Site-Directed Mutagenesis of Putative Active-Site Amino Acid Residues of 3,2-*trans*-Enoyl-CoA Isomerase, Conserved within the Low-Homology Isomerase/Hydratase Enzyme Family†

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ABSTRACT: During β-oxidation of unsaturated fatty acids, mitochondrial 3,2-*trans*-enoyl-CoA isomerase (mECI) converts 3-*cis*- or 3-*trans*-enoyl-CoA intermediates into their 2-*trans* isomers. The cDNA-derived amino acid sequence of mECI shows weak but significant homologies to the peroxisomal trifunctional enzyme (pTFE), the α-subunit of the fatty acid degradation complex from *Escherichia coli* (FadB), the mitochondrial 2-enoyl-CoA hydratase (mECH), the naphthoate synthase encoded by the *menB* gene from *Bacillus subtilis* (MenB), and the 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp. (CBDH). These proteins form the isomerase/hydratase enzyme family. Tyr-150, Arg-151, and Asp-211 of the mECI are the only amino acids with protic side chains conserved within the enzymes with isomerase activity (pTFE and FadB). These amino acids are exchanged in the remaining enzymes of the isomerase/hydratase family. Glu-165 is conserved in all enzymes with isomerase and/or hydratase activity (pTFE, FadB, and mECH). We argue that these amino acids are possibly involved in the proton transfer at the active site of mECI. To test this hypothesis, mECI was functionally expressed in *E. coli*. The recombinant enzyme (rmECI) exhibits the same specific activity as the enzyme from rat liver. Exchange of the candidate active-site amino acids by site-directed mutagenesis revealed that Tyr-150 is not involved in isomerase catalysis. The exchange of Arg-151 and Asp-211 leads to a reduced expression of the recombinant enzyme accompanied by a reduced specific activity. The replacement of Glu-165 by Gln leads to a strongly reduced enzymatic activity. We therefore propose that Glu-165 is an active-site amino acid of 3,2-*trans*-enoyl-CoA isomerase involved in the proton shift during isomerization of 3-enoyl-CoA compounds. Interestingly, the rmECI, which contains the 11 C-terminal amino acids of the highly charged mitochondrial targeting sequence, exhibits a strong "salting-in" effect, leading to a selective precipitation of rmECI after desalting of the bacterial lysate. This property was utilized for the purification of the recombinant enzyme.

Mitochondrial 3,2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8) (mECI)[1] is a key enzyme in the degradation of unsaturated fatty acids. During mitochondrial β-oxidation of unsaturated fatty acids with double bonds at odd-numbered carbon atoms, 3-*cis*-enoyl-CoA intermediates are formed which are converted by the mECI to their 2-*trans* isomers (Stoffel et al., 1964a). These products are the substrates of the mitochondrial 2-enoyl-CoA hydratase and are further metabolized by the remaining well-known enzymes of the mitochondrial β-oxidation spiral. 3-*trans*-Enoyl-CoA compounds are also isomerized to 2-*trans*-enoyl-CoA compounds by the mECI, but with a 10-fold lower rate (Stoffel & Ecker, 1969). Besides their origin from naturally-occurring 3-*trans* fatty acids, 3-*trans*-enoyl-CoA intermediates have been postulated to be products of a NADPH-dependent 2,4-dienoyl-CoA reductase in the oxidation of unsaturated fatty acids with *cis*-configurated double bonds at even-numbered carbon atoms (Kunau & Dommes, 1978). The mECI has been isolated as a 60-kDa dimer from rat liver mitochondria. It consists of two identical 30-kDa subunits (Stoffel & Grol, 1978). An additional 3,2-*trans*-enoyl-CoA isomerase activity has also been detected in rat liver peroxisomes and assigned to the 77-kDa peroxisomal trifunctional enzyme (Palosaari & Hiltunen, 1990).

Comparison of the cDNA-deduced amino acid sequence of mECI (Müller-Newen & Stoffel, 1991) with all sequences of the Swiss-Prot data base using the BLAST program (Altschul et al., 1990) revealed low but significant homologies (25–27% identity) to three different enzymes involved in the β-oxidation of fatty acids. These enzymes, which are likely to have evolved from a common ancestor, define the isomerase/hydratase enzyme family (Figure 1): (a) the 30-kDa monofunctional mitochondrial 2-enoyl-CoA hydratase (EC 4.2.1.17) (mECH) from rat liver, which hydrates 2-*trans*-enoyl-CoA intermediates to L-3-hydroxyacyl-CoA compounds (Minami-Ishii et al., 1989); (b) rat liver peroxisomal trifunctional enzyme (pTFE), which exhibits 3-hydroxyacyl-CoA dehydrogenase, 2-enoyl-CoA hydratase (ECH), and 3,2-*trans*-enoyl-CoA isomerase (ECI) activity (Osumi et al., 1985); and (c) the product of the *fadB* gene in *Escherichia coli*, which encodes the α-subunit of the fatty acid degradation multienzyme complex (Dirusso, 1990). This 78-kDa protein exhibits the same enzymatic activities as the 77-kDa pTFE with an additional 3-hydroxyacyl-CoA epimerase activity (Yang et al., 1988). The amino acid sequences of the smaller monofunctional proteins (mECI and mECH) align to the N-terminal

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[1] Abbreviations: ECI, 3,2-*trans*-enoyl-CoA isomerase; ECH, 2-enoyl-CoA hydratase; pTFE, peroxisomal trifunctional enzyme; mECI, mitochondrial ECI; mECH, mitochondrial ECH; rmECI, recombinant mECI; CBDH, 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp.; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IPTG, isopropyl thiogalactopyranoside.

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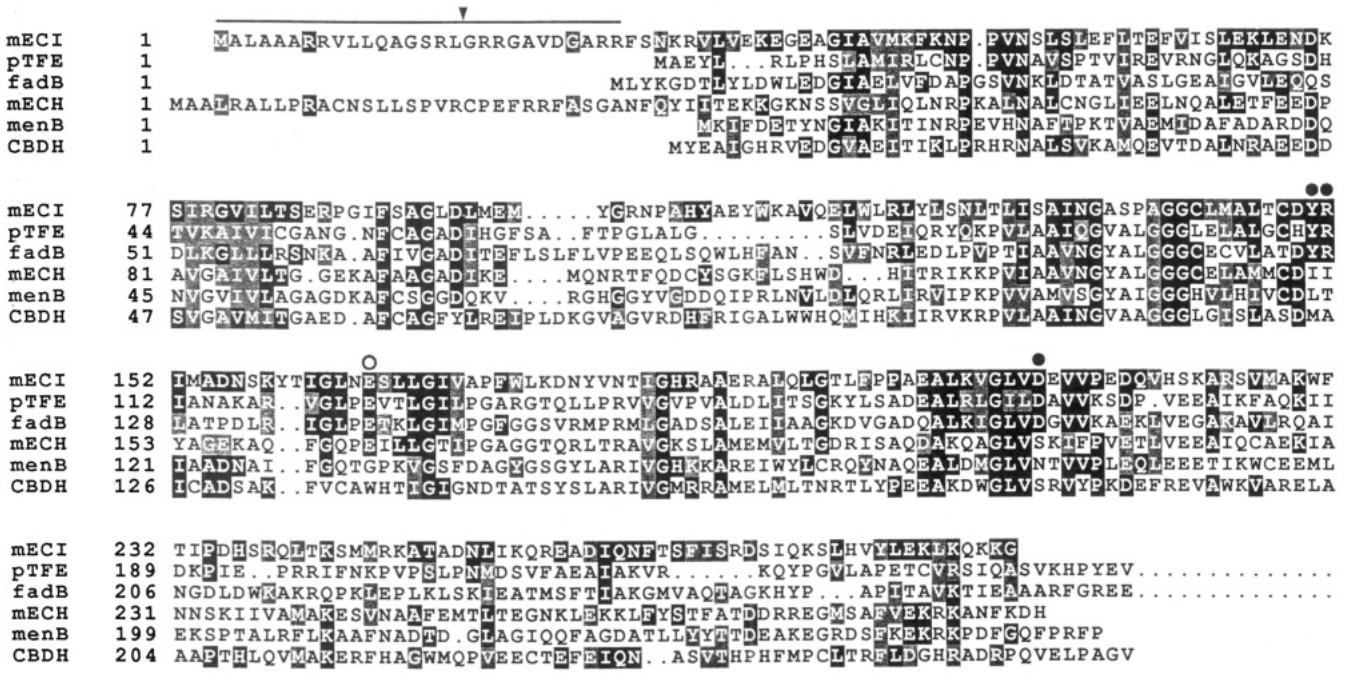

FIGURE 1: Alignment of the low-homology isomerase/hydratase enzyme family. mECI, rat liver mitochondrial 3,2-*trans*-enoyl-CoA isomerase (complete sequence of 289 amino acids); pTFE, rat liver peroxisomal trifunctional enzyme (amino acids 1-261 out of 723 amino acids); FadB, α-subunit of the *E. coli* fatty acid oxidizing multienzyme complex (amino acids 1–269 out of 729 amino acids); mECH, rat liver mitochondrial 2-enoyl-CoA hydratase (complete sequence of 290 amino acids); MenB, naphthoate synthase from *Bacillus subtilis* (complete sequence of 262 amino acids; CBDH, chlorbenzoyl-CoA dehalogenase from *Pseudomonas sp.* (complete sequence of 268 amino acids). The alignment shows the sequence identities and similarities of the members of the isomerase/hydratase family to mECI. Identical amino acids are marked white on black, similar amino acids white on gray. Polar amino acids conserved solely in enzymes with 3,2-*trans*-enoyl-CoA isomerase activity are marked with closed circles (Tyr-150, Arg-151, and Asp-211); the only polar amino acid conserved in enzymes with 3,2-*trans*-enoyl-CoA isomerase and/or 2-enoyl-CoA hydratase activity is marked with a open circle (Glu-165). The mitochondrial targeting presequence (Palosaari et al., 1991), which is cleaved during import of mECI into mitochondria, is marked with a bar. The arrowhead shows the start of the sequence of recombinant mECI.

parts of the multifunctional proteins (pTFE and FadB). Therefore, it is highly probable that the ECI and ECH activities are associated with the N-terminal domains of the multifunctional proteins. The C-terminal domains of the multifunctional proteins, on the other hand, show the expected homologies to monofunctional mitochondrial 3-hydroxyacyl-CoA dehydrogenase.

The sequences of two recently cloned enyzmes show a pattern of conserved residues typical for the isomerase/hydratase family. These proteins seem to be evolutionarily related and are regarded as members of this enzyme family. While these enzymes are not involved in β-oxidation, both use CoA derivatives as substrates: (d) the naphthoate synthase from *Bacillus subtilis*, encoded by the *menB* gene, which catalyzes the ring closure reaction of *o*-succinyl-CoA benzoate during vitamin K biosynthesis (Driscoll & Taber, 1992) and (e) the 4-chlorobenzoyl-CoA dehalogenase (CBDH) from *Pseudomnas* sp., which catalyzes the dehalogenation of 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA (Babbitt et al., 1992).

The mECI catalyzes the deprotonation of the α-methylene group and the protonation of the γ-carbon atom of 3-*cis*-enoyl-CoA substrates with the geometric isomerization and shift of the double bond to the α,β-position. The comparison between the conserved amino acids of the isomerase/hydratase enzyme family might be sufficient to define those amino acid residues within the active site of mECI that perform the proton shift during isomerization of 3-enoyl-CoA compounds. Assuming that the enyzmes of the isomerase/hydratase family have evolved from a common ancestor by divergent evolution, it is possible that their members share a similar protein folding pattern. Amino acids conserved in all members of the enzyme family are probably responsible for the formation of the hydrophobic substrate binding pocket, for CoA-binding, and for overall protein folding. Glycine and hydrophobic amino acids are predominantly found in those positions (Figure 1). Amino acids which are involved in the proton transfer during the catalysis of isomerization are required to possess protic side chains and should be strictly conserved in the enzymes exhibiting ECI activity (mECI, pTFE, and FadB). Because of the high mutation frequency within this enzyme family and the different structural needs at different active sites, it is reasonable to assume that these amino acids are exchanged in enzymes with different activities (MenB, CBDH, and ECH). We looked for amino acids of mECI with protic side chains which are invariant in all enzymes with ECI activity. Residues matching these criteria are Tyr-150, Arg-151, and Asp-211 (Figure 1).

The reactions catalyzed by ECI and ECH are very similar. Both enzymes use enoyl-CoA compounds as substrates, and a deprotonation (ECI) or protonation step (ECH) at C2 is involved in the mechanism of both reactions. Furthermore, in the polyfunctional enzymes (pTFE and FadB), ECI and ECH activities are located together on one polypeptide chain, possibly with a common active site for both activities. The amino acid facilitating deprotonation or protonation at C2 might be conserved in all enzymes with ECI or ECH activity (mECI, mECH, pTFE, and FadB). The only amino acid with a protic side chain solely conserved in these enzymes is Glu-165 of mECI (Figure 1).

To test whether these amino acids are indispensable for the catalytic activity of mECI, recombinant wild-type and mutant proteins were expressed in *E. coli*. In rat liver, mECI is synthesized with a N-terminal mitochondrial targeting sequence which is cleaved during mitochondrial import. How-

ever, the recombinant enzyme (rmECI) contains the 11 C-terminal amino acids of the targeting sequence. The highly charged partial presequence drastically changes the ionic strength dependence of the solubility of the protein but does not affect the catalytic activity. rmECI is soluble only in buffers with moderate ionic strength. This "salting-in" effect was utilized for the purification of the recombinant enzyme applying an unusual desalting step.

Five mutant proteins (Tyr-150/Ile + Arg-151/Ile double mutant and Tyr-150/Phe, Arg-151/Gln, Asp-211/Asn, and Glu-165/Gln mutant proteins) were expressed in *E. coli* and subsequently characterized. The exchange of Tyr-150 did not affect the enzymatic activity of rmECI. The exchanges of Arg-151 and Asp-211 led to a lowered expression of recombinant protein, accompanied with a reduced specific activity. The activity of the Glu-165/Gln mutant was reduced at least 5×10^3-fold, suggesting the participation of Glu-165 in the proton shift during isomerization of 3-enoyl-CoA compounds. That Glu-165 of mECI is conserved in the polyfunctional enzymes (pTFE and FadB) as well as in mECH suggests that the respective glutamic acid residues are also involved in ECH catalysis.

MATERIAL AND METHODS

All recombinant DNA techniques, if not otherwise stated, were carried out according to the methods of Sambrook et al. (1989) and Ausubel et al. (1987). Enzymes and coenzyme A were purchased from Boehringer, and chemicals for the synthesis of 3-*cis*-dodecenoic acid were purchased from Aldrich. α-^{32}P-dATP was obtained from Amersham. All other chemicals were purchased from Sigma.

Construction of mECI Expression Vector (pETrmECI) and Site-Directed Mutagenesis. The pGEM3Z vector (Promega), containing the whole mECI cDNA, was first cut with *Xma*III, which cleaves the cDNA in the sequence coding for the mitochondrial targeting peptide. The protruding 5′-ends were filled in with dNTPs using the Klenow fragment of DNA polymerase. A 878-bp cDNA fragment was released by cleavage with *Bam*HI downstream from the stop codon and ligated into the pET8c expression vector (Studier et al., 1990), which had previously been cleaved with *Nco*I, blunt ended with Klenow fragment, and cleaved with *Bam*HI. Thus, the *Nco*I site of the pET vector forms the start codon, which is followed by the cDNA encoding the partial signal sequence and the complete coding sequence of mature mECI. The resulting vector, pETrmECI, was used for wild-type rmECI expression and as a template for the generation of the mutant rmECI vectors. Site-directed mutagenesis was performed by the SOE-PCR (splicing by overlapping extension) method (Ho et al., 1989). The flanking 5′- and 3′-primers were designed to hybridize with the vector DNA, 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) hybridizes with the sense strand 145 bp downstream from the *Bam*HI site. The following primers and their respective antisense primers were synthesized (Applied Biosystems 380A DNA synthesizer) to introduce the mutated sequences: Tyr-150/Ile + Arg-151/Ile sense (acc tgt gac ATc aTT ata atg gcg), Glu-165/Gln sense (a gga ttg aat Cag agc ctg ct), Asp-211/Asn (ggt ttg gtg Aat gag gtg gt), Tyr-150/Phe (acc tgt gac tTc agg ata atg), Arg-151/Gln (c tgt gac tac CAg ata atg gcg) (mismatched bases are marked by capital letters). From the resulting PCR products, the 380-bp *Sma*I/*Kpn*I mECI cDNA fragments harboring the point mutation were released by cleavage with the respective restriction enzymes and ligated into the *Sma*I/*Kpn*I-digested wild-type pETrmECI vector. DH5α cells were transformed with the ligated vector DNAs, and the plasmid DNAs from recombinant clones were prepared. The sequence of the inserts of wild-type and mutant vectors were controlled by sequencing (Pharmacia T7 sequencing kit) (Sanger et al., 1978). The vector DNAs were used for transformation of BL21(DE3)pLysS host cells for the expression of wild-type rmECI or the respective mutant proteins.

Preparation of the E. coli Lysates after Expression of rmECI and Its Mutated Forms. M9ZB medium supplemented with ampicillin (50 μg/mL) and chloramphenicol (30 μg/mL) was used for all expression experiments. An 8.5-mL portion of an overnight culture of host cells containing the pETrmECI or respective mutant vector was used to inoculate 250-mL cultures. The cells were allowed to grow at 37 °C with strong aeration until the OD_{600} of 0.8 was reached. The culture was supplemented with additional ampicillin (50 μg/mL), and expression of rmECI was induced by adding 2.5 mL of 0.1 M IPTG to a final concentration of 1 mM. After 3 h of the solution being vigorously shaken at 35 °C, the cells were harvested by centrifugation (10 min, 4000*g*). The bacteria pellet was washed with 20 mL of PBS containing 0.02% sodium azide and 5 mM 2-mercaptoethanol and resuspended in 22 mL of the same buffer. Bacteria were lysed by shock freezing in a methanol/dry ice bath. To the viscous lysate were added 2.5 mL of 5 M NaCl, 5 μL of RNAse A (10 mg/mL), 50 μL of DNAseI (10 mg/mL), and 50 μL of lysozyme (50 mg/mL) and the mixture was incubated for 45 min at 35 °C. The lysate was centrifuged (15 min, 10000*g*), and the supernate was used for enzyme assays, immunoblotting, and purification of the recombinant protein.

Purification of rmECI and the Glu-165/Gln Mutant. The respective lysates (25 mL) were desalted using a Sephadex G 25 (Pharmacia) column (bed volume 125 mL) equilibrated with low-salt buffer (25 mM sodium phosphate, pH 6.5, 5 mM 2-mercaptoethanol, and 0.02% sodium azide). The turbid eluate was left overnight at 4 °C. The precipitated protein (enriched rmECI) was collected by centrifugation (15 min, 15 000*g*), washed once with 2.5 mL of low-salt buffer, and subsequently dissolved in 2 mL of high-salt buffer (25 mM sodium phosphate, pH 6.5, 5 mM 2-mercaptoethanol, 0.02% sodium azide, and 0.25 M sodium chloride). After centrifugation (15 min, 15 000*g*), the supernate was applied to a FPLC-HiLoad-Superdex 200 16/60-column (Pharmacia) equilibrated with high-salt buffer (flow, 1 mL/min). The rmECI-containing fractions eluted after 70 min. The purity of the rmECI was checked by gel electrophoresis (Laemmli, 1970), and the protein amount was determined by the Coomassie G250 assay (Sedmak & Grossberg, 1977), using BSA as protein standard. N-Terminal sequencing of 15 μg of rmECI was performed by automated Edman degradation (Applied Biosystems protein sequencer 477 A).

Partial Protease V8 (Endoproteinase Glu-C) Digestion of Wild-Type rmECI and Glu-165/Gln Mutant Protein. Nine micrograms of purified wild-type rmECI and 9 μg of purified Glu-165/Gln mutant protein in 60 μL of 25 mM ammonium bicarbonate were incubated 14 h at 25 °C with 0.25 μg of Protease V8 (endoproteinase Glu-C, sequencing grade, Boehringer) in the presence of 0.05% SDS. The peptides were visualized after separation on a high-resolution SDS-polyacrylamide gel (16.5% T, 3% C) (Schägger & von Jagow, 1987) by silver staining (Blum et al., 1987).

RNA Preparation from E. coli and Northern Blot Analysis. Three hours after induction of transcription, RNA was prepared from a 15-mL culture of BL 21 (DE3) pLysS cell clones transformed with the wild-type or respective mutant expression vectors. Any residual DNA contamination was removed by digestion with RNAse-free DNAse. Twenty micrograms of RNA from each culture was separated on a denaturing 1% agarose gel containing formaldehyde and transferred to a nylon membrane (Gene Screen plus, NEN). The membrane was hybridized with a randomly ^{32}-P-labeled 808-bp *Eco*RI fragment of the mECI cDNA in 50% formamide at 42 °C (10^5 dpm/mL), washed with 2 × SSC, 0.1% SDS at 60 °C, and exposed for autoradiography for 1 h to Kodak XAR-5 film.

Substrate Synthesis and ECI Activity Assay. 3-*cis*-Dodecenoic acid was synthesized from 1,4-butinol and octyliodide as described (Stoffel et al., 1964b). The purity and the identity of the fatty acid was controlled by gas chromatography of the methyl ester in comparison with a fatty acid methyl ester standard mixture (Tschöpe, 1963). Pure 3-*cis*-*dodecenoic* acid (9.5 mg, 0.05 mmol) and 13 mg (0.017 mmol) of coenzyme A (lithium salt) were converted to 3-*cis*-dodecenoyl-CoA by the mixed anhydride method (Goldman & Vagelos, 1961). The substrate was purified by HPLC chromatography on a reversed-phase C_{18}-column (Nucleosil 300-5 C_{18}, Macherey & Nagel) applying a linear acetonitrile gradient. 3-*cis*-Dodecenoyl-CoA eluted with 50% acetonitrile.

The assay buffer contained 25 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 1 mM 2-mercaptoethanol, 0.1 mg/mL BSA, and 35 μM 3-*cis*-dodecenoyl-CoA. The enzymatic reaction was started by addition of 10 μL of solution containing wild-type rmECI or mutant protein to 990 μL of assay buffer in a quartz cuvette of 10-mm light path at 22 °C, and the increase of absorbance at 263 nm was measured. One unit corresponds to 1 μmol of isomerized substrate per minute under these conditions. In the case of wild-type rmECI and Tyr-150/Phe-mutant protein, a 1:20 dilution of *E. coli* lysate was added to achieve a linear increase of absorbance. The low-activity mutants were assayed with undiluted lysate. The assay of purified rmECI was carried out with defined protein amounts. Determination of the K_m values was performed using the same assay buffer but in the absence of BSA and with varying substrate concentrations ranging from 5 to 35 μM.

BSA was routinely added to the enzyme assay buffer because it increases the sensitivity of the ECI assay 5-fold. The reason for this effect is not well understood, but it is observed with many enzymes utilizing acyl-CoA compounds as substrates. One possible explanation is that the acyl-CoA binding properties of BSA reduce the detergent effect of medium- and long-chain acyl-CoA compounds. Since the presence of BSA influences the concentration of free acyl-CoA, it was omitted in the assay buffer used for the K_m value determination (Bartlett et al., 1985). Furthermore, the substrate concentrations used for the assays were held below the critical micelle concentration of acyl-CoA compounds (Powell et al., 1981).

Immunoblotting. Immunoblotting was performed as described (Towbin et al., 1979). After expression under identical conditions, 1, 2, and 10 μL of lysate of wild-type rmECI and 10 μL of the respective lysates of mutant proteins were applied to a SDS 15% polyacrylamide gel and electroblotted onto nitrocellulose membrane (Schleicher & Schüll). The membrane was incubated with anti-mECI serum (1:500 diluted) raised against the native mECI from rat liver. Binding of antibodies was visualized after incubation with a second antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase) and staining with 5-bromo-4-chloro-3-indolyl phosphate as substrate.

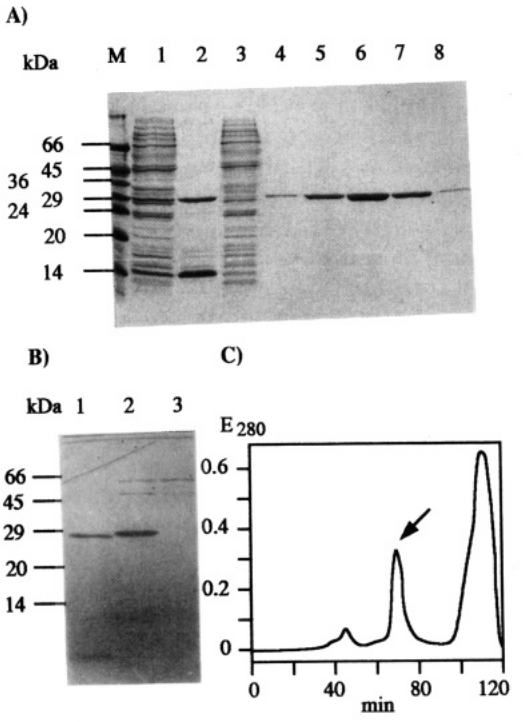

FIGURE 2: Purification of rmECI. (A) Demonstration of purification steps by Coomassie-stained SDS-PAGE M, molecular weight standards. Lane 1, *E. coli* lysate after rmECI expression; lane 2, enriched rmECI from the redissolved pellet after the desalting step; lane 3, supernate after the desalting step; lanes 4–8, FPLC gel filtration fractions after 66, 69, 72, 75, and 78 min. (B) Immunoblot with anti-mECI serum. Lane 1, crude extract from rat liver mitochondria; lane 2, *E. coli* lysate after rmECI expression; lane 3, lysate from bacteria transformed with mock vector. (C) Elution profile of rmECI purification from enriched rmECI by FPLC gel filtration on a Superdex 200 16/60 column (flow, 1 mL/min). The rmECI peak is marked by an arrow.

RESULTS

Expression of mECI in E. coli, Purification, and Functional Assay of the Wild-Type Recombinant 3,2-trans-Enoyl-CoA Isomerase. *E. coli* seemed to be a suitable host for expression of active mECI, because the enzyme undergoes no complex posttranslational modifications, except the cleavage of the presequence required for the targeting and import into mitochondria (Müller-Newen & Stoffel, 1991). An 878-bp cDNA fragment encoding the whole mature protein and, additionally, the 11 C-terminal amino acid residues of the mitochondrial targeting sequence was cloned into the *Nco*I and *Bam*HI sites of the pET8c expression vector and yielded the vector pETrmECI. Lysates were prepared from *E. coli* host cells transformed with pETrmECI after induction of expression. The immunoblot analysis of bacterial lysate shows that the recombinant enzyme is detected by polyclonal anti-mECI-IgG. Because of the residual presequence, rmECI migrates slightly slower during gel electrophoresis than the protein prepared from rat liver (Figure 2B). The corresponding protein band at the height of the 29-kDa marker protein is clearly visible in Coomassie-stained gel after SDS-PAGE of bacterial lysate (Figure 2A, lane 1). The *E. coli* lysate shows high ECI activity (7 units/mL), whereas in control lysates from bacteria transformed with the original pET8c vector, no activity was detectable. The enzyme activity was assayed in an optical test with 3-*cis*-dodecenoyl-CoA, the physiological substrate of mECI during oleic acid oxidation.

The shift of the isolated double bond into conjugation to the carbonyl group of the thioester leads to an increase of absorbance at 263 nm ($\epsilon = 6700$ L mol^{-1} cm^{-1} (Stern, 1955)). A double-reciprocal plot of initial isomerization rates at different substrate concentrations yielded a K_m of 37 ± 4 μM. This value is in the range of the K_m values determined for mECI from rat liver (50 μM) and bovine liver (32 μM) (Euler-Bertram & Stoffel, 1990).

The purification of rmECI takes advantage of an unusual property of the recombinant protein. The rmECI shows a strong "salting-in" effect, which means that the recombinant enzyme is only soluble in buffers with moderate ionic strength. This property was not observed with the enzyme prepared from rat liver and is probably caused by the presence of the residual charged presequence. Therefore, rmECI could be selectively precipitated by desalting of the bacterial lysate (Figure 2A, lanes 2 and 3). The precipitate was collected by centrifugation and the protein pellet could be dissolved in a small volume of buffer containing 0.25 M NaCl. The enriched rmECI was purified to homogeneity by gel filtration (see Materials and Methods and Figure 2A,C). The yield was 6–8 mg of pure rmECI per liter of culture, with a specific activity of 64 units/mg, which is comparable with the specific activity of the enzyme purified from rat liver (62 unit/mg). The k_{cat} value of rmECI (130 ± 6 s^{-1}) is not distinct from the k_{cat} of the native enzyme from bovine liver (128 s^{-1}) (Euler-Bertram & Stoffel, 1990). N-Terminal sequencing by Edman degradation (Edman & Begg, 1967) of purified rmECI confirmed the expected amino acid sequence (GRRGAVDG) and revealed that the N-terminal methionine residue is posttranslationally cleaved by *E. coli*.

Introduction of Directed Point Mutations and Functional Analysis of the Five mECI Mutants: Tyr-150/Ile + Arg-151/Ile, Tyr-150/Phe, Arg-151/Gln, Glu-165/Gln, and Asp-211/Asn. The point mutations were introduced into mECI cDNA by SOE (splicing by overlapping extension), using the PCR (Ho et al., 1989). The 380-bp *Sma*I/*Kpn*I fragment of mECI cDNA in pETrmECI was replaced by the respective *Sma*I/*Kpn*I fragments harboring the respective mutations generated by SOE-PCR. The sequence of the newly introduced DNA was verified for the presence of the directed mutation and absence of PCR-generated random mutations by DNA sequencing. The first proteins investigated were the Tyr-150/Ile + Arg-151/Ile double mutant and the Glu-165/Gln and Asp-211/Asn mutants. Immunoblot analysis of the *E. coli* lysates indicated that the Tyr-150/Ile + Arg-151/Ile double mutant and the Asp-211/Asn mutant are expressed to a lower extent compared with wild-type rmECI (Figure 3, lanes 4 and 10). Because of the reduced expression, these mutant proteins could not be purified by the desalting procedure described above. The yields of both mutant proteins were estimated to 10% of the wild-type protein by comparison of immunoblots obtained from different amounts of bacterial lysate after expression of wild-type and mutant proteins. Total enzyme activity of the lysate of the Asp-211/Asn mutant (0.03 units/mL) was 0.5% of wild-type activity, which corresponds to 5% of the specific activity of the wild type. No actvity was detectable in the lysate of the Tyr-150/Ile + Arg-151/Ile double mutant above control levels (cells transformed with original pET8c vector). The replacement of Tyr and Arg by Ile was chosen because the Ile residues appear at these positions in the mECH and we assumed that these exchanges would not disturb the overall structure of the protein. However, the low expression of these mutants indicatees that the structure of the protein is affected by these amino acid exchanges, leading

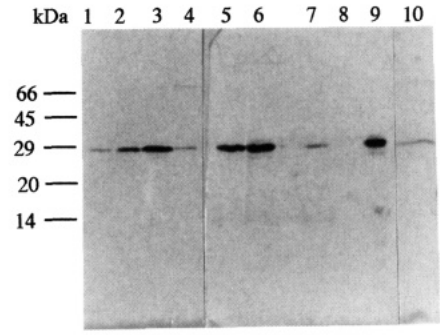

FIGURE 3: Immunoblot of wild-type and mutant forms of rmECI with anti-mECI serum. The respective *E. coli* lysates were applied for SDS-PAGE after expression under identical conditions. Lane 1, 1 μL of wild-type lysate; lane 2, 2 μL of wild-type lysate; lane 3, 10 μL of wild-type lysate; lane 4, 10 μL of Tyr-150/Ile + Arg-151/Ile lysate; lane 5, 10 μL of Tyr-150/Phe lysate; lane 6, 10 μL of Glu-165/Gln lysate; lane 7, 10 μL of Arg-151/Gln lysate; lane 8, mock vector lysate; lane 9, 10 μL of wild-type lysate; lane 10, 10 μL of Asp-211/Asn lysate.

either to an inefficient translation or, more likely, to misfolded protein which becomes the target of intracellular proteases. To decide whether either the Tyr-150 or the Arg-151 exchange of the double mutant protein is responsible for the low expression and activity, the individual mutations were introduced separately. Tyr-150 was replaced by Phe and Arg-151 by Gln, which is more hydrophilic compared to Ile. The lysate of the Tyr-150/Phe mutant showed an expression level and activity (5 units/mL) comparable to those of the wild-type protein, whereas the expression of the Arg-151/Gln mutant resembled the double mutant, exhibiting a very low but detectable activity (0.02 unit/mL, specific activity below 5% of wild type) (Figure 3, lanes 5 and 7). This demonstrates that the properties of the double mutant, including the low expression effect, are caused by the exchange of arginine. The K_m value of the Tyr-150/Phe mutant was determined as 66 ± 7 μM, which indicates a slightly reduced substrate affinity in comparison with the wild-type protein. The exact determination of the K_m values of the low-expression/low-activity mutants out of the bacterial lysate was not possible, because the measured activities of these mutants are near the threshold of detection and cannot be determined at lower substrate concentrations.

To exclude that the low expression of the Tyr-150/Ile + Arg-151/Ile, Arg-151/Gln, and Asp-211/Asn mutant proteins is caused by a low transcription of the cDNA from the expression vectors, the RNAs from cultures of the respective transformed bacteria were prepared after induction of transcription. Analysis of the RNAs by agarose gel electrophoresis shows that the expected 1-kb wild-type or mutant rmECI mRNAs are visible with similar intensities, while the band is missing in the RNA from bacteria transformed with mock vector. The RNAs were blotted to nitrocellulose and hybridized with a ^{32}P-labeled mECI cDNA fragment. The signals appearing after autoradiography confirm that the 1-kb RNA bands correspond with the rmECI mRNAs and that the cDNAs of the wild-type and mutant proteins are transcribed to a similar extent (Figure 4).

The Glu-165/Gln mutant was expressed in *E. coli* in amounts comparable with wild-type rmECI (Figure 3, lane 6). However, no activity above the control level was detectable in the lysate. Since all bacterial lysates show a considerable absorbance at 263 nm, which is the wavelength of the absorption maximum of the 2-*trans*-enoyl-CoA product measured in the optical assay, the threshold of detection of ECI activity is relatively high (0.005 units/mL). To control

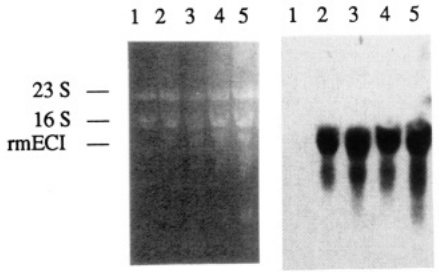

FIGURE 4: Agarose gel electrophoresis of bacterial RNAs and corresponding Northern Blot hybridization analysis with a mECI cDNA probe. A 20-μg sample of *E. coli* RNA was applied to the gel after induction of transcription of the following: lane 1, mock vector; lane 2, wild-type rmECI; lane 3, Tyr-150/Ile + Arg-151/Ile mutant; lane 4, Arg-151/Gln mutant; and lane 5, Asp-211/Asn mutant. 16 S and 23 S denote the bacterial ribosomal RNAs with sizes of 1.5 and 3 kb, respectively. rmECI marks the RNA encoding rmECI, approximately 1 kb in size.

whether the Glu-165/Gln mutant enzyme exhibits any residual ECI activity, the mutant protein was isolated by the rapid purification procedure described above. During purification, the Glu-165/Gln mutant protein and the wild-type rmECI showed exactly the same retention time in the final FPLC gel filtration on a Superdex G200 column, indicating that the mutation does not affect the dimerization of the protein and apparently does not lead to major structural alterations. The Glu-165/Gln amino acid exchange was confirmed by partial protease V8 (endoproteinase Glu-C) digestion and peptide mapping of the purified wild-type rmECI and the Glu-165/Gln mutant protein. The peptides were separated by SDS-PAGE and visualized by silver staining. The two peptides resulting from cleavage of the wild-type protein at Glu-165 are missing in the lane of the Glu-165/Gln mutant-derived peptides (Figure 5B). The remaining peptide patterns give no hints for further differences in the primary structure. Activity assays with the purified mutant protein revealed that the Glu-165/Gln exchange causes at least a 5×10^3-fold reduction of enzymatic activity: 6 μg of purified mutant enzyme in 1 mL of assay mixture gave no increase of absorbance at 263 nm, whereas 0.02 μg of purified wild-type protein gave rise to an increase of absorbance of 0.0086/min (Figure 5C). The drastic reduction of the enzymatic activity of the mutant protein suggests a participation of Glu-165 in the proton shift during isomerization of 3-enoyl-CoA compounds.

DISCUSSION

In the low-homology isomerase/hydratase enzyme family, four amino acids of mECI show a conservation pattern consistant with their possible involvement in the proton transfer during ECI catalysis. Tyrosine-150, arginine-151, and aspartic acid-211 are conserved in all enzymes with ECI activity (mECI, pTFE, and FadB) but are exchanged in enzymes with different activities. Glutamic acid-165 is conserved in all enzymes with ECI or ECH activity (mECI, pTFE, FadB, and mECH). To study the role of these particular amino acids in mECI catalysis, we first established an expression system for the active recombinant enzyme. Functional expression was achieved in *E. coli*, using a T7 RNA polymerase-directed expression system. Due to the cloning strategy, the N-terminus of the recombinant mECI contains the residual 11 C-terminal amino acids of the mitochondrial targeting sequence. The unchanged K_m value and specific activity of rmECI compared with the native, mature protein from liver indicates that the residual presequence does not affect the enzymatic activity. However, the residual presequence drastically changes the ionic strength-dependent solubility of the enzyme. In contrast to the enzyme purified from rat liver, rmECI is only soluble in aqueous buffers of moderate ionic strength. Lowering of the ionic strength leads to the precipitation of the enzyme. The four positive charges of the residual presequence apparently promote the aggregation caused by ionic interactions. It is possible that this "salting-in" effect is a general property of mitochondrial proteins possessing their highly charged presequences. This might explain the requirement for chaperones binding to mitochondrial proteins during their transport through the cytosol.

The mutagenesis experiments show that the conserved amino acids of the isomerase/hydratase enzyme family are of particular structural and functional importance. Of the four amino acid exchanges, only the replacement of Tyr-150 had no substantial effect on the expression or activity of the recombinant enzyme. Interestingly, the recent cloning of the *faoA* gene of *Pseudomonas fragii* (Sato et al., 1992) supports our result that Tyr-150 is not required for the catalysis of the ECI reaction, because in the gene product phenylalanine appears at the respective position instead of tyrosine. The *faoA* gene encodes the α-subunit of the fatty acid oxidizing complex and exhibits the same activities as the α-subunit of the fatty acid degradation complex of *E. coli* (FadB), including ECI and ECH activity (Imamura et al., 1990). This new member of the isomerase/hydratase enzyme family and the *fadB* gene show similar homology to the mECI. The remaining amino acids chosen for the mutagenesis experiments (Arg-151, Glu-165, and Asp-211) are conserved in the *faoA* gene.

The replacement of Asp-211 by Asn and Arg-151 by Ile or Gln leads to a considerably reduced amount of recombinant enzyme in the bacterial lysate, accompanied by a reduced specific activity. Northern Blot analysis of bacterial lysates demonstrates that the lowered expression is not caused by a lowered transcription of the mutated expression vectors. Therefore, the cause for the low expression is either an inefficient translation of the mRNA or degradation of misfolded protein by bacterial proteases. One cannot discriminate whether the reduced activity results only from a structural alteration or whether these amino acids are involved in the active site of mECI. Both amino acids Arg-151 and Asp-211 are conserved solely in enzymes with ECI activity. We suppose that these amino acids contribute to the formation of the binding site for 3-enoyl-CoA-compounds. The effect on the protein structure of their replacement by amino acids with nonionic side chains suggests that they might be involved in the formation of salt bridges.

The replacement of Glu-165 by Gln leads to a strongly reduced enzymatic activity of rmECI, indicating that Glu-165 is an active-site amino acid involved in the proton shift during ECI catalysis. The fact that this amino acid is conserved in the polyfunctional enzymes (FadB, FaoA, and pTFE) as well as in mECH has some implications for the ECI and ECH catalysis of both monofunctional and polyfunctional enzymes.

The results of studies on the kinetic isotope effect in the mECH reaction revealed that the reverse reaction, the dehydration of L-3-hydroxyacyl-CoA compounds (the equilibrium constant of the ECH reaction is near 1.00) to their 2-*trans*-enoyl-CoA derivatives, follows an E_2 mechanism (Bahnson & Anderson, 1991). By analogy, it can be assumed that the protonation at C4 and deprotonation at C2 during isomerization of 3-enoyl-CoA compounds, catalyzed by the

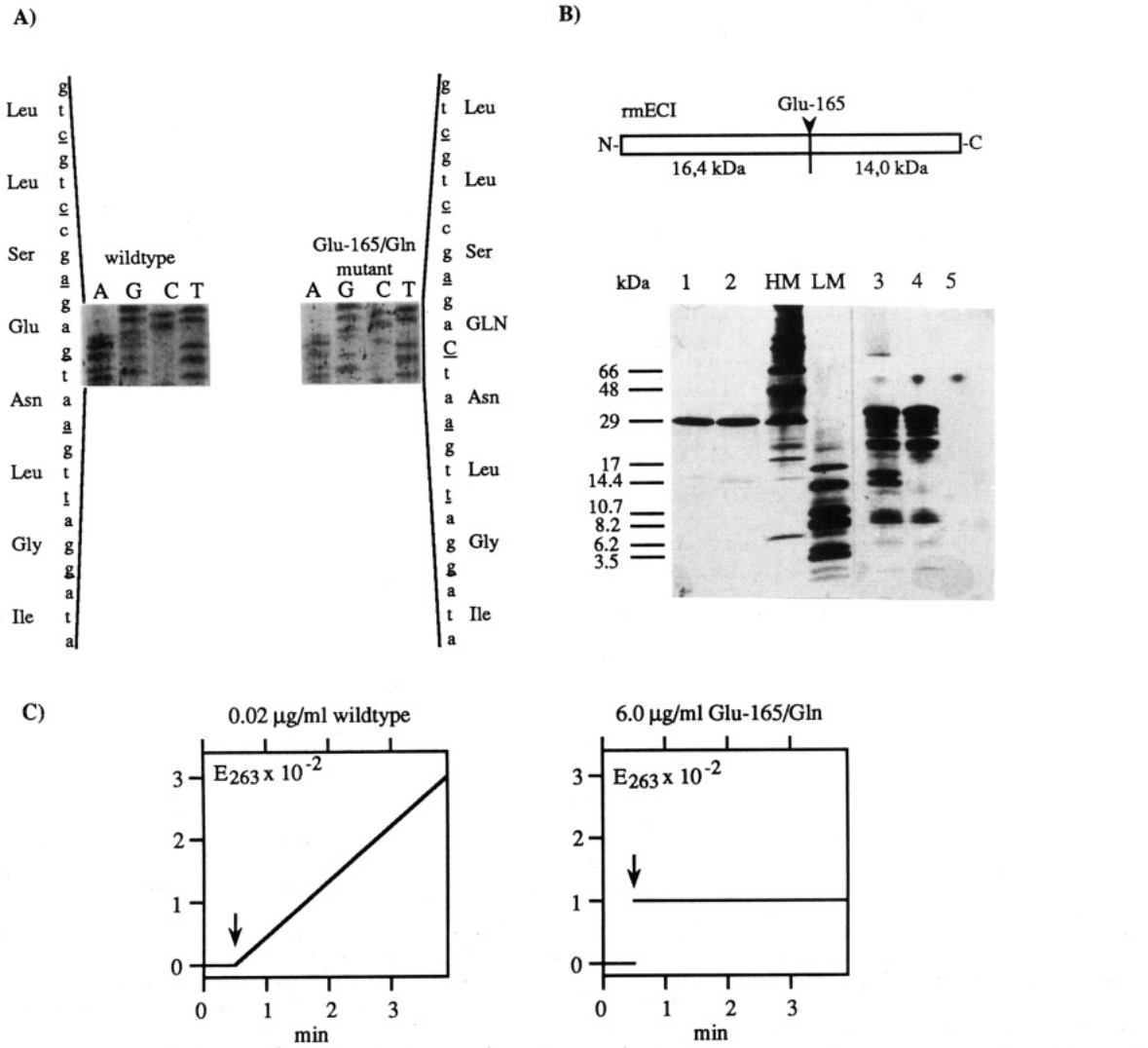

FIGURE 5: Characterization of the Glu-165/Gln mutant. (A) Dideoxy sequencing of the wild-type (pETrmECI) and mutant expression vector (pETrmECI/Q). The introduced G–C transversion leads to the Glu–Gln exchange (marked with capital letters). (B) Partial protease V8 (endoproteinase Glu-C) digestion of wild-type and Glu-165/Gln mutant rmECI. Silver-stained high-resolution SDS-PAGE of the following: lane 1, purified wild-type rmECI; lane 2, purified Glu-165/Gln mutant protein; HM, high molecular weight marker; LM, low molecular weight marker; lane 3, protease V8 partial digest of purified wild-type rmECI; lane 4, protease V8 partial digest of purfied Glu-165/Gln mutant protein; lane 5, protease V8 control. The two additional peptide bands in lane 3 result from cleavage of the wild-type protein at Glu-165 (see scheme of rmECI above the gel). These bands are missing in the mutant protein-derived peptides due to the Glu-165/Gln exchange. (C) Comparison of the enzymatic activity of wild-type rmECI and Glu-165/Gln mutant proteins in the optical assay with 35 µM 3-*cis*-dodecenoyl-CoA as substrate. Addition of 20 ng of wild-type rmECI (arrow) leads to a constant increase of absorbance at 263 nm. The addition of 6 µg of Glu-165/Gln-mutant protein (arrow) leads only to an elevated absorbance at 263 nm due to the protein concentration. Subsequent addition of wild-type protein leads to the increase of absorbance caused by the enzymatic activity (not shown).

related mECI, are also concerted. In this case, a second amino acid with a protic side chain must participate in the proton shift. The conservation of Glu-165 in mECH suggests that the respective glutamic acid is also involved in the active site of mECH, possibly with an identical function as in ECI catalysis.

The Δ^5-3-keto steroid isomerase (KSI) from *Pseudomonas testosteroni* catalyzes a reaction very similar to the ECI reaction, the conversion of Δ^5-3-keto steroids to Δ^4-3-keto steroids (Talalay & Wang, 1955). Here, as in the ECI reaction, an isolated C–C double bond is shifted into conjugation with a carbonyl function. However, the amino acid sequence of KSI shows no significant homologies to mECI or another member of the isomerase/hydratase enzyme family. In the KSI reaction, Asp-38 removes an axial 4β-proton of androst-5-ene-3,17-dione. The carbanionic intermediate is stabilized by protonation of the 3-carbonyl group by the hydroxy group of Tyr-14 to form 3,5-dienol intermediate. The 6β-position is reprotonated by Asp-38, and deprotonation of the carbonyl oxygen by Tyr-14 yields the conjugated Δ^4-3-keto steroid (Kuliopulos et al., 1989). In analogy to the KSI mechanism, the mECI mechanism may also proceed with proton retention via a carbanionic intermediate. In this case, it is possible that Glu-165 is the only amino acid involved in the proton transfer, analogous to Asp-38 of the KSI. The role Tyr-14 plays in KSI catalysis is not shared by the conserved Tyr-150 of mECI, because Tyr-150 can be exchanged without substantial loss of enzymatic activity. The conservation of Glu-165 of mECI in ECH, pTFE, and FadB suggests that at the polyfunctional enzymes, ECI and ECH catalysis occur at one active site, possibly with the respective glutamic acid facilitating both activities.

Final insight into the structure–function relationships of mECI and the other enzymes of the isomerase/hydratase enzyme family might come from solution of the X-ray crystal structures of wild-type and mutant enzymes. For the interpretation of the structure, the mutagenesis experiments presented here will be of interest.

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