Identification of Catalytic Residues in Human Mevalonate Kinase*

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Availability of a recombinant form of human mevalonate kinase would facilitate studies on inherited mutations in this enzyme. Such an enzyme, available in a stable, highly purified form and in substantial amounts, could also be useful for investigation of the structure/function correlations that account for phosphohemevalonate production or for feedback regulation by geranyl and farnesyl pyrophosphates. Using cDNA that encodes the human enzyme (11), little is known about the active site amino acids that are important to enzyme function. Group-specific reagents have been employed to demonstrate that mevalonate kinase contains reactive cysteine (6) and lysine (12) residues. Recently, the first identification of an active site amino acid was accomplished when protein chemistry and mutagenesis work indicated that lysine-13 influences ATP binding (13).

The pGEM-3Z plasmid harboring the full-length cDNA encoding human mevalonate kinase was a gift from the Bristol-Myers Squibb Co. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Pfu polymerase from Stratagene, Inc., and deoxyoligonucleotides from Operon, Inc. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Pfu polymerase from Stratagene, Inc., and deoxyoligonucleotides from Operon, Inc. Fluorescein-labeled deoxyoligonucleotides were synthesized by the Protein and Nucleic Acid Shared Facility at the Medical College of Wisconsin. The pET-3d expression plasmid along with Escherichia coli strain BL21(DE3) were provided by Novagen. Isopropyl-1-thio-β-D-galactopyranoside was purchased from Research Products International. Kits for DNA purification were products of QIAGEN, Inc. Fast-Q anion exchange resin was a product of Pharmacia Biotech Inc. Toyopearl Ether 650-S hydrophobic interaction chromatography medium was from TosoHaas, Inc. β- Mevalonic acid lactone, farnesyl pyrophosphate, and all other biochemicals and reagents were purchased from Sigma unless otherwise specified.

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A summary of portions of this work has appeared (Potter, D., and Miziolek, H. M. (1997) FASEB J. 11, A887).

Experimental Procedures

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Catalytic Residues in Human Mevalonate Kinase

Methods

Construction of the Wild-type HMK/pET-3d Expression Plasmid—Insertion of cDNA into pET-3d utilized an NcoI site, which is positioned just downstream from a T7 promoter. Because the cDNA for HMK does not contain an NcoI site at its N terminus, it was necessary to create one. The pGEM-3Z/HMK construct was used as a template for a polymerase chain reaction in which the codon for leucine 2 (TTG) was changed to CAG, thus creating a single base mutation that created an NcoI site (CATGQ) that overlapped the start codon of mevalonate kinase. After ligation of the modified cDNA into the pET vector, the N-terminal coding sequence of the resulting HMK/pET-3d plasmid was confirmed by sequencing in both directions using an LKB-A.L.F. automated DNA sequencer and fluorescent-labeled primers.

Construction of Plasmids Encoding Mutant Mevalonate Kinases—All mutations were generated by overlap extension polymerase chain reaction (14). The coding sequences of the mutagenic primers follow: E19D, 5′-CCTTCATGGGAGCTCATTGAC-3′; E193Q, 5′-CAAGGGCGAGGATGATTCACGGG-3′; E193Q, 5′-CAAGGGCGAGGATGATTCACGGG-3′; E193Q, 5′-CAAGGGCGAGGATGATTCACGGG-3′; D204A, 5′-GGAGTGCCCATGCTGAGCACC-3′; D204N, 5′-GGAGTGCAAATGCTGAGCACC-3′; and E296Q, 5′-CCTCGTGCCGAAGAGGTCATGAC-3′. Each mutant was sequenced to verify the presence of the desired mutation and the absence of polymerase chain reaction-generated mutations.

Purification and Kinetic Characterization of Wild-type and Mutant Human Mevalonate Kinases—Purification of protein from extracts of E. coli transformants was performed through the Fast Q chromatography step as described for rat mevalonate kinase (13). The fractions containing mevalonate kinase were brought to an ammonium sulfate concentration of 1.5 M; mevalonate kinase does not precipitate under these conditions. The protein was centrifuged to remove insoluble contaminants, then loaded onto a Toyopearl Ether 650-s column (0.7×10 cm) equilibrated with 1.5 M (NH₄)₂SO₄ in 50 mM HEPES, 0.5 mM dithiothreitol, pH 7.5. Mevalonate kinase was eluted with a decreasing linear salt gradient (1.5 M to 0.5 M (NH₄)₂SO₄). Protein purity and subunit molecular weight were determined by SDS-PAGE (15). Routine protein concentrations were determined using the method of Bradford (16) with bovine serum albumin as a standard. The protein concentration in pure samples can be calculated from absorbance at 280 nm (ε = 0.98 ml/mg cm⁻¹). Enzyme activity was measured in the presence of 10 mM MgCl₂ at 30 °C, pH 7.0, in HEPES buffer (100 mM) using a spectrophotometric assay described elsewhere (6). The initial velocities at various substrate concentrations were fitted to the Michaelis-Menten and Lineweaver-Burk equations using the program Enz-Fitter (17).

ATP-SAP Binding Studies—The spin-labeled ATP analog, ATP-SAP, was synthesized as described previously (18). In a typical binding study, the concentration of ATP-SAP was kept constant at 30–40 μM and the concentration of protein (either wild type or mutant) was varied. Under the conditions used in this type of experiment, only unbound ATP-SAP produces a signal. Therefore, the fraction of ATP-SAP not bound in each sample was calculated by dividing the amplitude of the high field spectral line produced in the presence of various concentrations of protein by the amplitude of the signal produced in the absence of protein. A Scatchard plot of the data was used to determine the dissociation constants and binding stoichiometry of ATP-SAP for both wild type and mutant proteins. Using increased gain and modulation amplitude, the spectrum of bound ATP-SAP was also measured; rotational correlation times were calculated from bound spectra using the program of Freed and co-workers (19).

Competitive displacement experiments were performed to measure ATP’s equilibrium dissociation constants for both wild type and mutant proteins. Various amounts of MgATP were added to a mixture containing ATP-SAP (30–40 μM) and wild-type or mutant enzyme (25–75 μM). The signal produced by the displaced ATP-SAP indicates the concentration of the free analog. This estimate, together with the previously determined dissociation constant of the displaced ligand (Kd(ATP-SAP)) was used in a calculation (20) that provides a value for the dissociation constant of the competing ligand (Kd(Ligand)). The curve shown in Fig. 4B represents a smooth fit to the experimental data and would extrapolate to background levels of bound probe (<10%) at high ATP concentrations. Reported Kd values (Table II) represent the average of individual Kd estimates calculated from displacement experiments over the range of 20–80% ATP-SAP bound.

RESULTS

Isolation of Human Mevalonate Kinase—The pET-3d/HMK construct allowed over expression of mevalonate kinase wild type and mutant proteins in E. coli at a level of approximately 10% of total cellular protein after isopropyl-1-thio-β-D-galactopyranoside induction. Soluble mevalonate kinase is visible as a 42-kDa band after SDS-PAGE of the bacterial lysate and fractions from subsequent purification steps (Fig. 1). Fast-Q anion exchange chromatography of the bacterial supernatant produced an enzyme that accounts for >90% of the total protein at this stage of the preparation. Human mevalonate kinase, however, eluted from this anion exchange resin at a higher ionic strength than does the rat enzyme, reflecting the difference in isoelectric points for the human and rat wild type enzymes (4.8 versus 5.5, respectively; data not shown). An attempt to purify human mevalonate kinase using phenyl agarose chromatography failed because this protein proved to be so hydrophobic that it could not be recovered from the phenyl column. An alternative strategy was developed, which successfully exploited a less hydrophobic resin (Toyopearl Ether 650-s) to produce essentially homogeneous protein. The recombinant enzyme exhibited a molecular mass of 78 kDa upon analytical Superoxide 12 gel filtration chromatography (data not shown), indicating that the enzyme exists in solution as a homodimer of 42-kDa subunits.

An identical strategy was used to purify the E19D, E193A, E193Q, D204A, D204N, and E296Q mutant proteins. The mutants E19A, E19Q, and I20A were expressed after isopropyl-1-thio-β-D-galactopyranoside induction of E. coli transformants, but these proteins were completely insoluble and not further characterized. It is likely that the region in which Ghu-19 and His-20 reside has structural importance, potentially stabilizing a folding intermediate. For soluble mutant proteins other than E19D, it was observed that the protein eluted from the anion exchange column at a phosphate concentration 5–10 mM lower than that required for wild-type enzyme elution. This result is not surprising, inasmuch as replacement of a carboxylate anion with a neutral residue decreases the overall anionic characteristics of the protein. No difference in hydrophobic interaction chromatography was observed for sol-

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2 The abbreviations used are: HMK, human mevalonate kinase; MVA, mevalonic acid; PAGE, polyacrylamide gel electrophoresis; ESR, electron spin resonance; ATP-SAP, adenosine 5′-O-(acetamidophosphor)-3′-thiotriphosphate; FPP, farnesyl pyrophosphate.

![FIG. 1. SDS-PAGE of human mevalonate kinase isolated from E. coli lysate. Each lane of the Coomassie-stained gel contains 5 μg of protein. Figure shows molecular weight standards (lane 1), crude bacterial lysate (lane 2), 100,000 × g supernatant (lane 3), the pooled activity-containing fractions from the Fast Q anion exchange column (lane 4), the pooled activity-containing fractions from the Toyopearl Ether 650-s column (lane 5), pure E193Q (lane 6), pure D204N (lane 7), and pure D204A (lane 8). All samples were loaded on the gel under reducing conditions.](image-url)
The rate of mevalonate kinase activity was determined as a function of ATP concentration using several fixed concentrations of FPP. The data shown were fit to a linear competitive inhibition model, yielding the apparent Michaelis constants for ATP and DL-mevalonic acid are 74 and 24 μM, respectively (Table II). With regard to regulation of mevalonate kinase, several reports indicate that the enzyme is subject to feedback inhibition by the downstream polyisoprenoid products, geranyl pyrophosphate and farnesyl pyrophosphate (FPP) (7, 8, 22). As an additional test of the functionality of our recombinant human enzyme, we tested the efficacy of FPP as an inhibitor. Our results indicate that human mevalonate kinase is highly susceptible to FPP inhibition (K_i = 10 ± 1 μM), and that this inhibition is competitive with respect to ATP (Fig. 2). The results of this kinetic characterization indicate that the recombinant mevalonate kinase represents a valid model for the tissue-expressed enzyme. Furthermore, as discussed below, the protein’s affinity for the inhibitor FPP underscores mevalonate kinase’s potential regulatory function in the polyisoprenoid pathway.

**Rationale for Mutagenesis of Mevalonate Kinase**—The catalytic function of carboxylate side chains in phosphotransferase reactions has been well documented for several enzymes, which, like mevalonate kinase, utilize alcohol groups as acceptors. This precedent is based on mutagenesis experiments on phosphofructokinase (23, 24), human hexokinase (25), and phosphoribulokinase (26), as well as on analysis of the crystal structure of glyceraldehyde kinase (27). Carboxylates have also been implicated as ligands to the cation of the MgATP substrates of these and other phosphotransferases, such as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (28) and adenylate kinase (29). The importance of carboxylates to the function of mevalonate kinase is suggested by the observed sensitivity of this enzyme to modification by the water-soluble carbodiimides, N-(dimethylaminopropyl)-N'-ethylcarbodiimide and N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide.3 However, relatively poor protection from inactivation is provided by the substrates, suggesting that these group-specific reagents may modify mevalonate kinase at multiple sites, thus precluding the assignment of a particular function to any one residue. In view of these results, we employed a more focused experimental approach that utilizes the ample sequence data available for mevalonate kinases from a variety of diverse species. Although human mevalonate kinase contains 41 carboxylate residues, comparison of this sequence to those of the protein from *Rattus norvegicus* (30), Arabidopsis thaliana (31), *Saccharomyces cerevisiae* (32), *Schizosaccharomyces pombe*,4 and *Methanobacterium thermoautotrophicum*5 indicated only four invariant carboxylates (Fig. 3), which include three glutamic acids (Glu-19, Glu-193, and Glu-296) and one aspartic acid (Asp-204). All of these residues are located in highly conserved regions of mevalonate kinases from a variety of diverse species. Although human mevalonate kinase contains 41 carboxylate residues, comparison of this sequence to those of the protein from *Rattus norvegicus* (30), Arabidopsis thaliana (31), *Saccharomyces cerevisiae* (32), *Schizosaccharomyces pombe*,4 and *Methanobacterium thermoautotrophicum*5 indicated only four invariant carboxylates (Fig. 3), which include three glutamic acids (Glu-19, Glu-193, and Glu-296) and one aspartic acid (Asp-204). All of these residues are located in highly conserved regions of mevalonate kinase, an observation that would be expected for functionally important amino acids. Such a small number of conserved carboxylates makes site-directed mutagenesis an attractive approach to assess the function of these acidic amino acids.

**Consequences of Mevalonate Kinase Mutations at Glu-19, Glu-193, Asp-204, and Glu-296**—Replacement of Glu-296 with glutamine resulted in an enzyme that displayed kinetic properties very similar to those of wild type protein (Table II). The initial replacement of Glu-19 with an alanine residue resulted in expression of a protein that was completely insoluble. This was not entirely unexpected, inasmuch as alanine’s small aliphatic side chain bears no negative charge and is not isosteric with that of glutamic acid. The isosteric substitution of glutamic acid for Glu-19 was therefore engineered and the mutant.

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**TABLE II**

| Sample | V_max | K_m(DL-MVA) | K_m(AMP) | K_d(AMP) | K_d(AMP+AP) | n |
|--------|-------|-------------|-----------|-----------|-------------|---|
| WT     | 37 ± 1| 24 ± 3      | 74 ± 9    | 4 ± 0.5   | 2 ± 0.2     | 0.8 |
| E19D   | 15 ± 1| 52 ± 7      | 113 ± 11  | 2 ± 0.6   | 3 ± 0.2     | 0.9 |
| E193Q  | 0.69 ± 0.05 | 1020 ± 87 | 1560 ± 148 | 1240 ± 260 | 7 ± 0.7 | 1.0 |
| D204A  | (7.2 ± 0.4) × 10^-4 | 12 ± 2 | 66 ± 2 | 4 ± 0.8 | 6 ± 0.7 | 0.9 |
| D204A* | (8.3 ± 0.2) × 10^-4 | 20 ± 3 | 25 ± 2 | 4 ± 0.7 | 0.7 ± 0.07 | 0.9 |
| E296Q  | 40 ± 3 | 11 ± 2 | 131 ± 19 | 12 ± 1 | 10 ± 1 | 0.8 |

n denotes the calculated number of binding sites per enzyme subunit. MVA, D-l-mevalonic acid. WT, wild type.

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3 D. Potter, unpublished data.

4 B. G. Barrell, M. A. Rajandream, and S. V. Walsh, GenBank accession number Z54308.

5 S. Sharma and J. N. Reeve, GenBank accession number U47134.
Comparison of mevalonate kinase amino acid sequences. Sequences were aligned using the program "Pileup" in the GCG Wisconsin Sequence Analysis Package version 8.1 (Genetics Computer Group, Inc., Madison, WI). The three conserved domains that contain invariant acidic amino acids are shown. Invariant amino acids are in uppercase bold type. Hyphens found in the human sequence, and periods represent gaps in the sequence. The GenBank™ accession number for each sequence follows: Homo sapiens (Human), M88466; R. norvegicus (Rat), M29472; A. thaliana (Arabid.), M29472; S. cerevisiae (S. cerevisiae), X77795; S. pombe, Z64808; S. cerevisiae (S. cerevisiae), X55875; M. thermoautotrophicum (Methan.), U47134.

![Figure 3](image)

**Fig. 3.** Comparison of mevalonate kinase amino acid sequences. Data for the Scatchard plot (A) were obtained by mixing various amounts of protein (0–100 μM) with a fixed amount of ATP+SAP (52 μM). The amounts of free ([ATP+SAP]f) and bound ([ATP+SAP]b) spin label were determined by measuring the amplitude of the high field EPR spectral line as described under "Methods." The data were normalized with respect to enzyme concentration ([MK]).

**Fig. 4.** Binding of ATP+SAP and ATP to wild-type human mevalonate kinase. Data for the Scatchard plot (A) were obtained by mixing various amounts of protein (0–100 μM) with a fixed amount of ATP+SAP (52 μM). The amounts of free ([ATP+SAP]f) and bound ([ATP+SAP]b) spin label were determined by measuring the amplitude of the high field EPR spectral line as described under "Methods." The data were normalized with respect to enzyme concentration ([MK]). The amount of competitive displacement of the spin label ([B]) was determined by adding ATP+SAP (44 μM) to mevalonate kinase (35 μM) in the presence of various concentrations of ATP. The amount of spin label bound at zero competing ligand is defined as 100%.

![Figure 4](image)
each of the mutant enzymes listed in Table II, confirming that no changes in subunit composition resulted from mutagenesis.

Calculation of free and bound ATP/SAP is accomplished by monitoring the change in signal amplitude that results from binding of the spin-label. Performing such an analysis with samples that vary in the ATP/SAP/mevalonate kinase ratio allowed construction of a Scatchard plot (Fig. 4A), which indicates that ATP/SAP binds to the wild-type human enzyme both stoichiometrically ($n = 0.8$) and with high affinity ($K_d = 2.2 \mu M$). The bound analog was displaced by addition of ATP, indicating that the spin-probe binds at the substrate site. By titrating samples prepared using a fixed ratio of ATP/SAP/enzyme with variable amounts of ATP, the competitive displacement of the analog was measured (Fig. 4B) and a $K_{d(ATP)}$ ($4 \mu M$; Table II) was calculated using a method described previously (20). The value of this equilibrium binding constant for ATP indicates intrinsic affinity for the nucleotide that is considerably higher than might be inferred from apparent $K_m$ values, which are influenced by both catalytic and binding parameters.

The E19D, E193Q, D204A/N, and E296Q mutant proteins were examined in similar spin labeling experiments. The results of the Scatchard analyses (Table II) indicate that all the mutant proteins tested exhibited dissociation constants for ATP/SAP that were similar to the wild type protein, and that all possessed a full complement of ATP binding sites. This is not a surprising result in the case of E19D, E193Q, and E296Q, which possess substantial catalytic activity when compared with wild type enzyme. These experiments, however, provide a rigorous test of the structural integrity of mutants such as D204A and D204N, which have greatly diminished activity. These mutants also displayed tight and stoichiometric binding to the ATP/SAP spin label, confirming the presence of an intact active site. This indicates that these mutants are not inactive due to misfolding or other gross structural perturbations. Thus, inactivation in the case of the Asp-204 mutants results from a localized but chemically crucial change in the active site microenvironment (see below).

**DISCUSSION**

The recombinant human mevalonate kinase that has become available due to development of the methodology described in this report is a useful model protein for study of this enzymatic reaction. The recombinant human protein exhibits a specific activity that easily matches the values reported for tissue-derived or recombinant forms of the rat (8, 13) and pig (6, 21) enzymes. Michaelis constants show some interesting differences. The $K_{m(DL-MVA)}$ ($24 \mu M$), similar to values reported for the enzyme in human fibroblast or lymphocyte extracts (35) or for purified pig mevalonate kinase (6), is more than 10-fold lower than estimates for the rat enzyme (8, 13). Similarly, the $K_{m(ATP)}$ ($74 \mu M$) is much lower than the value ($1.75 \mu M$) reported for the rat enzyme (8) and slightly lower than values measured in human cell extracts ($0.4 \mu M$) or with pig enzyme ($0.3 \mu M$). These smaller differences may be due to the use of phosphate buffer, which may be expected to compete with ATP, in work with human cell extracts (35) and with the pig enzyme (6).

An even more notable difference between recombinant human mevalonate kinase and other mammalian enzymes is related to the inhibition of these enzymes by downstream metabolites in the biosynthetic pathway for isoprenoids and steroids. Farnesyl pyrophosphate exhibits a $K_i$ ($10 \mu M$) for human mevalonate kinase that is much lower than the estimate ($2.5 \mu M$) for the rat enzyme (8) or the estimate ($2 \mu M$) for geranyl pyrophosphate inhibition of the pig enzyme (7). Thus, human mevalonate kinase activity should clearly be influenced by changes in the physiological levels ($10^{-7}$ to $10^{-6}$ M) of these metabolites (9).

In reporting work on the cDNA-deduced sequence of the A. thaliana enzyme, Riou et al. (31) proposed that the region between residues 189 and 210 exhibited significant homology between mammalian, plant, and yeast enzymes. Although there are not lengthy invariant sequences in this stretch, our mutagenesis data confirm the importance of this region of the protein. Both Glu-193 and Asp-204, each of which exhibits a significant effect on enzyme function, map within this sequence. Thus, of the four regions of mevalonate kinase in which substantial homology has been perceived, two have now been implicated as contributing to the active site. In addition to our mapping of Glu-193 and Asp-204 within the residue 189–210 region, our work implicating Lys-13 in ATP binding (13) indicates that the homologous residue 10–30 sequence is also important to enzyme function. This region has also been suggested to be a peroxisomal targeting sequence (36). The two remaining homologous regions (residues 133–156 and 327–341) have been proposed to represent nucleotide binding motifs (30, 37). No direct experimental proof to support this hypothesis has yet become available. However, given the recombinant forms of mevalonate kinase that are now available to facilitate structural work on this enzyme, the function of these two regions is much more amenable to direct investigation.

There are considerable differences in our ability to assign precise functions to the four invariant acidic amino acids that represent the focus of this report. Clearly, the carboxyl function of Glu-296 is not important to substrate binding or catalysis, inasmuch as its substitution does not have significant consequences in this regard. Evaluation of Glu-19 is somewhat more difficult. This invariant residue is unlikely to have a crucial function in reaction chemistry. Substitution by aspartate has a minimal impact on kinetic parameters, whereas precedent (24) suggests that a large (>102-fold) effect would be expected for such a substitution if a catalytically important acidic amino acid was involved. Reasonably conservative substitution by glutamine or alanine results in production of an unstable, insoluble protein. Because this residue maps within the region of high homology that is closest to the N terminus, it is possible that elimination of the Glu-19 carboxyl affects folding of the remainder of the protein. The adjacent invariant His-20 may also be important in this context; we have observed that the H20A enzyme is also insoluble upon expression in E. coli.3 The location of residues 19 and 20 within a region of high homology would also be consistent with a function in stabilization of the protein’s quaternary structure. Mevalonate kinase is a dimeric enzyme, and it is possible that perturbations within the dimerization interface may preclude formation of a stable, soluble enzyme. Evaluation of Glu-193 is based on characterization of E193Q by both kinetic and physical binding studies. Kinetic studies indicate this substitution produces significant effects on several parameters. The 50-fold diminution in $V_{max}$ is a significant effect that slightly exceeds the magnitude of the observed increases in $K_m$ values for ATP (−20-fold) and mevalonate (−40-fold). These observations are quite similar to effects reported (38) upon elimination of the aspartate carboxyl, which provides a ligand to cation of the MgATP substrate of 6-phosphofructo-2-kinase (28). Moreover, elimination of the carboxyl group that interacts indirectly (by the intermediacy of a water molecule) with the cation of the MgATP substrate of either the ATP-dependent E. coli or pyrophosphate-dependent Propionibacterium freudenreichii 6-phosphofructo-1-kinase also causes larger effects on $K_m$ of the phosphoryl acceptor than on $K_m$ of the phosphoryl donor (24, 39). Physical (ESR) experiments indicate that E193Q mevalonate kinase efficiently and
stoichiometrically forms a binary complex with the spin-labeled ATP analog (ATPγSAP), exhibiting a $K_d$ (ATPγSAP) (7 μM) approaching the value (2 μM) measured with wild-type enzyme. Presumably, binding interactions due to the acetamidopropyl moiety (which may occupy the binding site of the phosphoryl acceptor) substantially contribute to this analog’s affinity, because E193Q’s equilibrium binding constant for ATP (measured in competitive displacement experiments) is elevated by >300-fold in comparison with the $K_d$ for wild-type enzyme (Table II). Because ATP lacks any adduct to the γ-moiety of ATP. Although other roles for Asp-204 cannot be strictly excluded, the magnitude of the observed effects of Asp-204 mutagenesis is comparable to that reported upon elimination of the catalytic base in other enzymes. Then, replacing the catalytic base in other enzymes (e.g., 6-phosphofructo-1-kinase, hexokinase) that catalyze phosphorylation of an alcohol and for which high-resolution structural data are available to confirm this functional assignment (33, 40). Thus, recombinant human mevalonate kinase has facilitated our formulation of a working hypothesis concerning the active site of this enzyme and provides us with a model system useful for future structural investigation and mechanistic tests of functional assignments.

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