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Enoyl-CoA hydratase and isomerase form a superfamily with a common active-site glutamate residue

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Mitochondrial 2-enoyl-CoA hydratase (mECH) and 3,2-*trans*-enoyl-CoA isomerase (mECI), two enzymes which catalyze totally different reactions in fatty acid β-oxidation, belong to the low-similarity hydratase/isomerase enzyme superfamily. Their substrates and reaction mechanisms are similar [Müller-Newen, G. & Stoffel, W. (1993) *Biochemistry 32*, 11405–11412]. Glu164 of mECH is the only amino acid with a protic side chain that is conserved in these monofunctional and polyfunctional enzymes with 2-enoyl-CoA hydratase and 3,2-*trans*-enoyl-CoA isomerase activities. We tested our hypothesis that Glu164 of mECH is the putative active-site amino acid responsible for the base-catalyzed α-deprotonation in the hydratase/dehydrase and isomerase reaction. We functionally expressed rat liver mECH wild-type and [E164Q] mutant enzymes in *Escherichia coli*. Characterization of the purified wild-type and mutant enzymes revealed that the replacement of Glu164 by Gln lowers the k_{cat} value more than 100000-fold, whereas the K_m value is only moderately affected. We have demonstrated in a previous study that Glu165 is indispensable for the 3,2-*trans*-enoyl-CoA isomerase activity. Taking these results together, we conclude that the conserved glutamic acid is the essential basic group in the active sites of 2-enoyl-CoA hydratase (Glu164) and 3,2-*trans*-enoyl-CoA isomerase (Glu165), and that these enzymes are not only evolutionarily but also functionally and mechanistically related.

Keywords. 2-Enoyl-CoA hydratase; 3,2-trans-enoyl-CoA isomerase; enzyme superfamily; active site; site-directed mutagenesis.

Mitochondrial 2-enoyl-CoA hydratase (mECH, crotonase) catalyzes the hydration of 2-*trans*-enoyl-CoA compounds, intermediates of fatty acid oxidation (Stern et al., 1953) to L-3-hydroxy-acyl-CoA, whereas 2-*cis*-enoyl-CoA derivatives are converted to the respective D-3-hydroxy-acyl-CoA compounds (Wakil, 1954). Rat mECH is a polypeptide of 28 kDa. N-terminal sequencing and the cDNA-deduced amino sequence revealed that the mature form consists of 261 amino acids (Minami-Ishii et al., 1989). Sedimentation equilibrium analysis and gel filtration suggest a homohexameric structure of the native form (Furuta et al., 1980). No cofactor is required to maintain the enzymic activity.

A double-isotope-effect study on the mechanism of the reverse reaction and the dehydration of 3-hydroxy-acyl-CoA compounds to 2-enoyl-CoA compounds, showed that the enzyme-catalyzed β-elimination is concerted (Bahnson and Anderson, 1991). Due to the *syn* geometry of the elimination (Willadsen and Eggerer, 1975), two mechanisms are possible; (a) a single active-site amino acid performs the protonation of the hydroxyl group followed by the deprotonation at C2 and the concerted release of a water molecule, and (b) two amino acids bearing protic side chains might be involved in the protonation and deprotonation steps (Fig. 1).

mECH shows similarities to different monofunctional or polyfunctional enzymes with 2-enoyl-CoA-hydratase (ECH) and 3,2-*trans*-enoyl-CoA isomerase (ECI) activity, which are found in mitochondria, peroxisomes and bacteria. Monofunctional mitochondrial 3,2-*trans*-enoyl-CoA isomerase (mECI) is a 29-kDa protein involved in the oxidation of unsaturated fatty acids, which catalyzes the isomerization of 3-*cis*-enoyl CoA compounds or 3-*trans*-enoyl CoA compounds to 2-*trans*-enoyl-CoA compounds (Stoffel et al., 1964). The polyfunctional enzymes mitochondrial trifunctional enzyme (mTFE; Uchida et al., 1992), peroxisomal trifunctional enzyme (pTFE; Palosaari and Hiltunen, 1990), and the large α subunit of *E. coli* fatty acid oxidation complex (fadB) gene product (Pramanik et al., 1979) with a molecular mass around 78 kDa, share ECH, ECI (except mTFE) and 3-hydroxyacyl-CoA dehydrogenase activities. The N-terminal part of the polyfunctional enzymes shows similarity to mECH and mECI, whereas the C-terminal part aligns to mitochondrial monofunctional 3-hydroxyacyl-CoA dehydrogenase. Therefore, it is reasonable to assume that the ECH and ECI activities are both located on the N-terminal part of the polyfunctional enzymes. These enzymes define the low-similarity hydratase/isomerase enzyme superfamily (Müller-Newen and Stoffel, 1991).

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Abbreviations. CBDH, 4-chlorobenzoyl-CoA dehalogenase; ECH, 2-enoyl-CoA hydratase; ECI, 3,2-trans-enoyl-CoA isomerase; fadB, large α subunit of *Escherichia coli* fatty acid oxidation complex; m, mitochondrial; IPTG, isopropylthiogalactoside; p, peroxisomal; SOE, splicing by overlapping extension; TFE, trifunctional enzyme.

Enzymes. 4-Chlorobenzoyl-CoA dehalogenase (EC 3.8.1.–); mitochondrial 2-enoyl-CoA hydratase (EC 4.2.1.17); 3,2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8); mitochondrial trifunctional enzyme (EC 1.1.1.35; EC 4.2.1.17; EC 5.3.3.8); naphthoate-synthase (EC 4.2.1.–); peroxisomal trifunctional enzyme (EC 4.2.1.17; EC 1.1.1.35; EC 2.3.1.16).

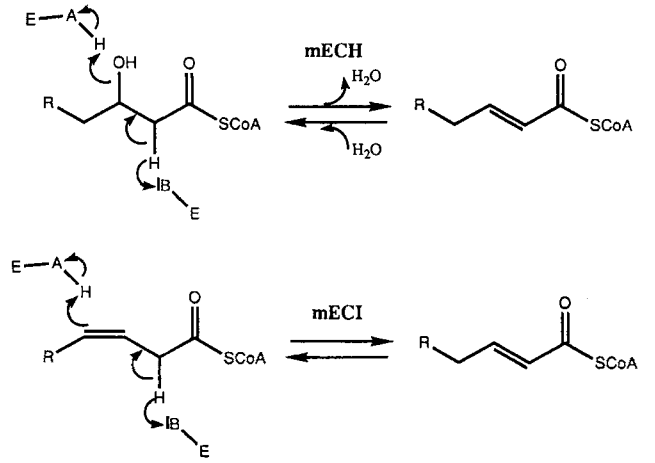

Fig. 1. Reactions catalyzed by mitochondrial 2-enoyl-CoA hydratase (mECH) and mitochondrial 3,2-*trans*-enoyl-CoA isomerase (mECI). Hypothetical active-site amino acid side chains which act as bases or acids and are part of the enzyme (E) are designated with B and A, respectively. Stereochemical details are not considered in the figure. Elimination of water proceeds with the concerted deprotonation at C2 and leaving of the hydroxyl group at C3, which is protonated to yield the eliminated water molecule. Isomerization is probably facilitated by the deprotonation at C2 and the protonation at C4. The conserved Glu164 of mECH and Glu165 of mECI may act as acid and corresponding base during the protonation/deprotonation step occuring at C2 of the substrates of both reactions.

Two additional enzymes are regarded as members of this enzyme family because they show the typical pattern of conserved amino acids (Fig. 2). These enzymes are not involved in fatty acid oxidation but also utilize CoA compounds as substrates; the naphthoate-synthase from *Bacillus subtilis* encoded by the *menB* gene (Driscoll and Taber, 1992), which catalyzes the ring-closure reaction of *o*-succinyl-CoA benzoate during vitamin K biosynthesis, and the 4-chlorobenzoyl-CoA dehalogenase (CBDH) from *Pseudomonas* sp., which catalyzes the dehalogenation of 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA (Babbitt et al., 1992). All enzymes of the hydratase/isomerase enzyme family are likely to have evolved from a common ancestor.

In a recent study, we exchanged by site-directed mutagenesis several conserved amino acids of mitochondrial 3,2-*trans*-enoyl-CoA isomerase. Enzyme kinetics of these mutant isomerases revealed that Glu165 is indispensable for catalytic activity and is, therefore, a putative active-site amino acid residue (Müller-Newen and Stoffel, 1993). This glutamic acid is the only amino acid with a protic side chain that is conserved solely in the enzymes with ECH and ECI activities (Fig. 2). The reactions catalyzed by mECH and mECI are very similar with regard to substrate molecules and enzyme mechanisms (Fig. 1). Therefore, we pursued the function of the glutamic acid (Glu164) of mECH which is involved in the catalysis of the hydration of 2-enoyl-CoA compounds.

Here we describe the functional expression of mECH in *E. coli*, the purification of the recombinant enzyme (rmECH), and the characterization of the [E164Q]rmECH mutant. The replacement of glutamic acid by glutamine leads to a drastical reduction of k_{cat} ($10^{5.2}$-fold) while K_m remains largely unaffected. From this result, we conclude that mECH and mECI are evolutionary, functionally and mechanistically related enzymes which share a common active-site amino acid pivotal for the protonation/deprotonation steps at the α-carbon atom of thioester during catalysis.

MATERIAL AND METHODS

All recombinant DNA techniques, if not otherwise stated, were carried out according to the methods of Sambrook et al. (1989). Oligonucleotides were synthesized using an Applied Biosystem 380A DNA Synthesizer. Enzymes, if not otherwise stated, were purchased from Boehringer, Mannheim.

PCR amplification of mECH cDNA from a rat liver cDNA library and construction of rmECH wild-type expression vector (pETmECH). Two oligonucleotide primers were derived from the published mECH cDNA sequence (Minami-Ishii et al., 1989), which contained a *Nco*I and a *Bam*HI site for convenient cloning of the amplified cDNA into the pET8c expression vector (Studier et al., 1990). The primer pair used was mECH 5′-primer (cgg cgc ttc gcc ATg ggt gct aac tt) and mECH 3′-primer (ggagaaaggtatggAtCc cagctc tca gtg). The introduced point mutations are marked by capital letters, the created restriction sites are underlined. PCR amplification was performed in a volume of 100 µl containing 10^7 plaque-forming units of a rat liver cDNA library (Stratagene), 20 pmol mECH 5′ primer and mECH 3′-primer, 125 µM each dNTP, 20 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.01% gelatine and 2.5 U Taq DNA polymerase (BRL). The temperature profile (2 min at 94°C; 30×1 min at 93°C, 2 min at 68°C, 2 min at 72°C; 5 min at 72°C) was performed in a DNA Thermo-Cycler (Perkin-Elmer-Cetus). The amplified 825-bp cDNA fragment was purified by agarose gel electrophoresis and digested with *Nco*I and *Bam*HI. Due to an internal *Nco*I site in the mECH cDNA, two fragments arose (167 bp and 630 bp) which were cloned subsequently into the *Nco*I/*Bam*HI-digested pET8c vector. The sequence of the insert of the resulting expression vector (pETmECH) was confirmed by dideoxy-sequencing (Pharmacia T7 Sequencing Kit; Sanger et al., 1977).

Construction of [E164Q]mECH mutant expression vector ([E164Q]pETmECH). The pETmECH DNA was used as a template for site-directed mutagenesis, applying splicing by overlapping extension (SOE-PCR; Ho et al., 1989). The flanking 5′-primer and 3′-primer were complementary to the vector DNA sequence, such that the 5′-flanking primer (ccagcaaccgcacctgt) hybridizes with the antisense strand 157 bp upstream from the *Nco*I site, and the 3′-flanking primer (gatatccggatatagttcct) with the sense strand 145 bp downstream from the *Bam*HI site. Sense and antisense primers were synthesized to introduce the point mutation (ttt gga cag cca Caa atc ctc ctg gg and the respective antisense oligonucleotide; the mutation is marked by a capital letter). The PCR conditions were the same as described above. From the resulting PCR product, a 548-bp fragment harbouring the point mutation was released by *Sac*I/*Hpa*I digestion and ligated into the *Sac*I/*Hpa*I-digested pETmECH vector. The sequence of the newly introduced DNA of the mutant expression vector ([E164Q]pETmECH) was controlled by DNA sequencing.

Expression of rmECH wild type and [E164Q]rmECH mutant in *E. coli*. 100 ml M9ZB medium supplemented with ampicillin (50 µg/ml) and chloramphenicol (30 µg/ml) were inoculated with BL21(DE3)pLysS cells, which had been previously transformed with wild-type or mutant expression vector (pETmECH or [E164Q]pETmECH). When the A_{600} of the culture reached 0.8, additional ampicillin (50 µg/ml) and isopropyl-thiogalactoside (IPTG, final concentration 1 mM) were added. After 3 hours at 35°C under strong aeration, the bacteria were harvested by centrifugation (4000 *g*, 10 min) and washed with 10 ml NaCl/P_i (1 l contains 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.4 g KH_2PO_4) adjusted to pH 7.4 with HCl and supplemented with 0.02% sodium azide and 5 mM mercaptoethanol. The bacterial pellet was resuspended in 2.5 ml NaCl/P_i

Fig. 2. Alignment of the low-similarity isomerase/hydratase enzyme superfamily. mECH, rat liver mitochondrial 2-enoyl-CoA hydratase (complete sequence of 290 amino acid residues; Minami-Ishii et al., 1989); mECI, rat liver mitochondrial 3,2-*trans*-enoyl-CoA isomerase (complete sequence of 289 amino acids; Müller-Newen and Stoffel, 1991); mTFE, α subunit of the rat liver mitochondrial trifunctional enzyme (amino acids 1−313 out of 727 amino acids; Kamijo et al., 1993); pTFE, rat liver peroxisomal trifunctional enzyme (amino acids 1−256 out of 723 amino acids; Osumi et al., 1985); fadB, α subunit of the *E. coli* fatty acid oxidizing multienzyme complex (amino acids 1−264 out of 729 amino acids; Dirusso, 1990); menB, naphthoate synthase from *Bacillus subtilis* (complete sequence of 262 amino acids; Driscoll and Taber, 1992); CBDH was from *Pseudomonas* sp. (complete sequence of 268 amino acids; Babbitt et al., 1992). The alignment shows the sequence identities and similarities of the members of the isomerase/hydratase family to mECH. Identical amino acids are marked white on black, similar amino acids black on grey. The enzymes exhibiting 2-enoyl-CoA hydratase and/or 3,2-*trans*-enoyl-CoA isomerase activity are marked by an asterisk (*). The only polar amino acid conserved solely in enzymes with 2-enoyl-CoA hydratase and/or 3,2-*trans*-enoyl-CoA isomerase activity is marked with an arrow (Glu164 of mECH).

and lysed by shock freezing (10 min in a methanol/dry ice bath) and thawing. DNAse, RNAse and lysozyme (20, 2 and 100 µg/ml, respectively) were added and the viscous suspension incubated for 30 min at 35°C. After centrifugation (15 min, 10000 *g*) the clear, slightly yellow supernatant (lysate) was characterized by gel electrophoresis (Laemmli, 1970), protein determination (Sedmak and Grossberg, 1977), activity assays (Stern, 1955) and used for the purification of rmECH.

Purification of wild-type rmECH and [E164Q]rmECH. The bacterial lysate was equilibrated with buffer A (25 mM Tris/HCl, pH 8.1, 5 mM mercaptoethanol, 1 mM EDTA, 0.02% sodium azide) using a PD-10 column (Pharmacia) and applied to a Resource-Q anion-exchanger column (Pharmacia; bed volume 6 ml), which was previously equilibrated with buffer A. Bound substances were eluted with a sodium chloride gradient (buffer B; buffer A plus 1 M NaCl) by FPLC (flow rate, 4 ml/min; for details, see Fig. 5). Wild-type rmECH eluted with 80 mM NaCl, the [E164Q]rmECH mutant with 70 mM NaCl. The volume of the fractions containing rmECH (4 ml) were reduced to 2 ml using Centricon-10 micro-concentrators (Amicon), and the concentrated protein solution was applied to a calibrated Superdex 200 16/60 column which was equilibrated with NaCl/P_i (flow rate, 1 ml/min). Homogenous wild-type rmECH as well as [E164Q]rmECH protein eluted after 67 min. The calibration of the gel-filtration column was performed using BSA (66 kDa, 74 min), ovalbumin (45 kDa, 80 min) and lysozyme (14.3 kDa, 112 min) as molecular-mass standards. N-terminal sequencing of 4 µg purified rmECH was performed by automated Edman degradation (Applied Biosystems protein sequencer 477 A).

Kinetic studies with wild-type rmECH and [E164Q]-rmECH. 10 µl [E164Q]rmECH lysate or 10 µl 1:100000 dilution (diluted with NaCl/P_i) of wild-type rmECH lysate were added to 990 µl 50 µM crotonyl-CoA dissolved in NaCl/P_i. The decrease of absorbance at 263 nm was immediately measured in a UV photometer (Zeiss, PMQ3). 1 U enzyme activity corresponds to 1 µmol hydrated substrate/min under these conditions. For the determination of K_m and k_{cat} values, the initial crotonyl-CoA concentrations were 10, 15, 20, 30, 50, 75 and 100 µM, whereas the amount of purified enzyme added was held constant at 20.7 µg [E164Q]rmECH protein and 0.00102 µg wild-type rmECH, respectively. The initial linear decrease of absorbance at 263 nm was measured. The kinetic data were analyzed by double-reciprocal plots ($1/v$ versus $1/[S]$).

RESULTS

Functional expression of wild-type rmECH and [E164Q]-rmECH mutant protein in *E. coli*. *E. coli* and the pET system were chosen for heterologous expression of mECH because the related mECI had been successfully expressed in the active form using this expression system (Müller-Newen and Stoffel, 1993). The mECH cDNA was amplified from a rat liver cDNA library by PCR. The primers were derived from the published sequence (Furuta et al., 1980). We introduced restriction sites for subse-

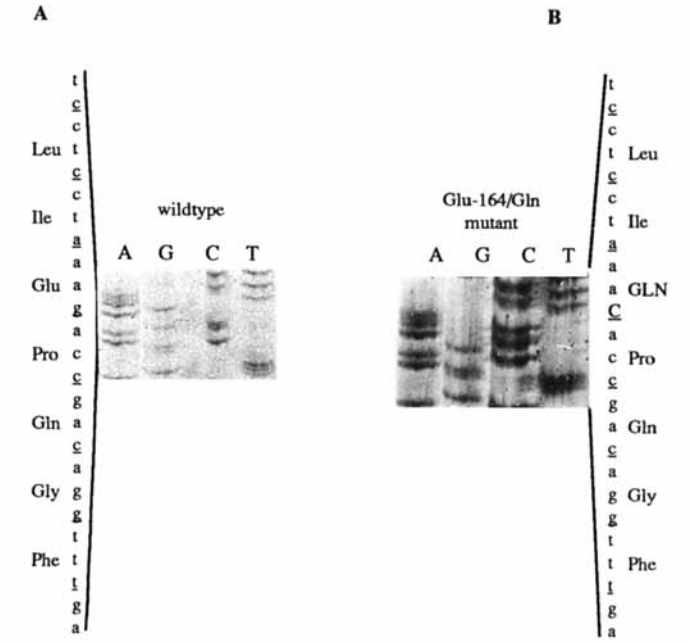

Fig. 3. Dideoxy sequencing of the wild-type (pETmECH) and mutant expression vector ([E164Q]pETmECH). The introduced G-C transversion leads to the Glu-Gln exchange (marked with capital letters).

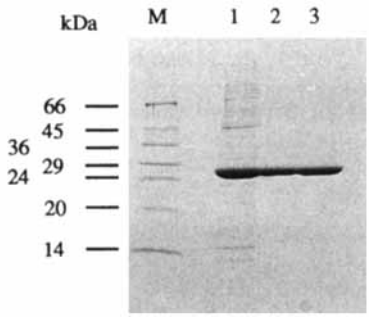

Fig. 4. SDS/PAGE analysis (15% acrylamide, Coomassie-stained) of lysate of bacteria transformed with wild-type expression vector pETmECH (lane 1) and purified wild-type rmECH and [E164Q]-rmECH mutant enzymes (lanes 2 and 3, respectively).

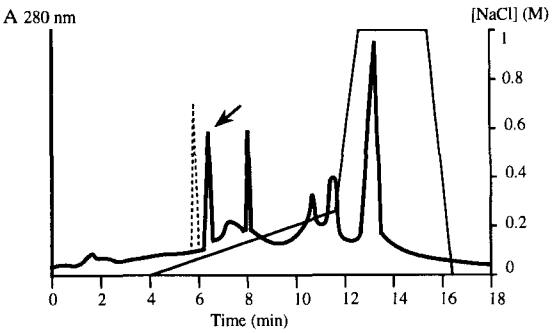

Fig. 5. Elution profile of anion-exchange chromatography of the lysate from bacteria harbouring pETmECH after expression of the recombinant enzyme. The rmECH peak is marked by an arrow. The dotted peak was observed instead of the rmECH peak when the lysate of the [E164Q]rmECH mutant protein expression was applied to the column (Resource Q, 6 ml bed volume).

quent ligation into the vector pET8c. The resulting rmECH expression vector (pETmECH) encodes the complete mECH (28 kDa) found in rat liver mitochondria with the starting methionine immediately followed by the first glycine of mature mECH. The G/C transversion leading to the expression of [E164Q]rmECH mutant enzyme was introduced by SOE-PCR (Ho et al., 1989), yielding the expression vector [E164Q]pETmECH. All PCR-derived vector DNA was tested for the presence of the directed mutation and the absence of PCR-generated random mutations by DNA sequencing (Fig. 3).

Lysates were prepared from *E. coli* cells transformed with pETmECH after induction of expression. SDS/PAGE analysis of the lysate reveals a predominant band below the 29-kDa marker protein (Fig. 4, lane 1) which is not visible in lysates from mock-transformed cells (not shown). The enzymic activity of the rmECH was detected using crotonyl-CoA as substrate. The rate of the hydration of the conjugated double bond was measured in an optical assay by the decrease of absorbance at 263 nm (ε_{263} = 6700 M^{-1} cm^{-1}; Stern, 1955). In order to obtain a linear decrease of absorbance, the lysate was diluted 100-fold before addition to the assay mixture. The specific activity of the wild-type lysate was 330 U/mg protein. No activity was detectable in the undiluted lysates of bacteria transformed with mock vector. SDS/PAGE analysis of lysates from cells transformed with the pETmECH ([E164Q] 2-enoyl-CoA hydratase) expression vector revealed the same protein pattern as lysate from wild-type expression, showing the predominant band below the 29-kDa marker protein. Enzymic activity was only detectable upon application of undiluted lysate for the activity assay. The specific activity of the lysate of [E164Q]rmECH mutant protein expression was 0.012 U/mg protein, which is 27500-fold lower than the specific activity of wild-type lysate.

Purification and characterization of rmECH wild-type protein and [E164Q]rmECH mutant protein. The recombinant rmECH wild-type and [Z164Q] 2-enoyl-CoA hydratase mutant proteins were purified to homogeneity by two chromatographic steps. The lysate was equilibrated with Tris/HCl buffer, pH 8.1, and applied to an anion-exchange column. Bound substances were eluted with a sodium chloride gradient. Interestingly, the mutant protein elutes with 70 mM sodium chloride, while the wild-type protein bearing an additional negative charge elutes with 80 mM sodium chloride (Fig. 5). The fractions containing rmECH were concentrated and applied to a calibrated gel-filtration column. Both rmECH wild-type and [E164Q]rmECH mutant proteins eluted with exactly the same retention time and appeared as a single band in SDS/PAGE analysis (Fig. 4, lanes 2 and 3). Comparison of the retention time of rmECH with that of molecular-mass marker proteins revealed a molecular mass of 80300 kDa, suggesting a homotrimeric structure of the native recombinant enzyme (with 28287 kDa/subunit). The purified [E164Q] mutant protein (0.035 U/mg) shows an 85000-fold lower specific activity compared with wild-type rmECH (2990 U/mg). The overall yield of recombinant enzyme was in the range of 6 mg/100 ml culture. N-terminal sequencing of 4 μg purified rmECH wildtype confirmed the expected sequence (GANFQYIITEKKGKN) demonstrating that the N-terminal *N*-formylmethionine residue is posttranslationally cleaved in *E. coli*.

The initial rates of the hydration of crotonyl-CoA catalyzed by purified wild-type rmECH and [E164Q]rmECH mutant proteins were determined by the optical assay described above. The substrate concentrations were varied over 10−100 μM at constant rmECH concentrations. The reaction was started by the addition of the enzyme. For the determination of the K_m and k_{cat} values of [E164/Q]rmECH mutant enzyme, 20.7 μg recombinant protein were added for each activity assay. In the case of wild-

type protein, 1 ng was sufficient to yield a linear decrease of absorbance. The evaluation of the double-reciprocal plots of initial velocities against substrate concentrations resulted in a K_m value of 71 μM for the wild-type rmECH and 15 μM for the [E164/Q] mutant protein. The specific activities, extrapolated to substrate saturation, were 7350 U_{max}/mg for wild-type rmECH and 0.048 U_{max}/mg for the [E164Q]rmECH mutant protein. Assuming the cDNA-derived molecular mass of 28287 Da for rmECH and one active site/monomer, the k_{cat} values are 3500 s^{-1} and 0.023 s^{-1}, respectively. Thus, the k_{cat} value of the [E164Q]rmECH mutant protein is $10^{5.18}$-fold lower than the k_{cat} of the wild-type protein.

DISCUSSION

Rat liver mitochondrial 2-enoyl-CoA hydratase can be efficiently expressed and purified in the two-step procedure described above; the yield is 6 mg functional recombinant enzyme from 100 ml bacterial culture. The recovery of the initial activity present in the lysate was 55%. The kinetic data of the recombinant enzyme (K_m = 70 μM, k_{cat} = 3500 s^{-1}) are similar to the reported data for the enzyme isolated from rat liver (K_m = 40 μM, k_{cat} = 2120 s^{-1}; Furuta et al., 1980). The *V/k* value of 5×10^7 M^{-1} s^{-1} confirms that the rate of the mECH-catalyzed reaction is nearly diffusion limited (Bahnson and Anderson, 1991). A discrepancy was observed between the reported molecular mass of the native rat liver enzyme (161000 Da; Furuta et al., 1980) and the molecular mass of the functional recombinant enzyme (80300 Da). These data imply that mECH possesses a homohexameric quaternary structure, whereas the rmECH shows a homotrimeric quaternary structure. Therefore, it can be assumed that the hexameric protein consists of two trimers and that the formation of the hexamer awaits an explanation.

Comparison of the kinetic data of the wild-type rmECH and [E164Q]rmECH mutant protein reveals that Glu164 of mECH, conserved in all enzymes with ECH or ECI activity of the hydratase/isomerase enzyme superfamily, is involved in the catalytic process at the active site of the enzyme. The replacement of the acidic glutamic acid side chain by the nonprotic glutamine side chain leads to an $10^{5.2}$-fold decrease of the k_{cat}value, while the K_m value is only moderately affected. Replacement of glutamate by glutamine at the active site might decrease the activity of the mutated enzyme even more if one takes into account that deamidation of glutamine could partially regenerate the active site of the wild-type enzyme. This finding suggests that Glu164 is not involved in substrate binding but in the protonation and deprotonation steps of the catalysis of the hydration of 2-enoyl-CoA compounds.

In support of our hypothesis, the respective glutamic acid is also indispensable for the activity of the evolutionarily related mECI (Müller-Newen and Stoffel, 1993). It is reasonable to assume that this glutamic acid is a common active-site amino acid of both mECH and mECI. A closer view at the differences and similarities of the events during mECH and mECI catalysis suggests a common function of the active-site glutamic acid for both enzymes. Both reactions need a protonation/deprotonation step at C2 of the substrate molecule (Fig. 1). The functionally important glutamic acid, conserved in both enzymes, is a good candidate for the mediation of this decisive step in the catalysis of both reactions. Two main differences are obvious when the mECH reaction is compared with the mECI reaction. First, there is the need for the access of a water molecule to the active site of mECH which is not required for the mECI reaction. Secondly, a protonation step occurs at the leaving hydroxyl group of the bound 3-hydroxy-acyl-CoA compound whereas the ECI reaction requires a protonation of C4 of the bound 3-enoyl-CoA compound. Amino acids mediating these steps need not necessarily be conserved.

The polyfunctional enzymes pTFE and fadB exhibit ECH and ECI activity. The involvement of the conserved glutamic acid in the active sites of the monofunctional enzymes mECH and mECI suggests that the respective glutamic acid is also involved in the catalysis of both reactions performed by the polyfunctional enzymes. Thus ECH and ECI catalysis must occur at a single active site on the N-terminal domain of these polyfunctional enzymes.

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