Identification and Functional Characterization of an Active-site Lysine in Mevalonate Kinase*

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We report the construction of an expression plasmid for rat mevalonate kinase and the overexpression of recombinant enzyme in *Escherichia coli*. The homogenous enzyme had a specific activity of 30 units/mg and an observed subunit molecular mass of 42 kDa. The Michaelis constants ($K_m$) for DL-potassium mevalonate (288 $\mu$M) and for ATP (1.24 mM) were in agreement with values reported for enzymes isolated from rat liver (Tanaka, R. D., Schafer, B. L., Lee, L. Y., Freudenberg, J. S., and Mosley, S. T. (1990) *J. Biol. Chem.* 265, 2391–2398). Recombinant rat mevalonate kinase was inactivated by the lysine-specific reagent, pyridoxal phosphate (PLP). ATP (5 mM) afforded protection against inactivation, suggesting reaction of PLP with an active-site lysine. Mapping, isolation, and Edman degradation of the ATP-protectable peptide from [3H]PLP-inactivated borohydride-reduced mevalonate kinase allow assignment of lysine 13, a residue invariant in known mevalonate kinase sequences, as the modification site. These results represent the first identification of an active-site residue in mevalonate kinase. The function of lysine 13 was evaluated by replacing this residue with methionine. $V_m$ of the mutant protein is diminished by 56-fold, suggesting that lysine 13 facilitates catalysis. $K_m$ values of wild-type and mutant proteins for ATP were determined in electron spin resonance competition experiments. The observed 56-fold diminution in affinity for the mutant enzyme supports an additional role for lysine 13 in stabilization of ATP binding.

Mevalonate kinase (EC 2.7.1.36) catalyzes the transfer of the $\gamma$-phosphoryl group of ATP to the C-5 hydroxyl oxygen of mevalonic acid (1) to produce a key intermediate in the pathways for biosynthesis of polyisoprenoids and sterols. Recently, the significance of this enzyme has been elevated by the documentation of defects that arise when production of 5-phosphomevalonate is either diminished or elevated from the usual physiological range. Mevalonic aciduria is an inherited metabolic disease that results from diminished mevalonate kinase activity in infants and children (2). At least one missense mutation (N301T) has been documented as the cause of the disease in the index patient (3). Recently, diminished mevalonate kinase activity has been implicated in a second disorder of sterol metabolism, Zellweger syndrome (4). However, the molecular basis for the decreased mevalonate kinase activity associated with this disease remains to be elucidated. In contrast, human hepatocytes that overexpress mevalonate kinase as a result of in-sertional activation by hepatitis B virus have an increased rate of cellular transformation. Presumably, this results from changes in prenylation of hepatocyte signal transduction effectors (5). While the above observations suggest that a detailed understanding of the factors that modulate production of 5-phosphomevalonate would be desirable, mevalonate kinase has been subjected to far less scrutiny than other enzymes in the pathway for polyisoprenoid and sterol biosynthesis.

Kinetic studies (6) suggest that mevalonate kinase catalyzes an ordered sequential reaction; mevalonic acid is the first substrate bound, and phosphomevalonate is the first product released. The downstream products of the polyisoprenoid pathway, farnesyl pyrophosphate and geranyl pyrophosphate are potent competitive inhibitors of mevalonate kinase with respect to ATP (7). The enzyme has been reported to contain reactive cysteine (6) and lysine (8) residues. However, despite the recent elucidation of cDNA sequences for mevalonate kinase from a variety of species, the identity and functional significance of those reactive amino acids remain to be determined.

Availability of a recombinant form of the enzyme would facilitate studies aimed at elucidation of the structure/function correlations that account for the reaction catalyzed by mevalonate kinase as well as for the features responsible for feedback inhibition. Using cDNA that encodes rat mevalonate kinase (9), we have developed a bacterial expression system that allows overexpression and facile isolation of the enzyme. The utility of this recombinant model system is illustrated by a series of experiments that account for the first identification and functional assignment of a mevalonate kinase active-site amino acid. A summary of a portion of this work has recently appeared (10).

**EXPERIMENTAL PROCEDURES**

**Materials**

The pGR50 plasmid harboring the full-length (1286 base pairs) cDNA that encodes rat mevalonate kinase was a gift from the Bristol-Myers Squibb Co. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs Inc. 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) was provided by Novagen. Isopropyl-$\beta$-thiogalactopyranoside was purchased from Research Products International Corp. Kits for DNA purification were products of QIAGEN Inc. DNA was sequenced using an AutoRead sequencing kit and a Pharmacia-LKB-ALF DNA sequencer, both from Pharmacia Biotech Inc. Fast Q anion exchange resin was also a product of Pharmacia.

Phenyl-agarose, DL-mevalonic acid lactone, pyridoxal phosphate (PLP)1

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1 The abbreviations used are: PLP, pyridoxal phosphate; HPLC, high performance liquid chromatography; ESR, electron spin resonance; ATP$\gamma$S, adenosine 5'-O-(3-acetamidopropyl)-3-thioribosphosphate; ATP$\gamma$S, adenosine 5'-O-(thioribosphosphate).
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Construction of the Rat Mevalonate Kinase pET-11a Expression Plasmid—A pET-11a expression plasmid was selected because of its utility in overexpression in E. coli of cDNA-encoded proteins. Insertion of a cDNA into pET-11a must occur at an NdeI site that is positioned just downstream from a T7 promoter. Since the cDNA for rat mevalonate kinase does not contain an NdeI site, it was necessary to create one by modifying a single base upstream from the start codon. The cDNA was cut with NcoI, and the 68 base pair oligonucleotide cassette composed of 4 deoxyoligonucleotides, the sequences of which were identical to the amino-terminal coding sequence of mevalonate kinase except for two changes: 1) a silent mutation at position 51 of the coding sequence (CAT → CAC) that eliminated an internal NcoI site and 2) an extra T at the −1 position. The extra T created an NdeI restriction site that overlapped the start codon, allowing ligation into the pET-11a multiple cloning site immediately downstream from the Shine-Dalgarno sequence. The 3’ end of the cassette contained an NcoI overhang, allowing ligation to the downstream coding sequence of the NcoI-digested cDNA. The sequences of the deoxyoligonucleotides follow, with non-wild-type bases underlined: 1) 5’-TAT GTT GTC AGA AGT CCT GCT GTG TCC TGC-3’; 2) 5’-AGG GAA AGT CAT TCT CCA CGG AGA ACA TGC TGT GTC C-3’; 3) 5’-CAT GGA CCA CAG CAT GTT CCT GTG GGA CTA-3’; 4) 5’-GAA TGA CTT TCC CTG GAG CAC ACA CCA GCA GGA CTT CTC ACA A-3’. An identical procedure was used to construct the K13M mutant with the exception that the oligonucleotide cassette contained an additional mutation that changed the lysine codon (AAA) to a methionine codon (ATG). To confirm the integrity of the plasmid, DNA was sequenced in both directions using a Pharmacia AutoRead sequencing kit and a Pharmacia-LKB-ALF automated DNA sequencer with fluorescein-labeled primers.

Purification and Kinetic Characterization of Wild-type Rat Mevalonate Kinase and the K13M Mutant Protein—E. coli transformants containing the pET-11a construct were grown in 0.5 liter of LB medium at 37 °C, induced with isopropyl-1-thio-D-galactopyranoside when the absorbance reached 0.8, and harvested after 6 h of incubation. The cells were suspended and lysed by passage through a French pressure cell at 10,000 p.s.i. The lysate was centrifuged at 100,000 × g for 1 h, and the supernatant was dialyzed against 2 liters of buffer containing 25 mM HEPES, pH 7.5, and 1 mM dithiothreitol. The dialyzed supernatant was loaded onto a column (1 × 20 cm) of Fast Q anion exchange resin equilibrated with the same buffer. The column was washed until the absorbance at 280 nm reached 0.1, and mevalonate kinase was eluted with a 0–100 mM linear gradient of potassium phosphate at pH 8.0. The fractions containing mevalonate kinase were pooled, and solid (NH₄)₂SO₄ was added to bring the final salt concentration to 1.0 M. The protein was then loaded onto a phenyl-agarose column (1.6 cm × 20 cm) of Fast Q anion exchange resin equilibrated with 100 milliunits of immobilized chymotrypsin. After a 2-h digestion at 37 °C, the immobilized chymotrypsin was removed, and trifluoroacetic acid (0.1%) was added to the peptide mixture. The mixture was loaded onto the Vydac column and eluted with a linear gradient of acetonitrile (20–40%) over a period of 50 min at 0.7 ml/min. A single radioactive peak was detected by counting samples of the eluted fractions. The radioactive peak correlated with a single symmetric UV peak, suggesting the recovery of a highly purified peptide, an expectation validated upon subsequent Edman degradation analysis.

Table 1. Purification of recombinant mevalonate kinase

| Purification step | Protein Units | Specific activity | Yield |
|------------------|---------------|------------------|-------|
| Supernatant       | 288           | 3.38             | 100   |
| Fast Q           | 62            | 14.4             | 92    |
| Phenyl-agarose   | 20            | 30.8             | 61    |

Inactivation and Covalent Modification of Rat Mevalonate Kinase with PLP and [3H]PLP—[3H]PLP was prepared as described previously (15). The inactivation reaction was carried out on ice using mevalonate kinase (1 nmol) in 100 μl of HEPES buffer (50 mM, pH 7.5). PLP (or [3H]PLP) was added to the enzyme solution at zero time, and at the indicated time points samples were removed from the reaction and reduced in the presence of 10 mM NaBH₄ (2 mM 10 mM Na₂CO₃). After 5 min of reduction the enzyme activity was determined as described above. As a control, a sample of mevalonate kinase was incubated with borohydride only; control activity was equivalent to untreated enzyme. The stoichiometry of PLP incorporation was determined on a sample of enzyme modified with nonradioactive PLP. The reduced PLP-mevalonate kinase adduct was separated from unbound PLP on a Sephadex G-50 column, and the quantity of PLP that co-eluted with rat mevalonate kinase was determined by measuring the pyridoxamine absorbance peak at 327 nm (ε₃₂₇ = 9400 M⁻¹ cm⁻¹).

Proteolysis of [3H]PLP-labeled Mevalonate Kinase and Isolation of the Labeled Peptide—Borohydride-reduced [3H]PLP-labeled mevalonate kinase (15 nmol) was denatured, reduced with dithiothreitol, and carboxymethylated with iodoacetic acid. The protein was precipitated with trichloroacetic acid (6%) and pelleted, and the pellet was dissolved in NH₂HCO₃ (0.4 M) plus urea (4.8 M). Following dilution with 5 volumes of water, trypsin (20 μg or 1%) was added once every hour and stirred at 37 °C for a total of 4 h of digestion. The digestion was quenched by addition of trifluoroacetic acid (0.1%).

The mevalonate kinase peptides that resulted from exhaustive trypsin digestion were separated by HPLC using a Vydac C-18 (218TP54) reversed phase column equilibrated in aqueous trifluoroacetic acid (0.1%). The peptides were eluted with a linear gradient of acetonitrile (0–60%) over a period of 90 min at a flow rate of 0.7 ml/min. The column eluent was monitored at 215 nm using an absorbance detector with [3H]radionuclide activity measured in tandem using a radioactivity detector.

The radioactive peptide eluted at 58 min was recovered by using a fraction collector and bypassing the radioactivity detector. Because the 58-min fraction contained contaminating nonradioactive peptides visible on the UV trace, further purification of the radiolabeled peptide was required. The 58-min fraction was subjected to further digestion with 100 milliunits of immobilized chymotrypsin. After a 2-h digestion at 37 °C, the immobilized chymotrypsin was removed, and trifluoroacetic acid (0.1%) was added to the mixture. The mixture was then subjected to the Vydac column and eluted with a linear gradient of acetonitrile (20–40%) over a period of 50 min at 0.7 ml/min. A single radioactive peak was detected by counting samples of the eluted fractions. The radioactive peak correlated with a single symmetric UV peak, suggesting the recovery of a highly purified peptide, an expectation validated upon subsequent Edman degradation analysis.

ATP-SAP Binding Studies—The spin-labeled ATP analog, ATP-SAP, was synthesized as described previously (16). All spectra were acquired at 22 °C using a Varian E-Line Century Series 9 GHz EPR spectrometer. Rotational correlation times were calculated from bound spectra using the program of Freed and co-workers (17, 18). In a typical binding study, the concentration of ATP-SAP was kept constant at 30 μM, and the concentration of protein (either wild type or K13M) was varied. Under the conditions used to obtain the spectra, only the signal produced by unbound ATP-SAP is observed. Therefore, the fraction of ATP-SAP free 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; [ATP-SAP]free = ([ATP-SAP]total − [ATP-SAP]bound). A Scatchard plot of the data was used to determine the binding constants and binding stoichiometry of ATP-SAP to both wild-type and mutant proteins.

Competitive displacement experiments were performed in order to measure ATP binding constants for both wild-type and K13M proteins. Varying amounts of MgATP were added to a mixture containing ATP-SAP (30 μM) and wild-type or mutant enzyme (75 and 150 μM, respectively). The signal produced by the displaced ATP-SAP was used in a calculation (19) that allows determination of the binding constant for the competing ligand (Kₛₐₜₑₜ[Sᵣₑᵩ]) in terms of the known binding constant of the displaced ligand (Kₛₐₜₑₜ[Sᵣₑᵩ]).

RESULTS

Identification of an Active-site Lysine in Recombinant Rat Mevalonate Kinase—Overexpression of rat mevalonate kinase using a pET-11a vector facilitates isolation of homogeneous enzyme (Table I; Fig. 1) in amounts adequate to support detailed study by chemical and physical approaches. The recombinant enzyme has a subunit molecular mass (42 kDa, Fig. 1) and kinetic characteristics (Table II) that are comparable to trypsin, immobilized chymotrypsin, and all other biochemicals and reagents were purchased from Sigma unless otherwise specified.
those reported for the tissue-isolated protein (20), suggesting that the recombinant protein represents a useful experimental model system.

An early study on the hog liver enzyme (8) suggests the presence of a reactive lysine, but no identification or functional assignment to this or any other active-site amino acid in mevalonate kinase has been reported. To address this issue, the recombinant rat enzyme was subjected to incubation with [3H]pyridoxal phosphate. Borohydride reduction of the mixture resulted in the time-dependent inactivation of the enzyme (Fig. 2) with incorporation of 2 mol of pyridoxal phosphate per mol of enzyme subunit. When a protective agent such as ATP was included in the incubation, the enzyme was completely protected from inactivation and only 1 mol of pyridoxal phosphate was incorporated per mol of subunit. Conversely, high levels of mevalonic acid (50 mM) provided only modest protection from inactivation. The high level of efficacy of ATP in blocking inactivation implicated the protected amino acid as an active-site lysine. The marked protection afforded by ATP was also reflected in the striking differences apparent upon comparison of tryptic peptide maps produced when [3H]pyridoxal phosphate was used to modify the enzyme in the presence or absence of ATP (Fig. 3). The distribution of radioactivity in this chromatogram is compatible with the equivalent labeling of two distinct sites in the sample (Fig. 3A), one of which is protected against modification in the presence of ATP (Fig. 3B). The “ATP-protectable” radioactive peak apparent at 58 min was recovered for further HPLC analysis. The presence of unlabeled contaminating tryptic peptides that flanked the radioactive peptide (detectable in the A215 nm HPLC map; data not shown) prompted a subsequent chymotryptic cleavage of the partially purified peptide. This digestion produced a shorter radiolabeled peptide obtainable in highly purified form under modified HPLC conditions. Edman degradation analysis allowed assignment of the 10 amino acids that comprise the chymotryptic peptide. The sequence obtained is in agreement with the deduced amino acid sequence near the amino-terminal end of mevalonate kinase (9). The release of a [3H]-labeled derivative in cycle 7, together with the absence of the phenylthiohydantoin-lysine that would appear at easily detectable levels in this step if no modification of mevalonate kinase had been performed, indicated that lysine 13 is the active-site target of pyridoxal phosphate. Identification of this residue, an invariant amino acid in all mevalonate kinase sequences reported to date, coupled with the observation of protection against pyridoxal phosphate modification by ATP, allows the first assignment of a mevalonate kinase active-site residue.

**Assignment of Function to Lysine 13; Kinetic Characterization**—The basic properties of the side chain of lysine 13, together with the observation that ATP protected this residue against modification by pyridoxal phosphate, not only argues for an active-site location for this lysine but also prompts speculation that lysine 13 could stabilize ATP binding because of interaction between the positively charged ε-amino group and the negatively charged phosphoryl groups of ATP. In a test of this hypothesis, site-directed mutagenesis was employed to substitute methionine for lysine at position 13. The mutant mevalonate kinase was expressed and isolated (Fig. 1) in a manner identical to that observed for wild-type enzyme. Kinetic characterization (Table II) indicated that elimination of the basicity at residue 13 depresses catalytic turnover by 56-fold. An effect of this magnitude suggests that lysine 13 facilitates catalysis but is not crucial to reaction chemistry.

Substrate $K_m$ values for the mutant enzyme change by approximately 1 order of magnitude, becoming numerically larger for mevalonate and smaller for ATP. Since for an ordered sequential reaction $K_m$ is a complex parameter that includes elements of both rate and binding constants, extrapolation from empirically determined apparent $K_m$ values to binding affinities may be misleading. For this reason, a more reliable evaluation of the possible function of the residue 13 side chain in stabilizing ATP binding was afforded by the determination and comparison of physical binding constants.

**Biophysical Characterization of Wild-type and Mutant Mevalonate Kinases**—The spin-labeled ATP analog, ATP-SAP (16), contains a paramagnetic PROXYL group tethered to the γ-phosphoryl of ATP-S. In buffered solution, this analog exhibits the sharp three-line ESR signal typical of nitroxide spin-labels free in solution (Fig. 4A). Upon binding to mevalonate kinase, the amplitude of signals attributable to free ATP-SAP diminishes and the broad spectral features assignable to an immobilized spin-label become apparent (Fig. 4B). The spectrum of the bound species exhibits a maximum hyperfine separation of 64.4 G. Spectral simulation of the bound component

![Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant mevalonate kinase isolated from E. coli lysate.](image)

**Summary of kinetic and binding properties of wild-type mevalonate kinase and K13M**

The sequence obtained by Edman degradation and the picomole recovery of each amino acid follows: Leu (17.2), Val (17.9), Ser (6.24), Ala (17.0), Pro (15.4), Gly (13.9), X, Val (13.2), Ile (18.5), and Leu (12.1). Assignment of a lysine as residue 7 correlates with release of tritium during this cycle. This assignment creates a sequence that exactly matches that of mevalonate kinase residues 7–16.

**Table II**

| Sample | $V_m$ ($\mu$mol/min/mg) | $K_{m,min}$ (MVA) | $K_{m,ATP}$ | $K_{m,SAP}$ | $K_{S,ATP}$ | $K_{S,SAP}$ | $n$ |
|--------|------------------------|------------------|--------------|--------------|--------------|--------------|-----|
| Wild type | 30.2 ± 5.7 | 288 ± 22 | 1240 ± 60 | 1.94 ± 0.39 | 3.12 ± 0.41 | 0.95 |
| K13M | 0.536 ± 0.02 | 2880 ± 250 | 166 ± 33 | 110 ± 13 | 49.3 ± 4.3 | 1.05 |
(17, 18) indicates a correlation time, \( \tau_c = 26 \text{ ns} \). Such a value corresponds to immobilization of the nitroxide on a spherical particle with a Stokes radius of 29 Å. Assuming a partial specific volume of 0.75 ml/g, a protein with such a Stokes radius would have a molecular mass of 83 kDa, in good agreement with a dimeric form for a mevalonate kinase composed of 42-kDa subunits.

Calculation of free and bound ATP\(\gamma\)SAP is accomplished by monitoring the change in signal amplitude due to the free analog. Performing such an analysis with samples that vary in the ATP\(\gamma\)SAP/mevalonate kinase ratio allowed construction of a Scatchard plot, which indicated that mevalonate kinase binds this analog both stoichiometrically \((n = 0.95)\) and with high affinity \((K_d = 3.1 \mu\text{M}; \text{Table II})\). The bound analog was displaced by addition of ATP (Fig. 5A), indicating that this probe binds at the substrate site. By titrating samples prepared using a fixed ratio of ATP\(\gamma\)SAP/enzyme with variable amounts of ATP, the competitive displacement of analog was monitored (Fig. 5A) and a \(K_{d,\text{ATP}}\) \((1.9 \mu\text{M}; \text{Table II})\) was calculated (19).

When the mevalonate kinase mutant that contains a methionine at position 13 was used for similar ESR binding experiments, a binding stoichiometry \((n = 1.05\) and \(\tau_c\) \((26 \text{ ns})\) comparable to that observed with wild-type enzyme were observed. These data represent a rigorous test of the integrity of the tertiary structure of the mutant, indicating that it contains a full complement of functional substrate binding sites, although the observed affinity for ATP\(\gamma\)SAP was diminished \((K_{d,\text{ATP},\gamma\text{SAP}} = 49 \mu\text{M}; \text{Table II})\). Moreover, when ATP was used to competitively displace the analog from the K13M mutant (Fig. 5B), data analysis generated a \(K_{d,\text{ATP}}\) \((110 \mu\text{M}; \text{Table II})\) that indicated >50-fold weakening of ATP binding to the mutant mevalonate kinase in comparison with that observed for wild-type enzyme. Thus, the collected data support the hypothesis that the lysine 13 \(\varepsilon\)-amino stabilizes binding of the substrate ATP.

**DISCUSSION**

The development of an expression system that produces a recombinant form of eukaryotic mevalonate kinase in the amounts documented in this report will undoubtedly expedite progress toward elucidation of the molecular basis for catalysis of 5-phosphomevalonate production. The high level of mevalonate kinase expression simplifies isolation of a homogeneous protein that exactly matches the amino acid sequence of the tissue-derived protein. The physical and kinetic properties of the enzyme expressed in *E. coli* argue strongly for the fidelity of this recombinant model and validate its expanded use in future studies.

In initial protein chemistry work on mevalonate kinase, we were guided by earlier reports on the sensitivity of the enzyme to various modification reagents (6, 8). The strategy of pursuing a reactive lysine was based on an expectation that pyridoxal phosphate, which may be expected to function as a group-specific modification reagent, could preferentially bind to the active site of a phosphotransferase due to the phosphoryl moiety of the reagent. The high degree of protection afforded by ATP against inactivation of mevalonate kinase by pyridoxal phosphate supported this expectation. This observation, coupled with the subsequent mapping of the protectable lysine as a residue invariant in the mevalonate kinase sequences, supported a provisional active-site assignment to lysine 13.

Another potential function of lysine 13 involves peroxisomal
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Fig. 4. Binding of ATPγSAP to mevalonate kinase. Conventional X-band ESR spectra are shown for ATPγSAP (30 μM) in buffer alone (panel A) or in the presence of 100 μM mevalonate kinase (panel B). The spectrum in panel B was recorded at a 2.5× higher gain than the spectrum in panel A in order to show the hyperfine spectral features. Spectral conditions: scan range, 100 G; modulation amplitude, 1 G for unbound and 5 G for bound ATPγSAP; modulation frequency, 100 kHz; microwave power, 5 milliwatts for unbound and 10 milliwatts for bound ATPγSAP. Each spectrum represents the average of four scans, 4 min each. Measurements were recorded at 22 °C.

targeting of mevalonate kinase. Rachubinski and Subramani (23) propose that peroxisomal targeting may be mediated by peroxisomal targeting sequence 2, which is characterized by the consensus sequence (R/K)(L/V/I)((X)?)(H/Q)/(L/A). Lysine 13 corresponds to the first residue of this peroxisomal targeting sequence 2 motif. However, Purdue and Lazarow (24) do not suggest an alternative to arginine as the first residue of this consensus sequence. Furthermore, Tsukamoto et al. (25) report that lysine cannot replace arginine in this targeting sequence. Additionally, the work of Glover et al. (26) suggests that ketoacyl-CoA thiolase is poorly targeted to the peroxisome when lysine replaces arginine in the targeting sequence. Nonetheless, Krisans and co-workers (27, 28) report the peroxisomal localization of mevalonate kinase, suggesting that lysine 13 may well function as part of a targeting sequence.

Regardless of whether lysine 13 is crucial to peroxisomal targeting, our data clearly implicate lysine 13 as an active-site residue. The magnitude in diminution of Vm observed upon conservative substitution to eliminate basicity at residue 13 suggests that lysine 13 significantly influences but is not crucial to catalysis. Any contribution to substrate binding would be difficult to evaluate on the basis of the contrasting changes in substrate Km values, which are not necessarily reliable indicators of substrate affinity. Thus, the availability of sufficient amounts of homogeneous protein for binding experiments was important to any evaluation of the lysine 13 function. The other important tool for which studies was a novel spin-labeled ATP analog that we recently reported in studies on another phosphotransferase (16, 22). The results of binding studies for ATPγSAP and of competition experiments with ATP clearly demonstrate that substitution to eliminate the basicity of lysine 13 impairs the binding of adenine nucleotides to mevalonate kinase. The >50-fold effect on ATP Kf values is compatible with hypotheses that lysine 13 either stabilizes the negative charge of the ATP by an electrostatic interaction with the phosphoryl groups or hydrogen bonds to another region in the nucleotide.

With respect to ATP binding, three domains that have highly conserved amino acid sequences exist in mevalonate kinase. One of these domains found in a central region of the protein has been proposed by Tsay and Robinson (29) to be part of an ATP binding consensus sequence. The consensus they propose, GXGXXAX(13–26)K, is found in Saccharomyces cerevisiae mevalonate kinase, with the lysine 24 residues downstream from the consensus alanine. However, when comparing sequences of mevalonate kinases from a variety of species, the lysine contained in this consensus sequence is not invariably conserved. In the rat and human enzymes it is 22 residues downstream from the consensus alanine, in the A. thaliana sequence it is 18 residues downstream, and in the sequences from Schizosaccharomyces pombe and Methanobacterium thermoautotrophicus no lysine is found within 26 residues of the consensus alanine.

A second domain that has been proposed as an ATP binding motif resides in the carboxyl-terminal region of both the human (3) and the rat proteins (9). This sequence conforms to the GXGXXGX(13–21)AXX consensus found in protein kinases. Human and rat mevalonate kinases contain the AXX motif 17 residues downstream from the last consensus glycine. However, the sequences from Arabidopsis thaliana, S. pombe, S.

Fig. 5. Displacement of bound ATPγSAP by ATP. ATPγSAP (30 μM) was added to wild-type mevalonate kinase (75 μM, panel A) or K13M (150 μM, panel B) in the presence of various concentrations of ATP. The amount of ATPγSAP displaced by ATP in each sample was determined by measuring the amplitude of the high field EPR spectral line. ATP was prepared as its magnesium complex by mixing 1 mol of ATP with 1.2 mol of MgCl2. Spectral conditions were as in Fig. 4A.
cerevisiae, and M. thermoautotrophicus do not contain such a motif.

The nucleotide binding sequences found in numerous proteins including protein kinases (30) as well as a variety of other nucleotide-binding proteins and phosphotransferases have been reported (21). A striking feature common to all these consensus sequences is the presence of either a lysine or arginine residue that interacts with a phosphoryl group on the nucleotide. This report on the function of the mevalonate kinase lysine 13 provides the only evidence for participation of an invariant basic residue in ATP binding. Without any experimental evidence to directly implicate the proposed central or carboxyl-terminal consensus sequences in nucleotide binding, it is difficult at present to evaluate their significance. However, our recombinant model may facilitate investigation of this issue.

Acknowledgments—Dr. Jennifer Runquist synthesized the [3H]pyridoxal phosphate used in these studies. Kelly Chun assisted in construction of the expression plasmid that encodes the K13M mutant. Edman degradation was performed under the direction of Dr. Liane Mende-Mueller at the Protein and Nucleic Acid Shared Research Facility, Medical College of Wisconsin. We thank Dr. G.W. Robinson and Bristol-Myers Squibb for supplying plasmid pGR50.

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