Identification of active site residues in mevalonate diphosphate decarboxylase: Implications for a family of phosphotransferases

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Abstract

A combination of sequence homology analyses of mevalonate diphosphate decarboxylase (MDD) proteins and structural information for MDD leads to the hypothesis that Asp 302 and Lys 18 are active site residues in MDD. These residues were mutated to replace acidic/basic side chains and the mutant proteins were isolated and characterized. Binding and competitive displacement studies using trinitrophenyl-ATP, a fluorescent analog of substrate ATP, indicate that these mutant enzymes (D302A, D302N, K18M) retain the ability to stoichiometrically bind nucleotide triphosphates at the active site. These observations suggest the structural integrity of the mutant MDD proteins. The functional importance of mutated residues was evaluated by kinetic analysis. The 10^3 and 10^5 -fold decreases in k_{cat} observed for the Asp 302 mutants (D302N and D302A, respectively) support assignment of a crucial catalytic role to Asp 302. A 30-fold decrease in activity and a 16-fold inflation of the K_m for ATP is documented for the K18M mutant, indicating that Lys 18 influences the active site but is not crucial for reaction chemistry. Demonstration of the influence of conserved aspartate 302 appears to represent the first documentation of the functional importance of a residue in the MDD catalytic site and affords insight into phosphotransferase reactions catalyzed by a variety of enzymes in the galactokinase, homoserine kinase, mevalonate kinase, phosphomevalonate kinase (GHMP kinase) family.

Keywords: mevalonate diphosphate decarboxylase; mevalonate pyrophosphate decarboxylase; GHMP kinase family; active site mapping; general base catalyst

The mevalonate pathway for isoprenoid biosynthesis is utilized in selected bacteria and the reactions are also part of the sterol biosynthetic pathway in eukaryotes. Production of isopentenyl diphosphate from mevalonic acid requires three consecutive ATP-dependent reactions, the last of which is catalyzed by mevalonate diphosphate decarboxylase (MDD; EC 4.1.1.33). This enzyme catalyzes the divalent cation-

dependent decarboxylation of mevalonate diphosphate (also referred to as mevalonate pyrophosphate, MPP) to isopentenyl diphosphate (or isopentenyl pyrophosphate, IPP) with concurrent hydrolysis of ATP to form ADP and inorganic phosphate (Reaction 1; Bloch et al. 1959).

Reaction 1

Several investigations have suggested the importance of the MDD-catalyzed reaction to cholesterol biosynthesis

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Abbreviations: MDD, mevalonate diphosphate decarboxylase; MK, mevalonate kinase; CDP, cytidine-5'-diphosphate; GHMP, galactokinase, homoserine kinase, mevalonate kinase, phosphomevalonate kinase; MPP, mevalonate diphosphate/pyrophosphate; IPP, isopentenyl diphosphate/pyrophosphate.

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(Ramachandran and Shah 1976; Sawamura et al. 1992). Experiments with spontaneously hypertensive rats indicated that low MDD activity is responsible for reduced cholesterol biosynthesis (Sawamura et al. 1992). Additionally, inhibition of MDD by a fluorinated substrate analog correlates with a diminution in cholesterol levels (Nave et al. 1985; Reardon and Abeles 1987).

Little is known about the enzymatic apparatus that supports conversion of mevalonate diphosphate to isopentenyl diphosphate. Recently the structure of the yeast MDD was solved by X-ray crystallography (Bonanno et al. 2001). The available structure does not contain any bound metabolites and thus provides limited information on the active site and mechanism of catalysis. However, the structure of yeast MDD supports assignment of this enzyme to the GHMP kinase family of enzymes (Krishna et al. 2001), an observation that was suggested as useful for guiding experimentation aimed at identification of the active site (Bonanno et al. 2001). The GHMP kinase family of enzymes includes mevalonate kinase (MK). Previous studies in our laboratory (Potter and Miziorko 1997; Potter et al. 1997; Cho et al. 2001) have implicated several residues in the phosphotransferase reaction catalyzed by mevalonate kinase. Observations based on a sequence homology analysis for MDD proteins from a variety of prokaryotic and eukaryotic species have been combined with information that emerged from the MDD (Bonanno et al. 2001) structure. The results generated by the combination of approaches prompt predictions of amino acid residues in the active site of MDD. This report describes the process for evaluation of two closely juxtaposed invariant residues as active site candidates as well as tests of the functional importance of these residues in mevalonate diphosphate decarboxylase.

Results

Characterization of recombinant yeast mevalonate diphosphate decarboxylase

Recently cDNA encoding yeast MDD (Toth and Huwyler 1996) has been engineered into a his-tagged expression construct (pSKB-2), supporting protein expression/isolation that led to crystallization and elucidation of a structure (Bonanno et al. 2001). Detailed characterization of the recombinant enzyme has not, however, been reported. Induction at 37°C does not result in substantial production of stable MDD. In contrast, strong expression is induced by addition of IPTG at 22°C; soluble protein is purified to homogeneity using affinity Ni-NTA agarose chromatography as described in Materials and Methods. The purity of the protein has been confirmed by SDS-PAGE (Fig. 1). The specific activity of purified recombinant yeast MDD is 6.4 units/mg; the typical yield of isolated protein from 1 L of induced *Escherichia coli* culture is 100 mg. A subunit molecular

Figure 1. SDS-PAGE of recombinant wild-type and mutant yeast mevalonate diphosphate decarboxylase proteins. Each lane of the Coomassie blue-stained gel contains approximately 10 μ g of protein. Samples include molecular weight standards (lane 1); total lysate of *E. coli* induced for expression of wild-type MDD (lane 2); 100,000 g supernatant (lane 3); NTA agarose purified MDD proteins (wild-type, lane 4; K18M, lane 5; D302A, lane 6; D302N, lane 7).

weight of 48 kDa is estimated for MDD under SDS-PAGE conditions (Fig. 1). Analytical gel-filtration experiments under nondenaturing conditions (Fig. 2) were used to estimate a native molecular weight of 107 kDa. The results are compatible with the suggestion that recombinant MDD exists as a homodimer in solution, consistent with the properties reported for eukaryotic MDD enzymes from tissue or cells (Cardemil and Jabalquinto 1985; Chiew et al. 1987; Cordier et al. 1999).

Figure 2. Analytical gel filtration of mevalonate diphosphate decarboxylase (MDD) under nondenaturing conditions. Chromatography was performed using a Pharmacia FPLC Superose 6 (1 $\times$ 30 cm) column. The figure depicts the elution properties of MDD (filled square) and various molecular weight standards (open circles): apoferritin, 443 kDa; pyruvate kinase, 237 kDa; alcohol dehydrogenase, 141 kDa; HMG-CoA synthase, 116 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa. Protein samples (100 μ g) were loaded on the column and eluted with 50 mM HEPES, 150 mM KCl (pH 7.5). The sample elution volume (V_e) is normalized to the column void volume (V_o), determined using Blue Dextran.

Strategy for prediction of active site residues in MDD

Deduced amino acid sequences for prokaryotic and eukaryotic proteins proposed to be mevalonate diphosphate decarboxylases were aligned using the BioEdit program (Hall 1999). Alignment indicated over 20 highly conserved residues with side chains that could support substrate binding or catalysis. Sequence alignment (Fig. 3) of some representative MDD proteins illustrates that Lys 18 and Asp 302 are invariant residues. A more extensive alignment (>25 deduced sequences) indicates that Asp 302 is the only strictly invariant acidic residue; acidic residues are commonly involved in phosphotransferase reactions as either general base catalysts or as ligands to cation of the M^{2+} -ATP substrate. Moreover, examination of combined alignment and protein structure information indicates that the Lys 18/Asp 302 MDD residues represent the only closely juxtaposed (2.75 Å; Bonanno et al. 2001) invariant acid/base pair. In MDD protein, this pair of residues maps in the interdomain hinge region associated with the ATP-binding site in the GHMP kinase family of proteins. For these reasons, a functional test of the closely juxtaposed residues Asp 302 and Lys 18 was implemented by mutagenesis of the cDNA that encodes these residues. Plasmids encoding K18M, D302A, and D302N mutants were engineered by PCR-based mutagenesis. Expression and isolation of these mutant proteins in highly purified form (Fig. 1) were performed using the protocol developed for the wild-type recombinant enzyme.

Biophysical characterization of wild-type and mutant MDD proteins

To test whether MDD mutant proteins retain active sites that are substantially comparable to wild-type enzyme, an at-

tempt was made to identify an active site probe that would be useful for comparison of these proteins. Previous studies on phosphotransferases (Runquist et al. 1996; Cho et al. 2001) suggested the utility of the fluorescent ATP analog trinitrophenyl-ATP (TNP-ATP), which has accordingly been evaluated as an active site probe for MDD. The binding of TNP-ATP to wild-type and mutant MDD proteins was studied by measuring the change in the emission spectra of the TNP-ATP upon binding to MDD (Fig. 4). The change of the maximum emission wavelength from 555 to 540 nm and substantial enhancement of fluorescence indicate the binding of the analog to the enzyme. Upon titrating the wild-type enzyme with TNP-ATP, a "blue shift" of the emission spectra is observed, indicating a relatively hydrophobic binding site for TNP-ATP. The stoichiometry for TNP-ATP binding to wild-type and mutant MDD proteins was obtained from the X-axis intersection points of Scatchard plots of the binding data; equilibrium binding constants were calculated from the slopes. An example of such a Scatchard plot for TNP-ATP binding to wild-type MDD is shown in Figure 5. An estimate of $K_d(\text{TNP-ATP}) = 0.49 \mu\text{M}$ and a binding stoichiometry (n) of 1.07 per subunit is calculated from such data. The comparable values for binding stoichiometry ($n = 0.97$ – 1.12) and affinity ($K_d = 0.13$ – $0.42 \mu\text{M}$) measured for mutant MDD proteins (Table 1) indicate that the mutants retain a functional substrate-binding site and, therefore, substantial overall structural integrity. Affinity for substrate ATP, $K_d(\text{ATP}) = 169 \mu\text{M}$, was measured in competitive displacement experiments (Mildvan and Cohn 1965), which involved titration of the TNP-ATP–enzyme complex with Mg-ATP. The binding constants for substrate ATP to wild-type and several mutant MDDs are summarized in Table 1.

Figure 3. Sequence alignment of mevalonate diphosphate decarboxylase proteins. The regions flanking Lys 18 and Asp 302 are displayed. All sequences were obtained from public databases. Alignment was generated using the BioEdit program (Hall 1999). Residues that are identical are shown with a black background. Similar residues are indicated by a gray background. Numbers appearing above the sequence alignment correspond to the amino acid numbering of *Saccharomyces cerevisiae* MDD. Asterisks indicate residues Lys 18 and Asp 302. Sequences corresponding to the following organisms and accession numbers are included: *Saccharomyces cerevisiae*, P32377; *Streptococcus pyogenes* M1 GAS, AAK33797; *Caenorhabditis elegans*, NP_496967; *Halobacterium* sp. NRC-1, D84217; *Homo sapiens*, P53602; *Lactococcus lactis*, E86675; *Mus musculus*, CAC35731; *Rattus norvegicus*, AAB00192; *Staphylococcus aureus*, BAB56753; *Arabidopsis thaliana*, T52625.

Figure 4. Fluorescence enhancement of TNP-ATP upon binding to MDD. The upper spectrum represents the fluorescence emission ($\lambda_{\text{max}} \sim 540$ nm) of a sample containing 2 μM TNP-ATP and 3.14 μM wild-type MDD in 100 mM Tris/Cl, 100 mM NaCl, 10 mM MgCl_2 (pH 7.0). The lower spectrum is measured for 2 μM TNP-ATP at the same buffer conditions, but in the absence of MDD. Excitation wavelength used in these experiments is 408 nm.

The dissociation constants for Asp 302 mutants to Mg-ATP are approximately fivefold lower than for the wild-type enzyme. This small effect may be explained by the reduction of the negative charge in the active site and thus higher affinity for the negatively charged ATP. A smaller but opposite effect is observed upon elimination of the positively charged side chain in K18M ($K_{d(\text{ATP})} = 270$ μM). Apparently, the positively charged ϵ -amino group of Lys 18 stabilizes the binary complex of ATP with enzyme. On the basis of similarities between wild-type and mutant MDD proteins in TNP-ATP binding to form a binary complex of enzyme with this substrate analog, it appears reasonable to interpret the results of kinetic characterization of these proteins without any obvious concern over major structural perturbations.

Kinetic studies of wild-type and mutant MDD enzymes

The $V_{\text{max}} = 6.4 \pm 0.17$ units/mg measured for recombinant wild-type MDD is in good agreement with the value of 6.0

Figure 5. Scatchard plot of the data for TNP-ATP binding to wild-type MDD. $[\text{TNP-ATP}]_b$ and $[\text{TNP-ATP}]_t$ represent the concentration of bound and free TNP-ATP, respectively. $[\text{MDD}]$ indicates the subunit concentration of wild-type MDD.

Table 1. Summary of equilibrium binding parameters for wild-type and mutant MDD enzymes to substrate analog TNP-ATP and to ATP

Enzyme	$K_{d(\text{TNP-ATP})}$ (μM)	n^a	$K_{d(\text{Mg-ATP})}$ (μM)
Wild type	0.49 ± 0.04	1.07 ± 0.12	169 ± 17
K18M	0.42 ± 0.03	0.97 ± 0.08	270 ± 20
D302A	0.13 ± 0.02	1.05 ± 0.24	30 ± 5
D302N	0.17 ± 0.03	1.12 ± 0.24	28 ± 4

K_d for TNP-ATP and ATP with MDD in 100 mM Tris/HCl, 100 mM NaCl (pH 7.4) in the presence of 10 mM MgCl_2 at 30°C. $K_{d(\text{TNP-ATP})}$ was calculated by Scatchard analysis of binding data obtained by titration of the enzyme with TNP-ATP. $K_{d(\text{Mg-ATP})}$ was calculated in competitive displacement experiments by titration of the TNP-ATP-enzyme complex with ATP (Mildvan and Cohn 1965).

^a The n values (binding stoichiometry) for TNP-ATP binding to wild-type and mutant MDD proteins were obtained from the X-axis intersection points of the Scatchard plots.

units/mg reported for MDD isolated from yeast cells (Dhe-Paganon et al. 1994), suggesting the utility of the recombinant protein as an experimental model. To evaluate the consequences of substitutions that replace the side chains of Lys 18 or Asp 302, measurements of the kinetic parameters for the K18M, D302A, and D302N mutants were performed. The data are summarized in Table 2. The most significant effects of mutagenesis on catalytic efficiency were observed when Asp 302 was replaced with alanine or asparagine. In both cases, catalytic activity could not be detected using the enzyme coupled spectrophotometric assay. Instead, a more sensitive radioisotope assay (Hulcher 1991; Sawamura et al. 1992) was employed. The results of these assays were used to determine V_{max} as well as the Michaelis constants for mevalonate diphosphate. More than a 10^3 -fold decrease in the enzyme activity was observed for D302N; a 10^5 -fold decrease in k_{cat} was observed for D302A. These observations of major k_{cat} effects upon D302 substitution support assignment of a crucial role to Asp 302 in catalysis. The higher activity observed for D302N may reflect the ability of this side chain to form a hydrogen bond with a solvent molecule or another active site residue that may weakly substitute for the catalytic function of Asp 302. Only modest differences in $K_{m(\text{MPP})}$ values (Table 2) are observed upon comparison of data for wild-type MDD and D302N or D302A mutants. Because of endogenous ATPase activity in these MDD proteins and the long incubations (several hours) required to detect activity in the Asp 302 mutants, slow degradation of ATP significantly influences the concentrations of this substrate when subsaturating levels must be used in the assays. Consequently, it is not possible to accurately determine $K_{m(\text{ATP})}$ values for Asp 302 mutants, but the reasonably close agreement between equilibrium binding constants for this substrate to wild-type and Asp 302 mutants (Table 1) does not suggest impaired affinity. In contrast, the large impact on catalytic efficiency

Table 2. Summary of kinetic properties of wild-type and mutant MDD enzymes

Enzyme	V_{max} (units/mg)	$K_{m(D,L-MPP)}$ (μ M)	$K_{m(Mg-ATP)}$ (μ M)	k_{cat} (sec^{-1})	$k_{cat}/K_{m(D,L-MPP)}$ ($s^{-1}\mu M^{-1}$)	$k_{cat}/K_{m(Mg-ATP)}$ ($s^{-1}\mu M^{-1}$)
Wild type	6.4 ± 0.17	123 ± 22	61 ± 6	4.9	4×10^{-2}	8×10^{-2}
K18M	0.20 ± 0.003	177 ± 7.8	1066 ± 109	0.15	8.7×10^{-4}	1.4×10^{-4}
D302A	$(2.14 \pm 0.08) \times 10^{-5}$	397 ± 34	—	1.64×10^{-5}	4.1×10^{-8}	—
D302N	$(1.74 \pm 0.14) \times 10^{-3}$	103 ± 27	—	1.34×10^{-3}	1.3×10^{-5}	—

Assays were performed in 100 mM Tris-Cl, 100 mM NaCl (pH 7.0) in the presence of 10 mM $MgCl_2$ at 30°C. A spectrophotometric assay (Cardemil and Jabalquinto 1985) was used for measurements on wild-type and K18M enzymes. A radioactive assay (Hulcher 1991) was used for measurements on Asp 302 mutants.

validates assignment of Asp 302 as an active site residue and suggests the crucial influence of Asp 302 on reaction chemistry. In the reaction catalyzed by MDD, an early step appears to be the phosphorylation of the C3 hydroxyl of mevalonate diphosphate (Dhe-Paganon et al. 1994). A role for Asp 302 as the catalytic base that deprotonates the C-3 hydroxyl of mevalonate diphosphate is compatible with the diminution in catalytic rate observed for Asp 302 mutants. The acceptor substrate's C3 hydroxyl would have to be closely juxtaposed to ATP to support in-line phosphoryl transfer. The location of MDD's Asp 302 in the same interdomain hinge region where bound ATP has been observed in mevalonate kinase (Fu et al. 2002) supports the chemical role proposed above for Asp 302. A similar role for an active site aspartate has been proposed for other phosphotransferases (e.g., phosphofructokinase, Berger and Evans 1992, and hexokinase, Aleshin et al. 2000).

The proximity of the γ carboxyl group of Asp 302 and ϵ -amino group of Lys 18 (2.75 Å in unliganded MDD; Bonanno et al. 2001) could influence pK_a values, stabilizing anionic oxygens. This residue could also contribute by orienting negatively charged substrates or the Asp 302 side chain. The substitution of methionine for Lys 18 affects the specific activity (≈ 30 -fold decrease) and $K_{m(ATP)}$ (16-fold inflation) of the enzyme, implicating Lys 18 as an active site residue but not suggesting a critical catalytic function.

Discussion

The utility of recombinant his-tagged yeast MDD for structural work (Bonanno et al. 2001) suggested that further characterization of this protein as well as functional investigation would be valuable. This report documents the optimized conditions for expression and isolation of substantial amounts of recombinant his-tagged yeast MDD. The procedure results in recovery of homogeneous protein in amounts that are approximately 50-fold higher than described for isolation of the protein from yeast cells (Dhe-Paganon et al. 1994), avian liver (Alvear et al. 1982), or pig liver (Chiew et al. 1987). The catalytic (e.g., specific activity) properties of the recombinant MDD are comparable

with those reported for other eukaryotic MDD proteins. Therefore, the recombinant protein seems to represent an effective experimental model for structure/function studies of eukaryotic MDD. Additionally, the utility of trinitrophenyl-ATP as an active site probe has been established; this fluorescent analog is a valuable tool for functional tests of ATP site integrity in recombinant MDD proteins as well as in other phosphotransferases. Because the active site of MDD has not previously been characterized in substantial detail, the utility of the recombinant protein for identification of functionally important residues was tested. The results outlined above demonstrate success in the functional evaluation of a pair of active site residues (Lys 18; Asp 302) and underscore the value of this recombinant protein as an experimental model.

Based on both amino acid sequence and protein fold homology (Bonanno et al. 2001; Krishna et al. 2001), mevalonate diphosphate decarboxylase is a member of the GHMP kinase family of proteins. There are contrasting reports on the importance of active site acidic residues to catalysis of phosphoryl transfer by these proteins. In the case of mevalonate kinase, elimination of the carboxyl side chain of Asp 204 resulted in a dramatic decrease in catalytic efficiency (Potter and Mizioro 1997). Subsequent structural results (Fu et al. 2002) indicated that this acidic residue is closely juxtaposed to both the γ phosphoryl group of bound ATP as well as the ϵ amino group of Lys 13. In contrast, structural work on homoserine kinase (Krishna et al. 2001) has been interpreted to suggest that an acidic side chain is not involved in catalysis of phosphoryl transfer. However, no direct test of the functional importance of acidic residues in homoserine kinase has been reported. Observations on other GHMP kinase proteins support the hypothesis that an active site Lys/Arg-Asp pair has functional importance. Galactokinase catalyzes the ATP-dependent phosphorylation of galactose to galactose-1-phosphate. Recently, structural results for a *Lactococcus lactis* galactokinase complex with α -D-galactose and inorganic phosphate (Thoden and Holden 2003) have been interpreted to suggest the importance of conserved Arg 36 and Asp 183 residues, which are closely juxtaposed and are also observed to be in

close (3.5 Å) proximity to the C1 hydroxyl of a bound galactose molecule. 4-CDP-methylerythritol kinase, another protein that exhibits the GHMP kinase fold, converts 4-(cytidine-5'-diphospho)-2-C-methyl-*D*-erythritol to 4-(cytidine-5'-diphospho)-2-C-methyl-*D*-erythritol-2-phosphate in an ATP-dependent reaction (Luttgen et al. 2000). The enzyme from *Thermus thermophilus* contains the conserved residues Lys 8 and Asp 125. On the basis of recent structural information (Wada et al. 2003), it has been established that the ϵ amino nitrogen atom of Lys 8 is positioned within 4 Å distance from carboxyl oxygen atom of Asp 125. Although the catalytic mechanism of this enzyme is still unclear, these two residues are proposed (Wada et al. 2003) for assignment to the active site.

Although both galactokinase and CDP-methylerythritol kinase contain basic/acidic residues that may be homologous to the lysine-aspartate pair found in mevalonate kinase and mevalonate diphosphate decarboxylase, no direct test of the functional importance of such residues in those proteins has been reported. In this context, the results documented above for Asp 302 and Lys 18 in yeast MDD seem quite informative because they reinforce the mechanistic results described for mevalonate kinase and contrast with the speculation outlined for homoserine kinase. As additional members of the GHMP kinase family are identified and functional tests of proposed active site residues are reported, it will be interesting to learn whether two branches of the family, distinguished by the presence or absence of a homologous acid/base pair of active site residues, become apparent.

Materials and methods

Materials

The pSKB2 plasmid containing full-length cDNA for yeast MDD and a histidine tag upstream of the N terminus of the enzyme was a gift from Drs. S. Burley and M. Romanowski (Rockefeller University). Deoxyoligonucleotides used for the mutagenic substitutions were synthesized by Operon Technologies. *E. coli* strain BL-21 (DE3) used for transformation was obtained from Novagen. Isopropyl- β -*D*-thiogalactopyranoside was purchased from Research Products International Corp. TNP-ATP (2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate) is a product of Molecular Probes. Materials used for DNA purification were products of QIAGEN Inc. Ni-NTA agarose was purchased from QIAGEN Inc. All other biochemical reagents were purchased from Sigma. Mevalonolactone, trimethylsilyl iodide, and other chemical reagents used for synthesis of mevalonate diphosphate were purchased from Aldrich.

Expression and isolation of recombinant wild-type and mutant yeast MDD proteins

A full circle PCR method, which employed a Stratagene QuikChange site-directed mutagenesis protocol, was used to gen-

erate the desired mutations. The following primers were used: K18M, 5'-CAACATCGCAACCCCTTATGTATTGGGGGAAAAG-3' and 5'-CTTTTCCCCCAATACATAAGGGTTGCGATGTTG-3'; D302A, 5'-GTTGCATACACGTTTGTGTCAGGTCCAAATG-3' and 5'-CATTTGGACCTGCAGCAAACGTGTATGCAAC-3'; D302N, 5'-GTTGCATACACGTTTAAATGCAGGTCCAAATG-3' and 5'-CATTTGGACCTGCATTAAACGTGTATGCAAC-3'.

The presence of the desired mutation and the absence of any unanticipated changes in coding sequence were confirmed by automated DNA sequencing, performed at the Protein and Nucleic Acid Shared Facility at the Medical College of Wisconsin. *E. coli* transformants containing the expression constructs for wild-type and mutant MDD proteins were grown in 1 L of LB medium at 22°C. Protein production was induced by addition of 1 mM isopropyl- β -*D*-thiogalactopyranoside when the A_{600} reached 1.5; bacteria were harvested after 10 h of induction. The cells were suspended and lysed by passage through a French pressure cell at 16,000 psi. The lysate was centrifuged at 100,000g for 1 h, and the supernatant was dialyzed against 10 L of 20 mM Tris/Cl buffer (pH 7.4), containing 0.5 mM DTT. The dialyzed supernatant was loaded onto a 10-mL Ni-NTA agarose column equilibrated with the same buffer. The column was washed until A_{280} was <0.015, and MDD was eluted with 10–200 mM linear gradient of imidazole (100 mL total volume) in 10 mM Tris/Cl, 100 mM NaCl, 0.5 mM DTT (pH 7.0). The fractions containing MDD were pooled and concentrated using an Amicon YM-10 filter and concentration device (Millipore). The buffer was exchanged to 10 mM Tris/Cl, 0.5 mM DTT (pH 7.0) using the same technique. The concentration of the protein was determined spectrophotometrically using an extinction coefficient ($\epsilon_{280\text{ nm}} = 55,340\text{ M}^{-1}\text{cm}^{-1}$) calculated from the deduced protein composition. Glycerol was added to a final concentration of 20%, and enzyme was frozen and stored at -80°C for further use. Freezing under these conditions did not affect the activity of the enzyme.

Estimation of the molecular weight of wild-type yeast MDD

The molecular weight of the MDD subunit was estimated by comparison of the electrophoretic mobility of the MDD band, measured under denaturing SDS-PAGE conditions, to the mobility of molecular weight standards. Gel filtration chromatography was used to estimate the molecular weight of MDD under nondenaturing conditions; a Superose 6 (1 × 30 cm; Pharmacia) FPLC column was employed. The elution volume (V_e) for wild-type MDD and for molecular weight standards was calculated from the flow rate and the elution time for each protein peak. Void volume (V_0) of the column was estimated using Blue Dextran. In these experiments, 100 μ g of the protein were loaded on the column, which was equilibrated and eluted with 50 mM HEPES, 150 mM KCl (pH 7.5).

TNP-ATP binding to wild-type and mutant MDD proteins

The recombinant wild-type and mutant enzymes were tested for structural integrity using trinitrophenyl-ATP, a fluorescent analog of ATP. The fluorescence enhancement of TNP-ATP upon binding to MDD was measured in 100 mM Tris/Cl, 100 mM NaCl, 10 mM MgCl_2 (pH 7.0), using a PTI spectrofluorimeter. Excitation wavelength used in these experiments was 408 nm. Emission spectra were scanned from 500 to 600 nm. For data analysis, values measured at the fluorescence emission peak of 540 nm for enzyme-

bound TNP-ATP were corrected for free TNP-ATP fluorescence. Thus, the fluorescence enhancement was used in all binding analyses. To calculate the stoichiometry for TNP-ATP binding to wild-type and mutant MDD proteins, the data were analyzed by Scatchard plots. The structural integrity of the mutant proteins was evaluated by comparing the binding stoichiometries and dissociation constants for TNP-ATP binding with the values measured for wild-type protein.

Kinetic characterization of wild-type and mutant enzymes

Measurement of enzyme activity was typically performed at 30°C in 100 mM Tris-Cl (pH 7.0) containing 10 mM MgCl₂ and 100 mM NaCl; product ADP formation was measured using a spectrophotometric assay previously described (Cardemil and Jabalquinto 1985). The substrate, mevalonate diphosphate, was synthesized by the method of Reardon and Abeles (1987). The initial velocity was determined on the basis of the rate of decrease in absorbance at 340 nm following addition of mevalonate diphosphate to the reaction mixture. One unit of enzyme activity corresponds to conversion of 1 μ mole of substrate to product in 1 min.

When the catalytic efficiency of the mutant enzyme was low, activity was measured using a radioisotope assay (Hulcher 1991; Sawamura et al. 1992). The radiolabeled substrate [2-¹⁴C] mevalonate diphosphate required for the assay was synthesized enzymatically from [2-¹⁴C] mevalonic acid lactone as previously described (Chiew et al. 1987; Pilloff et al. 2003). Recombinant forms mevalonate kinase (Potter and Miziorko 1997; Potter et al. 1997) and phosphomevalonate kinase (T. Herdendorf and H. Miziorko, unpubl.) were employed for this synthesis. C18 Sep-Pak cartridges (Waters) were used in this assay for separation of radiolabeled product, isopentenyl diphosphate, from unreacted substrate. Measurements of the radioactive product were made using a Beckman LS3801 scintillation counter.

For estimates of the maximum velocity (V_m) and Michaelis constant (K_m), the reaction velocities at various substrate concentrations were fit to the Michaelis-Menten equation using the Grafit program (Erithacus Software Ltd.).

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