6 weeks and last 3 to 7 days.
Fever rises abruptly, often over 40°C and the temperature then gradually returns to normal. There is no strict periodicity and patients may have long symptom-free periods. These fever episodes are associated with malaise, chills, headache, arthralgias, arthritis, nausea, abdominal pain, diarrhea, skin rash, hepatosplenomegaly and lymphadenopathy [56, 57]. The attacks in HIDS can be triggered by infections, minor trauma, childhood immunizations, menses or physical and emotional stress, but usually occur without any clear precipitating event [56]. In contrast to MA, HIDS patients usually have no developmental features [56].

The first detailed description of this disorder was published in 1984 [58] and its name was derived from the constitutively elevated level of serum IgD, usually accompanied by elevated levels of serum IgA, which is still the diagnostic hallmark of HIDS [56, 58]. The disease has been reported also as very-early-onset juvenile arthritis and etiocholanolone fever [59, 60]. Most of the reported patients are of Dutch origin and therefore the disease is also known as Dutch-type periodic fever. It should be noted, however, that periodic fever is a widely occurring phenomenon among children. The fact that the majority of currently identified HIDS patients are of Dutch origin is most likely the result of a heightened awareness of the disorder in the Netherlands and the inclusion of a specific laboratory test for IgD levels in Dutch patients with periodic fever. Both MA and HIDS have a recessive mode of inheritance and are rather rare.

With the availability of molecular analysis of the MVK gene and biochemical analysis of MK enzymatic activity in HIDS patients, it became apparent that not all patients diagnosed with HIDS have MK deficiency [61, 62]. Patients with MK deficiency were designated as having classic-type HIDS, whereas patients without MK deficiency were denoted as having variant-type HIDS [62]. Subtle differences in symptoms, signs and laboratory findings were noted upon comparison of these HIDS variants. In general, patients with classic-type HIDS were younger at the onset of the disease and had more additional symptoms during the fever episodes [61, 62].

Despite the increased knowledge about the genetic and biochemical basis, no effective treatment exists for HIDS and MA at present. A clinical trial in HIDS patients with thalidomide had only limited efficacy [63]. Also a therapeutic trial in which two MA patients were treated with low doses of lovastatin in order to block the production of mevalonate (speculated to be pathogenic) was unsuccessful and had to be stopped because of the development of severe clinical crises [51]. Statins are potent competitive inhibitors of HMG-CoA reductase and are widely used to treat atherosclerosis and familial hypercholesterolaemia. This drug blocks the synthesis of mevalonate and as a consequence lowers the endogenous synthesis of isoprenoids. The use of statins have been shown to trigger adaptive reactions giving rise to an up to 200-fold increased reductase activity [3]. The outcome of this trial strongly suggests that the symptoms of MA and HIDS are not caused by an excess of mevalonate but by a shortage of isoprenoid endproducts. The enormous difference in urinary mevalonate excretion in HIDS and MA patients also argues against a causative role of mevalonate in the pathogenesis of the clinical crises. MA patients have much higher mevalonate levels, but fever episodes occur as frequently as in HIDS. Despite the negative experience with the use of statins in MA [51], the efficacy of the drug is now tested in HIDS [63]. Also other therapeutic regimens in MA, including endproduct supplementation, seem to have little if any beneficial effect [51].

HIDS and MA are classified often as auto-inflammatory (or noninfectious inflammatory) disorders [64-66], which also include systemic-onset juvenile chronic arthritis (sJCA, MIM 604302), adult-onset Still’s disease, chronic infantile neurologic cutaneous
arthropathy syndrome (CINCA), periodic fever, aphthous stomatitis, pharyngitis and adenopathy syndrome (PFAPA), Crohn's disease (inflammatory bowel disease, IBD, MIM 266600), Blau's syndrome (MIM 186580), Behcet's syndrome (BS, MIM 109650), familial Mediterranean fever (FMF, MIM 249100), TNF-receptor associated periodic syndromes (TRAPS, MIM 142680) and familial cold auto-inflammatory syndrome or Muckle-Wells syndrome (FCAS, MIM 120100 or MWS, MIM 191900). All these syndromes are characterized by spontaneous attacks of systemic inflammation without an apparent infectious or autoimmune etiology [64]. A subgroup of these diseases are the hereditary periodic fever syndromes, which include HIDS, MA, FMF, TRAPS and FCAS [64, 65]. For most of the syndromes the molecular basis has been solved at least partly. It is interesting to study how the functions of the gene products defective in the other syndromes are related to the function of MK [66].

4.2. Molecular basis of mevalonate kinase deficiency
The first step in the elucidation of the molecular basis of MA was the isolation of the human cDNA encoding MK [32]. Subsequently, the deduced sequence was used for the identification of an MA-causing mutation in the MVK gene, N301T. The mutant allele was subsequently expressed in COS-7 cells and showed a markedly decreased enzyme activity varying from 5 to 20% of the activity of wild type MK [32]. Analysis of additional MA patients identified 13 different mutations (figure 3) [34, 44, 45].

The genetic basis of HIDS was identified independently in 1999 by two groups including our own and a Dutch/French consortium using different approaches. Both groups ended up with the same gene, MVK, coding for the enzyme MK. Houten et al. [12] performed organic acid analysis of urine from one HIDS patient collected during an episode of fever and found elevated levels of mevalonate. This patient had all the symptoms of HIDS except for an elevated IgD. Subsequent organic acid analysis in two fully characteristic HIDS patients also revealed elevated levels of mevalonate during episodes of fever. The elevated levels of mevalonate suggested a defect in the metabolism of mevalonate and enzyme measurements revealed a marked deficiency of MK enzyme activity. Subsequent mutation analysis yielded several disease-causing mutations in the MVK gene [12]. Drenth et al. [13] performed linkage analysis and obtained linkage at chromosome 12 position q24. After narrowing the region to 9 cM, they considered MVK a good candidate gene and identified mutations in this gene in several HIDS patients [13]. Since the human MVK cDNA sequence had already been cloned, both groups performed mutation analysis at the cDNA level. This revealed a common missense mutation, a G>A transition at nucleotide 1129 changing the valine at position 377 into an isoleucine (V377I) [12, 13], in ~90% of the HIDS patients [52, 53, 67]. Most patients studied are compound heterozygotes for this mutation. Two additional common missense mutations were identified, an A>C transversion at nucleotide 59 and a T>C transition at nucleotide 803, which change the histidine at position 20 into a proline (H20P) and the isoleucine at position 268 into a threonine (I268T), respectively. Since the latter two mutations had been identified also in MA patients and no V377I mutations have been detected in these patients, this strongly suggested that the V377I mutation is responsible for the HIDS phenotype of MK deficiency [12, 13, 34, 52, 53, 67]. Indeed, thus far only 5 patients clinically diagnosed with HIDS have been identified without this mutation [13, 52, 53, 68]. Most of the alleles found in these patients were identified only in HIDS patients, suggesting that V377I is not the only mutation which causes the HIDS phenotype.
An additional study revealed that homozygotes for the V377I mutation are underrepresented in HIDS. This is based on the fact that the distribution of the V377I mutation within the group of patients carrying MVK mutations is significantly different from the expected Hardy-Weinberg equilibrium distribution. In addition, the carrier frequency for the V377I mutation in the Dutch population is 1:153. This predicts an incidence between 1 and 6 V377I homozygotes per year, which is far more than observed. Thus, homozygotes for V377I might have another, yet unknown (milder) phenotype of MK deficiency or have no disease-phenotype at all [69].

The human MVK gene was previously reported to be a single copy gene located at chromosome 12 position q24 [32, 70]. We [52] and others [53] have resolved the genomic organization of the MVK gene, which spans approximately 22 kb and consists of 11 exons. At this moment, we perform mutation analysis mainly at the genomic level. Figure 3 is a schematic representation of the MVK gene and includes all 29 currently identified mutations in both MA and HIDS patients. These include 4 nonsense mutations, 23 missense mutations and 2 one-nucleotide insertions. In addition, two deletions at the cDNA level, corresponding to skipping of exon 2 or 4, have been described [12, 13, 32, 34, 44, 45, 52, 53, 68].

![Figure 3. Schematic representation of the human MVK gene and overview of all different mutations identified in patients diagnosed with HIDS or MA. The sizes of the introns are indicated in kb and the numbering of the exons corresponds to the cDNA position. The open reading frame is indicated in gray. The mutations include 23 missense mutations (H20N, H20P, L35S, L39P, S135L, A148T, P167L, G202R, T209A, G211A, R215Q, T243I, L264F, L265P, I268T, S272F, R277C, N301T, G309S, V310M, G326R, A334T, V377I), 4 nonsense mutations (K13X, W62X, Y149X, R388X) and two one nucleotide insertions. Furthermore, two deletions of an exon (exon 2 and 4) have been reported at the cDNA level.](image_url)

Evidence for the disease-causing nature of the identified mutations was obtained by characterization of mutant proteins by immunoblotting of fibroblast lysates of patients using an MK-specific antibody and heterologous expression of the mutant proteins in E. coli. All characterized mutations result in MK proteins with markedly decreased enzyme activity when expressed in E. coli and often decreased protein levels in fibroblast lysates (H20P, T243I, L264F, L265P, L268T, V310M and A334T) [34, 44, 45]. The V377I mutation, however, had considerable residual activity when expressed in E. coli [12, 43], but MK protein in fibroblast lysates of HIDS patients was hardly detectable as shown by immunoblotting [12]. However, when fibroblast cell lines originating from HIDS patients and harboring the common V377I MVK allele were switched from 37°C to 30°C culturing temperature, they displayed a progressive increase in MK enzyme activity. As shown by temperature inactivation...
experiments this resulted in a protein nearly as stable as in control cell lines, which indicates that it is primarily the maturation of the V377I protein which is affected. In accordance, when the HIDS cell lines were cultured at 39°C, MK activity decreased further. A similar phenomenon occurs in vivo. MK activity in PBMCs drops 2-6-fold when HIDS patients experience febrile attacks.

4.3. Laboratory findings and pathogenesis

In search for the pathophysiology of the MK deficiency disorders several abnormalities have been identified. An enhanced urinary excretion of leukotriene E₄ (LTE₄) was noted in MA patients, which strongly correlated with excretion of mevalonate in urine [71]. This was not due to an impaired degradation of cysteinyl LTs, but the result of an increased systemic generation of LTE₄ via the 5-lipoxygenase pathway [72]. Cysteinyl LTs (LTC₄, LTD₄ and LTE₄) are mediators of edema and increased vascular permeabilization during acute inflammation. Periodic systemic capillary leak syndrome (Clarkson disease) is a disorder characterized by unexplained attacks of a marked increase in capillary permeability. Especially the presence of edema during the severe clinical crises in MA suggests the occurrence of capillary leak [71]. Thus, the cysteinyl LTs could play an important role in the pathophysiology of MA [71]. In addition, LTE₄ excretion in HIDS was elevated also, but only during fever episodes [73].

Analysis of the fatty acid composition of plasma cholesterol esters and phospholipids, and erythrocyte phosphatidylethanolamine and phosphatidylcholine in MA patients revealed decreased levels of linoleic acid and markedly increased levels of arachidonic acid [51]. A similar phenomenon is observed when selected cell lines are cultured in the presence of HMGR inhibitors (statins), which causes an increase in the biosynthesis of arachidonic acid from linoleic acid [74, 75] and, as a result, an elevation of arachidonic acid in cellular phospholipids and secreted lipids [74].

Most MA patients exhibit a decreased level of ubiquinone-10 in plasma [51]. LDL samples taken from an MA patient during a clinical crisis show decreased concentrations of ubiquinol-10, which may sensitize the LDL particles to oxidation by Cu²⁺ as seen in other patients during a clinical crisis [76]. The deficiency of ubiquinone-10 could account for the progressive cerebellar atrophy observed in MA patients, since the cerebellum is the region of the brain most vulnerable to lipid peroxidation [76]. Increased susceptibility to oxidative stress could also account for the formation of cataracts in these patients. However, chronic exposure of lenses to mevalonate has been shown to induce cataracts too [77].

Other biochemical abnormalities related to isoprenoid biosynthesis observed in fibroblasts or lymphoblasts derived from MA patients are a decreased (but still substantial) biosynthesis of cholesterol, dolichol, ubiquinone-10 and glycosylated macromolecules [76, 78]. On the other hand, protein prenylation was normal as studied by measuring membrane bound isoprenylated proteins [79], and also cholesterol biosynthesis can be entirely normal depending on the culture condition [78, 80]. Thus, it appears that MA en HIDS cells are able to compensate for their defect in MK. This is possible because they have increased activity of HMGR and the LDL receptor pathway [78, 80]. HMGR, which converts HMG-CoA into mevalonate, is believed to perform the main rate-limiting step in isoprenoid biosynthesis and is among the most highly regulated enzymes in nature [3]. This increased activity of HMGR was unsuppressible by exogenous LDL cholesterol and was further upregulated under cholesterol-free culture conditions [80], suggesting that the high basal HMGR activity in MA
is not due to a shortage of sterol endproducts. Accordingly HMGR mRNA levels are normal in MA cells, indicating that the sterol-dependent SREBP pathway (transcriptional) is not activated [79]. The increased HMGR activity, however, was downregulated when the medium of MA cells was supplied with FOH, GGOH, or mevalonate [79]. This suggests that one of the non-sterol dependent regulatory mechanisms causes the increase in HMGR activity. These mechanisms act post-transcriptionally and involve higher mRNA translation efficiency and decreased protein turnover.

It appears that MA and HIDS cells are able to compensate for reduced MK activity by elevating their intracellular mevalonate levels, since addition of extra mevalonate to the medium downregulated HMGR activity in MA fibroblasts [79]. In this case the elevated HMGR activity as observed in MA fibroblasts serves mainly to compensate for the leakage of mevalonate (or mevalonolactone) out of the cell. Since HMGR is not elevated in HIDS fibroblasts, this leakage is only minimal, reflecting a lower level of mevalonate in the cell. Accordingly, MA fibroblasts are more sensitive to simvastatin than HIDS fibroblasts, whereas HIDS fibroblasts are more sensitive to HMGR inhibition than control fibroblasts. This was measured by the loss of membrane-bound isoprenylated proteins after treatment of cells with statins [79]. The elevation of intracellular mevalonate concentrations allows a normal pathway flux under the following three conditions; (1) MK is not saturated with substrate. When MK would be saturated any elevation in mevalonate has no effect; (2) HMGR is able to provide mevalonate levels that are high enough for MK to function at a (near) normal rate. HMGR has to compensate for the leakage of mevalonate out of the cell; (3) HMGR is not subject to noncompetitive product inhibition by mevalonate. This is not the case because HMGR is insensitive to any form of product inhibition [81].

These conditions suggest a unique mechanism for the pathogenesis of a metabolic disorder. Normally, the pathogenesis may be caused by toxic accumulation of some intermediate or a shortage of end-products. A shortage of end-products may be due either to an accumulation of an intermediate, which is a noncompetitive inhibitor of the previous enzyme in the pathway, or a residual activity that is too small for keeping up with the pathway flux, resulting in a saturation of the deficient enzyme with substrate. In the case of HIDS and MA, elevated mevalonate appears to compensate for the deficiency of MK under normal conditions. However, a decrease in the residual MK activity in HIDS caused by increased temperature (e.g. fever) will have a huge impact [82]. Since the steady-state levels of MK protein are very low, the enzyme approaches saturation. Thus, the relative increase in intracellular mevalonate level must be much higher than in controls in order to keep the pathway flux normal, which leads to a temporary decrease in the pathway flux and in the production of isoprenoids. This is reflected by a compensatory increase in HMGR activity indicating that MK becomes progressively rate-limiting [82]. A temporary decrease in the production of isoprenoids will affect especially high-turnover or newly-synthesized isoprenoids, such as ubiquinone-10 in plasma, which is decreased in most MA patients [76], prenylated small G-proteins like Rho [83], which are involved in multiple cellular processes like signal transduction or cytoskeletal organization and the isoprenylated guanylate-binding proteins (GBP), which are synthesized in response to interferon-γ (IFN-γ) and LPS [84]. It seems conceivable that this shortage of non-sterol isoprenoids is responsible for the pro-inflammatory phenotype of HIDS and MA. Thus, even minor elevations in temperature, due to exercise or infections could set off a chain of events, with MK becoming progressively rate
limiting, leading to a temporary deficiency of anti-inflammatory isoprenoids, followed by inflammation and fever.

The observed differences in stability and temperature sensitivity of mutant MKs may explain why HIDS patients display episodic fever [82]. Since such fever episodes are prominent not only in HIDS but also in MA, the same may be true for MA. Indeed, the fact that mevalonate excretion in urine of both HIDS and MA patients correlates with disease severity points to a similar mechanism [51, 54]. Unfortunately, the extremely low and already hardly detectable MK activity levels in MA cells do not allow demonstration of a similar temperature-sensitive phenomenon. However, the finding that the increase in temperature does not only affect the mutant MKs, but also the wild-type MK activity renders it highly plausible [82]. Even a small additional decrease in MK activity in MA cells may have far reaching consequences, for two possible reasons. MK is saturated with substrate, thus an additional increase in mevalonate concentration has no effect. This may be the case when a MVK mutation results in reduced MK protein levels. Additionally, HMGR already appears maximally induced to compensate for the MK deficiency. A further induction to establish even higher mevalonate levels may not be possible. This may be true for the A334T mutation that alters the affinity of the enzyme for its substrate mevalonate. These reasons could form an explanation for the reported fatal outcome of a fever episode in several MA patients [51].

The difference in activity of HMGR also provides an explanation for the observed differences in mevalonate excretion between HIDS and MA. In MA, there appears to be a constitutive derepression of HMGR. This is reflected by the elevated reductase activity in fibroblasts and PBMCs from MA patients [80, 82] and the level of excreted mevalonate (>800 mmol/day) which greatly exceeds the level of normal whole body cholesterol biosynthesis (4 mmol/day, equivalent to 24 mmol of mevalonate/day) [44, 51]. In HIDS, HMGR activity may be derepressed only at the onset of and/or during a fever episode or only in tissues where the relative MK expression is low. This is illustrated by the fact that HMGR in HIDS fibroblasts is within the normal range whereas in PBMCs it is elevated significantly. Preliminary results with multiple tissue RNA dot blots seem to confirm the hypothesis that the ratio between MK and HMGR expression is variable and lowest in the appendix, bone marrow and peripheral blood leukocytes. This also indicates that these tissues may be the most sensitive to lowered MK activity.

In accordance with the in vitro observations mentioned above, squalene, cholesterol and bile acid levels are usually (near) normal in patients with MA [51, 85]. In one of three patients tested, however, a decreased rate of biosynthesis of primary bile acids was noticed [86]. This again indicates that MA patients are capable of compensating for the reduced MK activity.

Whereas studies on the pathogenesis of MA have mainly focused on biochemical aspects, studies on HIDS pathophysiology were focused on the dysregulation of the immune system observed in these patients. There are several hematological abnormalities observed during a fever attack, which are indicative of an acute phase response, including granulocytosis, increased erythrocyte sedimentation rate and elevation of several acute phase proteins such as C-reactive protein, serum amyloid A (SAA), fibrinogen, soluble type II phospholipase A2 and α1-acid glycoprotein (AGP) [56]. The concentration of AGP is increased continuously during attacks and remissions, as is the glycosylation of AGP, indicating a persistent state of inflammation [87].

38
As mentioned above, in HIDS there is a constitutive polyclonal elevation of serum IgD, usually with concomitant high levels of IgA (subclass IgA1)[88]. An elevated serum IgD and IgA has been reported also in MA [53, 89]. Plasma cells residing in the bone marrow are probably the source of both the IgA and IgD, suggesting continuous, systemic stimulation of the immune system [88]. The physiological role of IgD and its role in the pathogenesis of HIDS, however, remains enigmatic. Although IgD is a potent inducer of cytokines in vitro [90], its elevation is more likely to be an epiphenomenon than the cause of the inflammatory state in HIDS, since there is no relation between IgD level and severity of the attacks, frequency of the attacks or disease activity itself. In accordance, fever attacks in children may precede the rise in IgD [91]. Recently, we reported a patient with all the signs and symptoms of HIDS and a deficient MK activity, but no serum IgD elevation. Serum IgA, however, was elevated in this patient [12]. Furthermore, IgD may be elevated in other auto-inflammatory diseases, notably PFAPA, FMF and BS, and various other diseases and conditions including infections, immunodeficiencies, autoimmune diseases, allergic diseases and malignancies [92].

The production of pro- and anti-inflammatory cytokines in HIDS has been studied both in vivo in plasma from patients and in vitro in the culture supernatant of whole blood or PBMC cultures [93-95]. During febrile attacks the serum levels of IFN-γ and interleukin-6 (IL-6) rise sharply [93, 94], and tumor necrosis factor-α (TNF-α) rises to high normal values, whereas IL-1α and IL-1β are not elevated [94]. The effect of the increased stimulation of mononuclear phagocytes by IFN-γ is reflected in a rise in urinary neopterin excretion simultaneously with the onset of fever. The neopterin excretion remains high for several days after normalization of the body temperature [93]. Of the tested anti-inflammatory cytokines, IL-1 receptor antagonist (IL-1ra) and soluble receptors for TNF-α (sTNFαR, p55 and p75) were elevated, whereas IL-10 remained normal [94].

In supernatants of unstimulated cultures of whole blood samples of HIDS patients obtained between or during attacks, IL-1β and IL-6 are not increased [94]. Although plasma levels of TNF-α stay within normal limits, its concentration is increased in the supernatants of unstimulated cultures of whole blood samples drawn between attacks. IL-1ra was elevated only when blood was obtained during fever. When stimulated with LPS, the supernatants of such cultures showed an elevated TNF-α level, which was even higher when the blood cells had been obtained during an attack. IL-1β and IL1-ra were only elevated in these stimulated cultures when blood was drawn during an attack [94]. Similar results were obtained in culture supernatants of isolated PBMCs obtained between attacks. Notably, spontaneous IL-1β, IL-6 and TNFα production by isolated PBMCs is elevated significantly in HIDS and rises further upon stimulation with LPS [95].

Taken together these data are compatible with macrophage activation during the febrile attacks [57]. This is reflected by an increased expression of CD64 (Fcγ-Receptor I) on monocytes and granulocytes during fever [96]. Between the febrile attacks the in vitro findings are still compatible with an increased activity of the mononuclear phagocytic compartment [57]. The cause of this macrophage activation is unknown. While activated Th1 cells are known to induce macrophage activation, the high IgD and IgA levels are more compatible with an increased activity of Th2 cells [97]. However, direct evidence of T-cell activation during attacks is lacking [57].

There are several other indications that isoprenoid biosynthesis plays a role in inflammation and can influence the immune system. For example, it has been reported that
administration of LPS, TNF-α, or IL-1β to Syrian hamsters triggers a rapid upregulation of hepatic HMGR and a downregulation of squalene synthase, the enzyme catalyzing the first committed enzyme step of sterol biosynthesis [98-100]. These observations suggest a higher demand for non-sterol isoprenoids during inflammation. Furthermore, it became clear that not all beneficial effects of treatment of atherosclerosis and familial hypercholesterolaemia with statins could be due to lipid-lowering, but were caused also by anti-inflammatory effects of these drugs. This led to many in vitro experiments showing that these statins either suppress or enhance the inflammatory response, depending on the cell type studied and the way in which these cells had been stimulated. Many anti-inflammatory effects of statins have been reported, including the reduction of lymphocyte proliferation, and of the expression of MHC class II molecules, matrix metalloproteinases, cytokines, and chemokines [101-107]. On the other hand, these compounds also have pro-inflammatory properties. Statins enhance endothelial expression of cellular adhesion molecules [108]. Interestingly, the secretion of IL-1β, IFN-γ and IL-18 by PBMC stimulated in vitro with inactivated Mycobacterium tuberculosis is augmented greatly by the inhibition of isoprenoid biosynthesis with statins [109]. The increased cytokine secretion was due to lack of isoprenoids, since addition of mevalonic acid reduced cytokine secretion to control levels [109]. The same phenomenon was noted when purified monocytes were used. Treatment of these cells with statins increased the production of IL-1β, TNF-α, macrophage chemotactic protein-1 (MCP-1) and IL-8 [110]. In addition, statins enhanced infiltration of mouse leukocytes into an inflamed peritoneal cavity [110]. A similar mechanism was shown to cause elevated cytokine secretion in HIDS and MA PBMC cultures [111]. Statins augmented IL-1β secretion by control stimulated PBMCs, which could be countered by bypassing HMGR with mevalonate, and, to a lesser extent, by FOH or GGOH. In the absence of statins, mevalonate itself did not change the already minimal IL-1β secretion of PBMC, indicating that mevalonate by itself does not induce cytokine secretion. As reported before, HIDS and MA PBMCs, spontaneously secrete more IL-1β than control MNC. This secretion increased even further in the presence of statins. Bypassing HMGR with mevalonate, FOH or GGOH tended to abort this increased IL-1β secretion. More importantly, in the absence of statins, IL-1β secretion by PBMCs from HIDS and MA patients was reduced when cultured in the presence of FOH [111]. These observations again indicate that not mevalonate but a shortage of one of the non-sterol endproducts is the pathogenic factor in HIDS and MA.

These results together with the above described biochemical pathogenetic mechanism, suggest that supplementation of isoprenoid precursors, such as mevalonate, FOH and GGOH, may be beneficial in the abortion and prevention of fever episodes in HIDS and MA. However, the toxicity of these compounds has not been tested in vivo. In vitro, FOH and GGOH have substantial cytotoxicity. Furthermore, they are able to downregulate HMGR enzyme activity. Since mevalonate kinase deficient cells depend on an elevated HMGR activity, studies toward the in vivo effects of isoprenoid precursor supplementation are necessary.

5 Regulation of mevalonate kinase activity

Besides the possible feedback inhibition by isoprenoid intermediates, the activity of MK is regulated mainly at the transcriptional level. Regulation of MK mRNA transcription was studied in rat livers after statin administration or other dietary modifications. Diets supplemented with cholestyramine (a bile acid sequestrant) alone, or cholestyramine and pravastatin, or lovastatin caused a 3-6-fold increase in MK activity and a 5-89-fold increase in...
Biochemical and genetic aspects of mevalonate kinase and its deficiency

HMGRR activity [17] (table 2). Diets supplemented with 5% cholesterol caused a decrease in the activities of both enzymes [17]. The changes observed in MK enzyme activity correlated with similar changes in the levels of MK protein and MK mRNA. From this it was concluded that MK activity was regulated principally by alterations in the rates of enzyme synthesis (and degradation) and not by enzyme activation/inactivation [17, 27]. Similar results were obtained for two other enzymes in the mevalonate pathway, phosphomevalonate kinase and isopentenyl pyrophosphate isomerase [112, 113].

Table 2. The effect of cholesterol-lowering agents, dietary cholesterol, or fasting on the activities of MK and HMGRR in rat liver.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MK relative enzyme activity</th>
<th>HMGRR relative enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular chow</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5% cholestyramine</td>
<td>3.1*</td>
<td>5.0*</td>
</tr>
<tr>
<td>+0.05% pravastatin</td>
<td>2.5*</td>
<td>9.2*</td>
</tr>
<tr>
<td>+0.1% pravastatin</td>
<td>4.1*</td>
<td>19*</td>
</tr>
<tr>
<td>+0.5% pravastatin</td>
<td>5.6*</td>
<td>41*</td>
</tr>
<tr>
<td>+1.0% pravastatin</td>
<td>5.7*</td>
<td>89*</td>
</tr>
<tr>
<td>+0.1% lovastatin</td>
<td>6.3*</td>
<td>17*</td>
</tr>
<tr>
<td>Fasting</td>
<td>1.3</td>
<td>0.1*</td>
</tr>
<tr>
<td>5% cholesterol</td>
<td>0.5*</td>
<td>0.1*</td>
</tr>
<tr>
<td>+0.5% cholic acid</td>
<td>0.5*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Enzyme activities are measured by Tanaka et al. [17] and expressed as the ratio of the experimental value divided by the value in untreated control animals. The activities in control animals for MK were 3.93 nmol/min/mg protein and for HMGRR 0.42 nmol/min/mg protein. ND, not determined. *, p<0.05.

The induction of both HMGRR and MK activity suggests a coordinate transcriptional regulation of enzymes involved in cholesterol biosynthesis. Indeed, all enzymes of isoprenoid and cholesterol biosynthesis have a coordinate regulation via a class of transcription factors called sterol regulatory element binding proteins (SREBPs). This is illustrated by the induction of expression of every enzyme involved in isoprenoid and cholesterol biosynthesis after overexpression of SREBP-1a or SREBP-2 in mice [114]. Additional evidence for a coordinate regulation of enzymes involved in isoprenoid biosynthesis came with the characterization of the 5'-untranslated region of the human MVK gene. The promoter region of the MVK gene contains two Sp1-binding sites and a sterol regulatory element (SRE). SREs are conditional positive elements that enhance transcription in the absence of sterols, whereas Sp1 sequences are binding sites for the general transcription factor Sp1. SREBPs are the proteins that enhance the transcription via binding to a SRE. The SRE in the MVK promoter has a 7/8 base pair match to the consensus sequence identified in the promoter of the HMG-CoA synthase, HMGRR and LDL receptor gene [115]. Analysis of MK mRNA expression in human fibroblasts showed induction of transcription when LDL was limited. Furthermore, when a promoter construct with the 5'-untranslated region was fused with a reporter gene and transfected into a cell line, an induction of reporter gene expression was observed when the transfected cells were grown in medium supplemented with lipoprotein deficient serum [115].
Chapter 2

HMG activity is induced 15-fold higher than MK activity in response to statin treatment (table 2). This can be explained by the multilevel regulation of HMG and is in accordance with its proposed role as rate-limiting enzyme in the pathway [3]. Indeed, induction of MK activity alone does not change the sterol content of cells, which was shown by overexpression of the ERG12 gene in yeast cells [31], indicating that MK is not a rate-limiting enzyme in isoprenoid biosynthesis [31]. However, a consequence of this relative greater induction of HMG is a significant rise in intermediary metabolites. In the case of overproduction, this may cause inhibition of MK by the polyisoprenyl pyrophosphates, GPP, FPP and GGPP. There are two possible beneficial effects of this feedback inhibition; (1) mevalonate accumulation in the cell does not inhibit HMG enzyme activity, since this enzyme is insensitive to product inhibition [81]; (2) mevalonate is the only metabolite that can diffuse out of the cell, since other intermediates contain negatively charged phosphates. Thus, this mechanism could serve as a sink and/or a buffer for mevalonate.

6 Subcellular localization of mevalonate kinase

The fact that MK was purified from a 100,000 x g supernatant of pig and rat liver suggested that the enzyme is located in the cytosol [16, 17]. Following the identification of peroxisomal-localized HMG activity it was found that the conversion of mevalonate to cholesterol could be catalyzed by highly purified rat liver peroxisomes in the presence of a cytosolic fraction [116-118]. This indicated that several steps of cholesterol biosynthesis are localized in peroxisomes. The first evidence that MK is localized in peroxisomes was obtained after immunoblotting peroxisomes isolated by equilibrium density centrifugation. Immunoreactive material was found both in cytosolic and peroxisomal fractions [119]. Additional evidence was provided by immunofluorescence with an MK-specific antibody. After transfection of CV-1 monkey kidney cells with a plasmid containing the MK cDNA, MK colocalized with catalase, the prototypical peroxisomal protein [120]. Endogenous MK expression in these cells was undetectable. The results were confirmed by immunocytoelectron microscopy on rat liver tissue, which revealed specific immunolabelling mainly in the matrix of peroxisomes, from which the authors concluded that MK is predominantly localized in peroxisomes, but is easily solubilized and released into the cytosol [120]. Labelling, however, was only visible in animals treated with cholestyramine and statins [120].

Biochemical evidence for the peroxisomal localization of MK was obtained by selective permeabilization of CV-1 cells with digitonin. Like catalase, MK displayed latency, which suggests that both enzymes are not present in the cytosol but in or associated with a membrane-bound organelle [121]. These results contrast to the almost totally released MK activity after selective permeabilization with digitonin in rat hepatoma H35 cells [122]. This indicates that MK is associated differentially with peroxisomes and may diffuse easily to the cytosol, which was suggested recently for farnesyl pyrophosphate synthase [122]. However, it remains to be seen whether this differential association is a regulatory feature, in which enzymes involved in isoprenoid biosynthesis are transported from the peroxisomal compartment to the cytosol, as suggested by Gupta et al. [122].

Further evidence for the involvement of peroxisomes in cholesterol biosynthesis came from clinical studies in patients suffering from peroxisomal biogenesis disorders, who have abnormally low plasma cholesterol concentrations [123-127]. In patients diagnosed with Zellweger syndrome, there is a generalized loss of peroxisomal functions due to a total deficiency in peroxisomal protein import, while in rhizomelic chondrodysplasia punctata
Biochemical and genetic aspects of mevalonate kinase and its deficiency

(RCDP) type 1 patients, there is only a partial loss of peroxisomal function due to an impaired import of proteins with a PTS2 [46]. As mentioned above, MK has a PTS2 consensus sequence. In contrast to these patients, newborn Pex5-/− mice, a well-characterized model for human Zellweger syndrome, had normal cholesterol levels and an elevated ubiquinone-9 content [128].

Mislocalization of peroxisomal enzymes to the cytosol leads in many cases to their degradation and/or inactivation. This phenomenon was studied also for MK. Its activity was normal in fibroblasts of 8 different patients diagnosed with Zellweger syndrome as analyzed by us [129], but significantly lowered in 3 other Zellweger patients analyzed by Krisans et al. [120]. Furthermore, MK activity was absent in 3 of 4 biopsied liver specimens from Zellweger patients [129]. Additional measurements revealed deficient MK activity in two livers from patients diagnosed with RCDP type 1 [130, 131]. In accordance with these results, the amount of MK-immunoreactive material in liver tissue of three patients with Zellweger syndrome was dramatically reduced [120]. Recently, however, MK activity was reported normal in livers of Pex5-/− mice. This led to a reevaluation of the conflicting reports in literature, showing that the previously reported deficient activity of MK in livers from human Zellweger patients reflect the bad condition of the liver, rather than mislocalization to the cytosol [132].

The rate of cholesterol biosynthesis from acetate was studied in cultured skin fibroblasts from 2 normal individuals, 16 patients with Zellweger syndrome (7 different complementation groups [46]) and 3 patients with RCDP [133]. In 11 of the 16 cell lines from patients with the Zellweger syndrome there was a significantly lowered cholesterol biosynthesis, ranging from 2 to 62% of the control values. The biosynthesis rates in the other Zellweger syndrome and RCDP cell lines did not differ significantly [133]. There was no obvious correlation between cholesterol biosynthesis levels and severity of the disease or the plasmalogen biosynthetic capacity, which is also impaired in peroxisome biogenesis defects [133]. These results were confirmed by studies of Mandel et al. [134], who concluded that the reduced cellular cholesterol content in fibroblasts from patients with the Zellweger syndrome was at least partially the result of a reduced cholesterol synthesis [134]. These results, however, are in contrast with other studies in which incorporation of octanoate, acetate and/or mevalonate was measured in fibroblasts from Zellweger and RCDP type 1 patients, a peroxisome deficient CHO cell line and primary and immortalized fibroblasts from Pex5-/− mice. These studies demonstrated comparable or even higher cholesterol biosynthesis rates in these cells [128, 135-137]. Ubiquinone biosynthesis was also equal in fibroblasts from Zellweger patients and control subjects [137]. Thus, studies of MK activity and isoprenoid biosynthesis in patients with peroxisomal biogenesis defects have many discrepancies and should be interpreted with great care. The reason for some of the conflicting reports is unclear. However, it can be concluded that the absence of functional peroxisomes does not lead to a deficiency of enzymes involved in cholesterol biosynthesis and that intact peroxisomes are not a prerequisite for isoprenoid biosynthesis. The reduced cholesterol levels may be an epiphenomenon of peroxisomal biogenesis disorders. Since these results do not exclude a peroxisomal localization under physiologic circumstances, conclusive evidence on the role of peroxisomes in isoprenoid biosynthesis is still lacking.

43
7. Future prospects

Although MK has been characterized biochemically and genetically both in health and disease, several questions have remained unanswered. Possible functions have been assigned to several conserved regions and amino acids of the protein on the basis of biochemical characterization of mutant proteins and the crystal structure of apo-MK. Validation of the resulting model for catalysis awaits the elucidation of the crystal structure of MK with at least one of its substrates bound. The remarkable finding of MK deficiency in inherited disorders with dysregulation of the immune system as one of the main symptoms suggests a role of MK in the regulation of fever and inflammation, and is another fertile area for research. Especially the study of isoprenylated proteins in patients with an MK deficiency is of interest since these proteins play a central role in diverse regulatory pathways and intracellular and cell-cell signaling. Further information on the physiological role of MK should come from animal models for HIDS or MA. Since MK "knock-out" mice may die very soon after birth like the 7-dehydrocholesterol reductase "knock-out" mice [138, 139] or may even turn out to be embryonic lethal just as the squalene synthase "knock-out" mice [140], the best approach would probably be the construction of "knock-in" mice for specific mutant alleles or conditional "knock-outs". Another important research area may be the development of sensitive methods to measure the intermediates of isoprenoid biosynthesis. This would enable a more detailed studies towards the effect of MK deficiency on the concentrations of these intermediates. It may be the only proper way for an unequivocal demonstration of reduced pathway flux as the cause of fever and inflammation in HIDS and MA. Furthermore it could help by the identification of metabolites important for the regulation of isoprenoid biosynthesis. Other areas of interest are the ambiguous subcellular localization of MK and the physiological role of the potential feedback inhibition of MK by GPP, FPP and GGPP.

References

Biochemical and genetic aspects of mevalonate kinase and its deficiency


Chapter 2

