Quantification of mevalonate-5-phosphate using UPLC-MS/MS for determination of mevalonate kinase activity

Lukas Reitzle a,1, Barbara Maier b,1, Silvia Stojanova a, Daniel Teupser b, Ania C. Muntau d, Michael Vogeser b, Sören W. Gersting a,b

a Department of Molecular Pediatrics, Dr. von Hauner Children’s Hospital, Ludwig-Maximilians-University, Munich 80337, Germany
b Institute of Laboratory Medicine, Ludwig-Maximilians-University, Munich 81377, Germany
c Department of Child and Adolescent Psychiatry, Psychotherapy and Psychosomatics, University Medical Center Hamburg-Eppendorf, Hamburg 20246, Germany
d University Children’s Hospital, University Medical Center Hamburg-Eppendorf, Hamburg 20246, Germany

ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 19 April 2015
Accepted 6 May 2015
Available online xxxx

Keywords:
Mevalonate kinase
MKD
Mevalonate kinase deficiency
HIDS
Hyperimmunoglobulinemia D and periodic fever syndrome
MA, mevalonic aciduria
Activity assay
Mevalonate-5-phosphate
UPLC-MS/MS

ABSTRACT

Objectives: Mevalonate kinase deficiency, a rare autosomal recessive autoinflammatory disease, is caused by mutations in the MVK gene encoding mevalonate kinase (MK). MK catalyzes the phosphorylation of mevalonic acid to mevalonate-5-phosphate (MVAP) in the pathway of isoprenoid and sterol synthesis. The disease phenotype corresponds with residual activity ranging from <0.5% for mevalonic aciduria to 1–7% for the milder hyperimmunoglobulinemia D and periodic fever syndrome (HIDS). Hence, assessment of loss-of-function requires high accuracy measurements. We describe a method using isotope dilution UPLC-MS/MS for precise and sensitive determination of MK activity.

Design and methods: Wild-type MK and the variant V261A, which is associated with HIDS, were recombinantly expressed in Escherichia coli. Enzyme activity was determined by formation of MVAP over time quantified by isotope dilution UPLC-MS/MS. The method was validated according to the FDA Guidance for Bioanalytical Method Validation.

Results: Sensitivity for detection of MAVP by UPLC-MS/MS was improved by derivatization with butanol–HCl (LLOQ, 5.0 fmol) and the method was linear from 0.5 to 250 μmol/L (R² > 0.99) with a precision of ≥89% and an accuracy of ±2.7%. The imprecision of the activity assay, including the enzymatic reaction and the UPLC-MS/MS quantification, was 8.3%. The variant V261A showed a significantly decreased activity of 53.1%.

Conclusion: Accurate determination of MK activity was enabled by sensitive and reproducible detection of MAVP using UPLC-MS/MS. The novel method may improve molecular characterization of MK mutations, provide robust genotype–phenotype correlations, and accelerate compound screening for drug candidates restoring variant MK activity.

© 2015 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Introduction

Mevalonate kinase deficiency (MKD) is a hereditary autoinflammatory disease, caused by mutations in the MVK gene, encoding mevalonate kinase (MK). MK is part of the cholesterol and non-cholesterol isoprenoid metabolism and is located one step downstream of HMG-CoA reductase (Fig. 1). The enzyme catalyzes the phosphorylation of mevalonic acid (MVA) to mevalonate-5-phosphate (MVAP) in the presence of ATP and Mg²⁺ [1]. This step is highly regulated and the enzyme is negatively controlled by a feedback loop of geranyl- and farnesylpyrophosphate [2,3]. In MKD, enzymatic activity of MK in patient cells is severely impaired and residual MK activity determines the clinical phenotype, ranging from the milder hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) to severe mevalonic aciduria (MA) [4]. The residual activity is <0.5% for MA and between 1 and 7% for HIDS in human fibroblasts [5]. The molecular basis of loss of function of MK is heterogeneous. Some MVK mutations induce structural derangements of the MK protein, such as increased aggregation and decreased thermal stability [6,7], whereas others show reduced affinity of MVA binding [1].

Clinically, MK loss of function leads to a disruption of the innate immunity signaling resulting in episodically reoccurring periods of fever. In HIDS, the febrile attacks are accompanied by skin rash, diarrhea, lymphadenopathy and arthralgia/arthritis. Although the number of febrile episodes decreases with age, in recent studies amyloidosis was observed in 2.9% of patients [8]. MA patients are more severely affected, presenting symptoms of dysmorphism, cataracts, cerebellar ataxia,
and psychomotor retardation, often leading to death in early childhood [9].

In clinical practice, HIDS and MA are diagnosed based on the clinical picture presented by the patients. Due to the unspecific clinical signs and symptoms the median time period between the first attack and diagnosis of the disease is 9.9 years [8]. If MKD is suspected, excretion of MVA in urine during episodes and IgD levels in serum can be measured, though in 12% of the cases IgD is in the normal range [10]. The diagnosis is confirmed by identification of disease-causing mutations in the MVK gene or determination of deficiency in MK enzyme activity in patients’ fibroblasts [4] or leukocytes [11]. The relation between genotype and phenotype is not yet fully understood. As most patients are compound heterozygous [5,10] in these cases MK activity in patient cells does not allow for estimation of the effect of each mutated allele on structure and function of the MK enzyme. Recombinantly expressed and purified proteins provide a means to investigate mutation-specific mechanisms of loss of function.

Currently, the most common activity assay for determination of MK activity in patients’ fibroblasts or leukocytes and for recombinant human MK is based on radiolabeled MVA [12]. The activity is calculated by the conversion of MVA to MVAP and mevalonate pyrophosphate, which are detected by liquid scintillation. The aim of our work was to develop a non-radioactive assay for the measurement of MK activity based on the enzymatic phosphorylation of MVA to MVAP and subsequent quantification of MVAP by UPLC-MS/MS, which sets the basis for high throughput applications.

**Materials and methods**

**Chemicals**

For expression and purification yeast extract and tryptone were obtained from Oxoid, d-maltose and ampicillin-Na salt from Serva and glucose from Braun. Isopropyl-thio-β-β-galactopyranosid (IPTG) was obtained from Thermo Fisher Scientific and phenylmethylsulfonyl fluoride (PMSF) from Sigma-Aldrich. For buffers used in purification and activity assay Tris–HCl was purchased from Applichem, HEPES, ATP and R-mevalonate lithium salt from Sigma-Aldrich and MgCl₂ from Merck.

R-mevalonic acid 5-phosphate lithium salt was obtained from Sigma-Aldrich and [13C,2H₃] DL-mevalonate-5-phosphate trilithium salt was obtained from AlsaChim. Stock solutions were prepared in methanol/water (1:1). Acetonitrile, methanol and water were from Biosolve. Butanol–HCl was from Sigma-Aldrich.

**Calibrators and quality control samples**

For quantification of mevalonate-5-phosphate calibrators (0.5, 2.5, 5.0, 25, 50, 125, 250 μmol/L) and quality control samples (1.5, 37.5, 100 μmol/L) were prepared in water/acetonitrile (1:1). Left over samples (see below), which were prepared for enzyme assay development, were mixed and used as an additional quality control sample (pool) for method validation. Calibrators and quality control samples were aliquoted and stored at −20 °C.

**Cloning and site-directed mutagenesis of the MVK gene**

The cDNA plasmid of MVK was obtained from the Mammalian Gene Collection. A PCR-based QuikChange site-directed mutagenesis kit (Stratagene) was used to replace the thymine at bp 780 by a cytosine resulting in the amino acid substitution Val261Ala (V261A), which is associated with a HIDS phenotype [13,14] (Fig. 2). cDNAs encoding wild-type MK and the variant V261A were cloned into the prokaryotic expression vector pMAL-c2X (New England Biolabs), generating MK N-terminally in fusion with the maltose-binding protein (MBP), linked by a factor Xa cleavage site.

**Expression and purification of human MK**

Wild-type and variant MBP-MK fusion proteins were expressed in Escherichia coli DH5α (Invitrogen). Bacteria were grown in 2 L of LB-medium completed with 100 μg/ml ampicillin and 0.2% glucose at 37 °C. Protein overexpression was induced by addition of 100 mmol/L of IPTG at an optical density at 600 nm of 0.5 and the temperature...
was reduced to 20 °C. Cells were harvested after 20 h and suspended in column buffer (containing 200 mmol/L NaCl, 10 mmol/L Tris–HCl, pH 7.4) supplemented with protease inhibitors (Complete Mini, Roche). After lysis by sonication, the lysate was centrifuged at 15,000 rcf for 20 min at 4 °C and the supernatant was filtered with Filtropur S 0.45 syringe filters (Sarstedt). Protein purification was performed with AKTApurifier (GE Healthcare) at 4 °C. The MBP-MK fusion protein was separated from the bacterial protein by MBPTrap (GE Healthcare) affinity chromatography. The MBP-MK fusion protein was eluted with maltose buffer (column buffer supplemented with 10 mmol/L maltose). The eluted protein fractions were loaded on a HiLoad 16/60 Superdex 200 column (GE Healthcare) to perform size exclusion chromatography (SEC). The fractions containing dimeric MBP-MK fusion protein (MK fusion protein) and the fractions containing protein aggregates were separated. The MBP tag of the fusion protein was cleaved off by factor Xa (Novagen) at a protease to protein ratio (U:μg) of 1:25 for 16 h at 20 °C. Cleavage was stopped with 1.0 mmol/L PMSF and the sample was centrifuged at 20,000 rcf for 10 min at 4 °C. The supernatant was loaded on an MBPTrap affinity chromatography column to separate MK (MK cut protein) from MBP. The unbound protein fractions, containing MK and factor Xa, were centrifuged at 20,000 rcf for 10 min at 4 °C and subjected to SEC to separate MK from factor Xa. The protein concentration of the MK fraction was determined using the Bradford protein assay (Biorad). Purity was analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE).

### Analysis of MK activity

Enzyme activity was determined by the conversion of MVA to MVAP. The buffer conditions were adapted based on a previously described radiolabeled assay [15]. Heterologously expressed and purified MK was diluted with activity buffer containing Tris–HCl, MgCl2 and ATP to a concentration of 0.005 mg/ml (MK cut protein) or 0.01 mg/ml (MK fusion protein), respectively. In order to identify the optimal conditions, Tris–HCl was varied from 1 to 100 mmol/L, MgCl2 from 1 to 20 mmol/L, ATP from 1 to 10 mmol/L and the pH was tested in a range from 3.8 to 13.5. The reaction was started by addition of 1 mmol/L R-mevalonate and carried out at 25 °C. After 2 min it was stopped via protein precipitation by adding 100 μL acetonitrile. The samples were centrifuged for 20 min at 20,000 rcf and the supernatant was further processes for UPLC-MS/MS analysis.

### Sample preparation for UPLC-MS/MS analysis

For quantification of mevalonate-5-phosphate 50 μL internal standard working solution (containing 50 μmol/L [13C3, 2H2]-DL-mevalonate-5-phosphate) were added to 50 μL enzyme assay sample, calibrator or quality control sample. After vortexing, the samples were evaporated to dryness under a gentle stream of nitrogen. Subsequently, 50 μL butanol–HCl was added to derivatize the carboxy acid group of...
MVAP and its internal standard to the corresponding butyl ester at 70 °C for 45 min. The samples were centrifuged for 2 min at 15,000 rcf and evaporated to dryness. The residue was dissolved in 1000 μL water/acetonitrile (1:1) and further diluted 1:100 with water/acetonitrile (1:1). The clear solution was transferred into a glass vial and placed into the UPLC autosampler.

**UPLC-MS/MS conditions**

Analyses were performed on a Waters UPLC system consisting of an autosampler, a column heater and a Waters Acquity UPLC coupled to a Waters Xevo TQ-S (Waters). The injection volume was 20 μL. The chromatography was carried out on a Waters Acquity UPLC HSS T3 column (2.1 × 100 mm; 1.8 μm), which was kept at 35 °C. The mobile phase consisted of two solvents, A (water) and B (acetonitrile). The run-time of the gradient program was 5 min with the following conditions: 10% B for 0.5 min, linear increase to 55% B over 2.5 min, further increase to 90% B in 0.5 min, hold for 0.5 min, return to the initial conditions in 0.2 min and re-equilibration. The flow rate was 0.4 ml/min.

Electrospray ionization in the negative mode was used. The following settings were applied: Capillary voltage, 1.56 kV; source offset, 50.0 V; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebulizer gas flow, 7.0 bar and collision gas flow 0.15 ml/min. Cone voltage and collision energy were optimized for the analyte (cone voltage, 50 V; collision energy, 12 eV) and the internal standard (cone voltage, 44 V; collision energy, 16 eV). For detection of mevalonate-5-phosphate butyl ester the mass transition 283.2 > 96.9 and for [13C, 2H3]-DL-mevalonate-5-phosphate butyl ester the mass transition 287.0 > 96.9 were monitored.

For evaluation the Waters TargetLynx Method Editor V4.1 SCN 843 was used with the following settings: smoothing method, mean; smoothing iterations, 2; smoothing width, 3; polynomial type, linear; origin, force; weighting function, none.

**Validation**

The method was validated according to the FDA Guidance for Industry for Bioanalytical Method Validation [16]. For linearity studies seven calibrators of mevalonate-5-phosphate (0.5, 2.5, 5.0, 25, 50, 125, 250 μmol/L) were analyzed in five independent series. Accuracy, intraassay and interassay imprecision were determined by replicate analysis (n = 5) of the quality control (1.5, 37.5, 100 μmol/L) and pool samples. Referring to the FDA the method was accepted as accurate, if the deviation of the mean value from the actual value was ≤ 15%, except for the lower limit of quantification (LLOQ) (≤ 20%) and it was assumed to be precise for coefficient of variation (CV) ≤ 15%, except for the LLOQ (≤ 20%).

A post column infusion experiment was performed to test the potential impact of sample matrix compounds on the ionization of analyte and internal standard, respectively [17]. In order to generate a constant MS/MS signal a pure solution of the butylester of MVAP and the corresponding internal standard (0.1 mg/L) were infused with a syringe pump (flow rate 5 μl/min) into the column effluent using a T-piece. Several processed samples were injected and the influence of the matrix to the background signal was monitored. Cross-talk effects were studied by injecting either analyte or internal standard and monitoring the other MS/MS channel for interference.

The stability of MVAP was examined at room temperature for 12 h and after three freeze and thaw cycles. Also the stability of the derivatized MVAP was tested for 1 day and 1 week.

The imprecision of the activity assay including the enzymatic reaction and the UPLC-MS/MS quantification was determined by replicate analysis. For this purpose the enzyme activity of the recombinant MK wild-type was measured eight times on three different days (n = 24) and the CV was calculated.

**Calculation of enzyme kinetic parameters**

The enzymatic parameters \( K_m \) and \( V_{\text{max}} \) of MK activity determined at varying concentrations of MVA, Mg\(^{2+} \), or ATP were calculated using the Michaelis–Menten equation of GraphPad Prism 5 (GraphPad Software).

**Results**

**UPLC-MS/MS analysis**

A representative chromatogram of the UPLC-MS/MS method is shown in Fig. 3. The retention time of mevalonate-5-phosphate and [13C, 2H3]-DL-mevalonate-5-phosphate was 2.4 min. The method was linear across the whole concentration range of 0.5 to 250 μmol/L (\( R^2 > 0.99 \)). The mean slope of the calibration lines (n = 5) was 0.059 (VK = 1.71). The lower limit of quantification (LLOQ) of the method was 5.0 fmol on column. The data of accuracy, intraassay and interassay imprecision of the UPLC-MS/MS method are presented in Table 1. The method showed high accuracy (± 2.7%) and the imprecision (CV) was ≤ 11% for all QC, which fulfills the FDA acceptance criteria. The matrix of the different samples tested in the post column infusion experiment had no influence on the MS signal at the retention time of derivatized MVAP. No cross-talk effect was observed since separate injection of analyte and internal standard showed no interference in the other MS/MS channel. MVAP was shown to be stable at room temperature for 12 h (97%) and after three freeze and thaw cycles (102%). Butylated MVAP and internal standard were stable for one week.

**MK activity assay**

The function of MK was significantly affected by the salt concentration and the pH of the buffer. No enzyme activity was detected for...
Tris-HCl concentrations below 20 mmol/L. For concentrations ≥ 100 mmol/L MK activity remained constant. The optimal pH ranged from 7.0 to 11.0. Values outside of this range resulted in markedly reduced enzyme activities (Fig. 4A). The activity was constant for ATP and Mg2+ concentrations above 2 mmol/L (Fig. 4B and C). The K_m values were K_m[Mg2+] 0.59 mmol/L (R^2 = 0.93) with a V_max[Mg2+] of 13.71 µmol MVA½/ C138 min/C2 mg MK½/C138 and K_m[ATP] 0.22 mmol/L (R^2 = 0.99) with a V_max[ATP] of 12.36 µmol MVA½/ C138 min/C2 mg MK½/C138. Based on these results, the following buffer concentrations have been used for subsequent experiments: 100 mmol/L Tris–HCl, 6 mmol/L MgCl2 and 4 mmol/L ATP at pH 7.0. The formation of MVAP was linear between 30 and 600 s (R^2 ≥ 0.99). The reaction was determined at varying substrate concentrations (Fig. 4D) and a K_m[MVA] value of 0.027 mmol/L (R^2 = 0.94) with a V_max[MVA] of 12.91 µmol MVA½/ C138 min/C2 nmol MK cut½/C138 was observed. The total imprecision of the enzyme assay combined with the quantification of MVAP, as determined by replicate analysis, was 8.3%.

MK variant V261A showed impaired enzyme activity

Affinity purification of wild-type and variant MK was performed by means of MBP-MK fusion proteins (MK fp). Subsequently, MBP was cleaved by proteolysis and dimeric MK (MK cut) was purified by SEC. However, the MBP-MK-V261A variant was not amenable to proteolytic cleavage. Therefore, the MBP-MK-V261A fusion protein was compared to the respective fusion protein of wild-type MK. Kinetic parameters are generally given as a function of the mass of the enzyme, however, MBP significantly contributes to the mass of MK fp. In order to compare activity of MK cut and MK fp, we analyzed enzyme kinetic parameters given as a function of amount of substance. Values of V_max [MK cut] 0.97 µmol MVA½/ (min × mg MK cut) and V_max [MK fp] 1.24 µmol MVA½/ (min × mg MK fp) revealed that enzyme function does not differ between MK cut and MK fp. Five independent measurements of enzyme activity were performed for WT MK fp and the MBP-MK-V261A variant with the conditions constituted as optimal. The variant showed significant impairment of specific enzyme activity of 53.1% (p < 0.001).

Discussion

The clinical spectrum of MKD comprises the milder HIDS phenotype, which is governed by recurrent febrile attacks, and severe MA with congenital dysmorphism, cataracts, cerebellar ataxia, and psychomotor retardation in addition to febrile episodes, associated with a shortened

Table 1

<table>
<thead>
<tr>
<th>Sample target concentration</th>
<th>LLOQ 0.5 µmol/L</th>
<th>QC 1 1.5 µmol/L</th>
<th>QC 2 37.5 µmol/L</th>
<th>QC 3 100 µmol/L</th>
<th>Pool ≈ 70 µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed mean concentration</td>
<td>0.49</td>
<td>1.46</td>
<td>37.6</td>
<td>100.8</td>
<td></td>
</tr>
<tr>
<td>Accuracy [%] (n = 5)</td>
<td>98.0</td>
<td>97.3</td>
<td>100.3</td>
<td>100.8</td>
<td></td>
</tr>
<tr>
<td>CV Intraassay [%] (n = 5)</td>
<td>10.7</td>
<td>6.67</td>
<td>2.77</td>
<td>1.20</td>
<td>2.47</td>
</tr>
<tr>
<td>CV Interassay [%] (n = 5)</td>
<td>8.23</td>
<td>8.84</td>
<td>0.42</td>
<td>3.58</td>
<td>5.18</td>
</tr>
</tbody>
</table>

Fig. 4. Enzyme activity of MK as a function of pH (A), ATP (B), MgCl2 (C) and R-mevalonate (D). (B–D) Enzyme kinetics followed a Michaelis–Menten model.
life-span. Interestingly, for both phenotypes residual function of the MK enzyme is severely impaired and confined to a narrow range of 0 to 10% in most of the cases. Therefore, precise determination of MK residual function is a prerequisite for robust prediction of a patient's clinical phenotype in clinical practice and for thorough characterization of MK mutations in basic research. In our approach, we applied isotope dilution UPLC-MS/MS as high-sensitivity technique for determination of low molecular weight molecules in order to detect MVAP as the product of the MK enzymatic reaction. In comparison to the radiochemical MK activity assay [12], mass spectrometry provides practical benefits. Handling of radioactive material is hazardous and requires adherence to highly regulated procedures including structural and administrative regulations. It was previously shown that MVAP and other intermediates of the isoprenoid biosynthesis could be quantified using HPLC-MS/MS (LLOQMVAP 4.17 μmol/L) and UPLC-MS/MS [3,18]. In comparison to previous studies, we were able to increase the sensitivity of quantification of MVAP substantially through derivatisation with n-butanol, making it suitable for the application in an enzyme activity assay. Detection of MVAP was linear for concentrations from 0.5 to 250 μmol/L. Quantification of MVAP was sensitive (LLOQ 5.0 fmol on column), accurate (±2.7%) and precise (CV < 11%). In a next step, we established the MK enzyme activity assay by adapting the conditions from the previously described radiochemical assay [12]. The phosphate buffer was replaced by Tris–HCl because of the low solubility of magnesium phosphate, which may lead to precipitation of Mg₅(H₂PO₄)₂ during sample preparation. The enzyme is sensitive to acidic conditions and Mg²⁺ in combination with ATP is necessary for the reaction [19]. These findings were substantiated in our study as MK activity depended on Mg²⁺ and ATP for concentrations below 2 mmol/L. Above these levels, the concentrations of Mg²⁺ and ATP had no influence on the velocity of the enzyme. The Kₘ(MVA) of 0.027 mmol/L determined by the method as presented here is comparable to the previously reported values for human recombinant protein [19–21], as well as to the values detected in human fibroblast [12,22].

As a proof of principle for application of this method to recombinant MK, we investigated the functional impairment of the mutation V261A, which is associated with an HIDS phenotype. The branched hydrophobic amino acid valine at position 261 is substituted by alanine, which also contains a hydrophobic side chain. However, alanine is structurally smaller with a straight chain shortened by two methyl groups as compared to valine. The residue 261 is located at the dimer interface of the MK protein (Fig. 2). The stability of the resulting protein was reduced, as it was only stable as fusion protein in the presence of the stabilizing MBP tag. Cleavage of the MBP-MK-V261A variant resulted in the formation of insoluble aggregated protein species. However, the recombinant dimeric MBP-MK-V261A fusion protein showed partially preserved specific activity of 53.1% as compared to the wild-type. In light of a residual activity of 1–7% determined in patient cells lines carrying the V261A mutation, the loss-of-function molecular phenotype is not exclusively caused by impairment of catalytic function. A major determinant of loss of function may rather be reduced structural stability causing elimination of the variant protein by the cellular quality control system. Earlier studies showed sensitivity of MK towards heat stress as a consequence of HIDS-causing mutations, indicating that loss-of-function due to a decrease of the MK protein level is a consequence of protein misfolding and/or aggregation [6,7]. Our data showing low stability but partially preserved specific activity support the notion of a protein misfolding molecular phenotype for the V261A variant. Protein misfolding induced by missense mutations is described in an ever-increasing number of inherited disorders [23]. For a number of these, conformational stabilization of misfolded proteins by small molecules acting as pharmaceutical chaperones has been shown and two small molecule compounds have been approved for treatment [24,25]. Consequently, small molecules targeting the MK protein may analogously improve MK stability and therefore increase the residual activity in patient cells. The novel method presented here is generally feasible for high throughput applications and may thus be used for screening of potential drug candidates.

In conclusion, the enzyme activity assay combined with isotope dilution UPLC-MS/MS presented in this study allowed for precise determination of specific activity of a variant recombinant MK protein, enabling the characterization of the impact of mutations on enzyme function. Moreover, adaptation of this method to the determination of MK activity in human cells may provide a robust tool for diagnosis and prediction of disease severity.

Acknowledgment

This research was funded by the Bavarian Genome Research Project (Junior Project #3). We wish to thank Heike Preiser for excellent technical assistance and Dunja Reiß for fruitful scientific discussion.

References

[21] Hinson DD, Chambless KL, Hoffmann GF, Kriis S, Keller RK, Gibson KM. Identification of an active site alanine in mevalonate kinase through characterization of a


