Glutamate-119 of the Large α-Subunit Is the Catalytic Base in the Hydration of 2-*trans*-Enoyl-Coenzyme A Catalyzed by the Multienzyme Complex of Fatty Acid Oxidation from *Escherichia coli*†

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ABSTRACT: Glu^{139} of the large α-subunit of the multienzyme complex of fatty acid oxidation from *Escherichia coli* was identified as the catalytic residue of enoyl-CoA hydratase [Yang, S.-Y., He, X.-Y., & Schulz, H. (1995) *Biochemistry 34*, 6441−6447]. To determine whether any of the other conserved protic residues is directly involved in the hydratase catalysis, the multienzyme complexes with either an α/Asp^{69} → Asn or an α/Glu^{119} → Gln mutation were overproduced and characterized. The catalytic properties of 3-ketoacyl-CoA thiolase and L-3-hydroxyacyl-CoA dehydrogenase of the mutant complexes were almost unaffected. The amidation of Asp^{69} and Glu^{119} caused a 7.6- and 88-fold decrease, respectively, in the k_{cat} of enoyl-CoA hydratase without a significant change in the K_m value of the hydratase as well as a 5.9- and 62-fold increase, respectively, in the K_m of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase with a very small decrease in the k_{cat} of the latter enzyme. The data suggest that the carboxyl group of Glu^{119} is particularly important to the catalytic activity of enoyl-CoA hydratase. Furthermore, the wild-type hydratase shows a bell-shaped pH dependence of the k_{cat}/K_m with pK_a values of 5.9 and 9.2, whereas the Glu^{119} → Gln mutant hydratase has only a single pK_a of 9.5. A simple explanation for these observations is that a deprotonated Glu^{119} and a protonated Glu^{139} are required for the high k_{cat} of the enoyl-CoA hydratase. The results of site-directed mutagenesis studies, together with the structural information about the spatial arrangement of two conserved glutamate residues of rat liver enoyl-CoA hydratase [Engel, C. K., Mathieu, M., Zeelen, J. P., Hiltunen, J. K., and Wierenga, R. K. (1996) *EMBO J. 15*, 5135−5145] to which Glu^{119} and Glu^{139} of the large α-subunit correspond, lead to the conclusion that the γ-carboxyl group of Glu^{119} serves as the second general acid−base functional group in catalyzing the hydration of 2-*trans*-enoyl-CoA.

The multienzyme complex of fatty acid oxidation from *Escherichia coli* consists of two 79 kDa α-subunits and two 41 kDa β-subunits (*1*). The small β-subunit is a 3-ketoacyl-CoA thiolase (EC 2.3.1.16), whereas the large α-subunit is a multifunctional protein, of which the C-terminal domain is a L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (*2*−*4*). The molecular mechanism of the dehydrogenase catalysis has recently been elucidated by our site-directed mutagenesis studies (*5*, *6*). There are two enzyme activities, enoyl-CoA hydratase (EC 4.2.1.17) and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8), associated with the N-terminal domain of the large α-subunit (*7*, *8*). The γ-carboxyl group of glutamate-139 has been identified as the catalytic residue of enoyl-CoA hydratase (*8*). Meanwhile, the counterpart of this glutamate residue in rat liver crotonase was found to play a similar role (*9*). Yang et al. (*8*) and Müller-Newen et al. (*9*) have independently reached a conclusion that the γ-carboxyl group of such a conserved glutamate residue functions as a catalytic base to abstract the α-proton from the substrate.

Enoyl-CoA hydratase catalyzes the second reaction of the fatty acid β-oxidation, i.e., the *syn* addition of water to α,β-unsaturated fatty acyl-CoA thioesters (*10*). The kinetic properties of enoyl-CoA hydratase from various sources were reported previously (*11*−*15*). Although the *syn* addition−elimination of water proved not to be superior to an *anti* process in chemical efficiency (*16*), Gerlt and Gassman (*17*) have argued that the *syn* stereochemical course of the hydration/dehydration reaction catalyzed by the hydratase might be required for efficient catalysis by natural selection because a single acid−base enzymatic group could interact both with the α-proton and with the β leaving group in a stepwise manner. Bahnson and Anderson (*18*) believe that the *syn* stereochemistry of the crotonase-catalyzed β elimination would permit a single active-site base to mediate both proton transfers in a concerted mechanism because a carboxylate can function as both proton donor and acceptor in a cyclic transition state. Later D'Ordine et al. (*19*) also stressed the point that only one general acid−base functional group is required for the catalysis of a β-elimination reaction. As a result, an economical use of catalytic groups by the enzyme became the prevailing theory of the enoyl-CoA hydratase catalysis. However, such a theory is still lacking convincing evidence, because identification of a functional group, such as the γ-carboxyl group of glutamate-139 of the large α-subunit that is essential to the hydratase catalysis (*8*), does not necessarily rule out the possibility that the

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removal of the β leaving group is facilitated by another catalytic acid−base functional group. Sequence alignments showed that, in addition to the glutamate previously identified as a catalytic residue (*8*, *9*), only two protic residues (one aspartate and one glutamate) are conserved in all 11 known enoyl-CoA hydratases (S.-Y. Yang, unpublished results). In order to determine whether an additional general acid−base functional group is involved in the catalytic process, those two conserved acidic residues were chosen for site-directed mutagenesis studies.

In this report, we provide evidence that the γ-carboxyl group of glutamate-119 of the large α-subunit of the *E. coli* fatty acid oxidation complex serves as a catalytic base of enoyl-CoA hydratase. A deprotonated glutamate-119 and a protonated glutamate-139 of the multifunctional fatty acid oxidation protein are required for catalyzing the hydration of crotonyl-CoA. On the basis of kinetic data and structural information we propose a mechanism for the hydration of 2-*trans*-enoyl-CoA catalyzed by the γ-carboxyl groups of two conserved glutamate residues of enoyl-CoA hydratases.

MATERIALS AND METHODS

Materials. NAD^+, NADH, CoASH, pig heart L-3-hydroxyacyl-CoA dehydrogenase, and all other standard biochemicals were obtained from Sigma. 3-*cis*-Tetradecenoic acid (*20*), crotonyl-CoA (*21*), and acetoacetyl-CoA (*22*) were synthesized according to published procedures. The CoA derivative of 3-*cis*-tetradecenoic acid was synthesized by the mixed anhydride procedure as detailed by Goldman and Vagelos (*23*). Bovine liver crotonase (*13*) and pig heart 3-ketoacyl-CoA thiolase (*24*) were purified according to published methods. *E. coli* BL21(DE3) pLysS and BMH71-18 *mutS* were obtained from Novagen and Clontech, respectively. Restriction endonucleases, T4 polynucleotide kinase, T4 ligase, and T4 DNA polymerase were supplied by Boehringer Mannheim. The Sequenase version 2.0 kit and radiochemicals were purchased from Amersham. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc.

Site-Directed Mutagenesis of the fadB Gene. The single-stranded DNA template for mutagenesis was prepared as described previously (*7*). The oligonucleotide-directed mutagenesis was carried out by an adaptation of the unique site elimination method (*25*). A synthetic oligonucleotide 5′-CAGGCATGC**A**C**GC**G**T**GGCGTAATC-3′ with an *Mlu*I site instead of a *Hind*III site was used as the selection primer. Oligonucleotides 5′-GTCGGTGCT**AAT**ATCACCGAA-3′ and 5′-GGTGGCTGC**CAA**TGCGTGCT-3′, containing codon changes from GAT(Asp) to AAT (Asn) and from GAA (Glu) to CAA (Gln), respectively, were used as the mutagenic primers. The selection primer and one of the mutagenic primers were simultaneously annealed to the template and then incorporated into a new strand of DNA as a result of the elongation catalyzed by T4 DNA polymerase. After this heteroduplex DNA was introduced into *E. coli* BMH 71-18 *mutS*, the replicative form of M13 was isolated from the transformants and digested by *Hind*III. Suitable mutant clones were selected for the presence of the unique *Mlu*I site. The desired mutation on the *fadB* gene was identified by dideoxy sequencing (*26*). The mutant *fadB* gene containing the desired mutation was reconstructed back into the *Bam*HI−*Sal*I site of the original expression plasmid pNDBA, which could produce the *E. coli* wild-type complex (*7*), to form a mutant expression plasmid. The mutant expression plasmids carrying the α/Asp^{69} → Asn and α/Glu^{119} → Gln mutation were designated as pNDBA-10 and pNDBA-11, respectively.

Overexpression and Purification of Mutant Fatty Acid Oxidation Complexes from E. coli. The mutant expression plasmids were transformed into *E. coli* BL21(DE3) pLysS according to the method of Chung et al. (*27*). The transformants were grown in 2YT medium to an absorbance of about 1.0 at 600 nm and then induced by 0.5 mM IPTG[1] for 4 h. Cells were harvested by centrifugation for 10 min at 3000*g*, 4 °C, and washed twice with ice-cold 200 mM potassium phosphate, pH 8.0. The preparation of cell extracts and the purification of the fatty acid oxidation complex were performed as described previously (*28*).

Protein Analysis and Enzyme Assays. Protein concentrations were determined by the method of Bradford (*29*). Proteins were separated by SDS−PAGE on a 4−20% gradient gel at pH 8.3 as described previously (*30*). The enoyl-CoA hydratase activity was measured by the direct assay method (*1*) with crotonyl-CoA as substrate. Assays of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase with acetoacetyl-CoA as substrate and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase with 3-*cis*-tetradecenoyl-CoA as substrate were also performed as described previously (*1*). Kinetic parameters of different component enzymes and pK_a values of enoyl-CoA hydratase of the wild-type and mutant complexes were estimated by analysis of the kinetic data with the computer program Leonora (*31*). All enzymes were assayed at 25 °C on a Gilford recording spectrophotometer (Model 2600). A unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product/min.

RESULTS AND DISCUSSION

Expression of the α/Asp^{69} → Asn and α/Glu^{119} → Gln Mutant Complexes. Expression of these mutant complexes by IPTG induction was as effective as the production of the wild-type complex, which constituted about a third of the total soluble proteins in *E. coli* cells (data not shown) (*7*). Four different enzyme activities were measured in cell extracts of the transformants containing plasmids pNDBA, pNDBA-10, or pNDBA-11, and are listed in Table 1. Activities of the β-oxidation enzymes present in the cell extract of the transformant containing a blank vector were undetectable or at least several hundred times lower than in the cell extract containing the wild-type complex (Table 1). This system is suitable for studying the impact of mutations on enoyl-CoA hydratase and the other activities of the complex. The activities of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase in cell extracts containing the mutant complexes were only slightly lower than the wild-type control levels. This indicates that replacement of either of these conserved acidic residues by the corresponding amide did not disrupt the structure of the fatty acid oxidation complex. The enoyl-CoA hydratase activity in cell extracts containing the α/Asp^{69} → Asn and α/Glu^{119} → Gln mutant complexes was about 11- and 110-fold lower, respectively,

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Table 1: Activities of Component Enzymes of the Fatty Acid Oxidation Complexes in IPTG-Induced Cell Extracts of *E. coli* BL21(DE3) pLysS Transformants Containing Different Plasmids

enzyme	specific activity[a] (units/mg of protein)			
	PND-1 (no complex)	pNDBA (wild type)	pNDBA-10 (α/Asp^{69} → Gln)	pNDBA-11 (α/Glu^{119} → Gln)
enoyl-CoA hydratase	0.08	42.7	4.03	0.39
L-3-hydroxyacyl-CoA dehydrogenase	0.02	8.70	8.25	5.35
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	0[b]	2.87	2.19	0.15
3-ketoacyl-CoA thiolase	0.01	2.39	2.31	1.47

[a] Values are averages of three determinations. [b] Zero implies activity of <0.005 unit/mg.

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                    116          139
                     |  *         |
MP 108    AVNGYALGGGCECVLATDYRLATPDLR--IGLPETKLGIMPG
TE  92    AIQGVALGGGLELALGCHYRIANAKAR--VGLPEVTLGILPG
PT 108    AIDGLALGGGLEVAMACHARISTPTAQ--LGLPELQLGIIPG
LT 104    AINGTCLGGGLELAISCQYRIATKDKKTVLGAPEVLLGILPG
EI 104    AINGASPAGGCLMALTCDYRIMADNSKYTIGLNESLLGIVAP
EH 104    AVNGYALGGGCELAMMCDIIYAGEKAQ--FGQPEILLGTIPG
CC 103    AVNGFALGGGCEIANSCDIRIASSNAR--FGQPEVGLGITPG
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FIGURE 1: Comparison of the amino acid sequence in the central region of the amino-terminal domain of the *E. coli* multifunctional protein (MP) (*4*) with those of homologous regions of rat peroxisomal trifunctional enzyme (TE) (*41*), plant glyoxysomal tetrafunctional protein (PT) (*48*), pig mitochondrial long-chain-specific bifunctional enzyme (LT) (*42*), rat mitochondrial Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (EI) (*49*) and enoyl-CoA hydratase (EH) (*43*), and crotonase from *Clostridium acetobutylicum* (CC) (*50*). Standard one-letter amino acid abbreviations are used. The glutamate residue that was substituted by glutamine in the *E. coli* mutant multifunctional fatty acid oxidation protein is indicated by an asterisk.

as compared with the wild-type control. In addition, the Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activity in the cell extract containing the α/Glu^{119} → Gln mutant complex was 19-fold lower than the wild-type control (Table 1). The ionizable carboxyl group of glutamate-119 appears to be more important than that of aspartate-69 to the hydratase catalysis. This was not a surprising observation, since glutamate-119 but not aspartate-69 is located in the center of the N-terminal region of the *E. coli* multifunctional fatty acid oxidation protein that is conserved throughout the hydratase family and plays an important role in the catalytic function (Figure 1) (*4*, *8*). The results of this study suggest that the γ-carboxyl group of glutamate-119 is a likely candidate for the general acid−base functional group of enoyl-CoA hydratase.

The Ionizable Carboxyl Group of Glutamate-119 Is Particularly Important to the Catalytic Activity of Enoyl-CoA Hydratase. The catalytic properties of the purified mutant complexes were characterized by steady-state kinetic measurements (*32*). The kinetic parameters of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase of the mutant complex were similar to those of the wild-type complex (Table 2). The data suggest that these point mutations did not cause a long-range structural perturbation in the large α-subunit or a change in the quaternary structure of the complex. The α/Asp^{69} → Asn and the α/Glu^{119} → Gln mutations resulted in 5.9- and 62-fold increases, respectively, in the K_m values of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase but only caused a slight decrease in the k_{cat} value of the isomerase. The results suggest that neither aspartate-69 nor glutamate-119 functions as a catalytic residue of the isomerase but that the latter residue is probably located around the substrate-binding site of the isomerase. It is noteworthy that (a) glutamate-119 was conserved in rat liver hydratase but not in the isomerase (see Figure 1) and (b) the hydratase and the isomerase of the large α-subunit of the *E. coli* fatty acid oxidation complex may share a substrate-binding site (*8*). These structural features seem to be consistent with the kinetic data obtained in this study (Table 2). Since the α/Asp^{69} → Asn and the α/Glu^{119} → Gln substitutions reduced the rate constant of enoyl-CoA hydratase by 7.6- and 88-fold, respectively, without a significant change in the K_m value for crotonyl-CoA, the γ-carboxyl group of glutamate-119 is most likely involved in the catalytic mechanism of the hydratase. The high catalytic efficiency of enoyl-CoA hydratase could be best interpreted by a cooperation of glutamate-119 with glutamate-139, a primary catalytic residue identified previously (*8*), in the catalytic process.

pH Dependence of k_{cat}/K_m of the Wild-Type and Mutant Enoyl-CoA Hydratases. The effects of pH on the kinetic parameters of the wild-type and the Glu^{119} → Gln mutant hydratases were studied over a broad range of pH values. The bell-shaped pH dependence of wild-type enoyl-CoA hydratase of the large α-subunit shows pK_a values of 5.9 ($\pm$0.06) and 9.2 ($\pm$0.06) (Figure 2), of which the latter most likely reflects ionization of the γ-carboxyl group of glutamate-139 because a protonated primary catalytic residue is required for transferring a proton to the α-carbon of crotonyl-CoA for converting the substrate to L-3-hydroxybutyryl-CoA. The unusually high pK_a of glutamate-139, the primary catalytic residue of the hydratase (*8*), is probably caused by the hydrophobic environment of the active site (*33*). As shown in Figure 1, its counterpart in rat liver enoyl-CoA hydratase is glutamate-164,[2] which has a reported pK_a of 8.5 and was shown to catalyze the exchange of the α-proton of butyryl-CoA with solvent (*34*). Therefore, it seems to be a general phenomenon that this conserved glutamate, the primary catalytic residue (*8*, *9*), has a γ-carboxyl group with a high pK_a value. Another pK_a of the wild-type hydratase is assigned to the ionization of the γ-carboxyl group of glutamate-119. This assignment is supported by the observations that the α/Glu^{119} → Gln substitution not only reduces the catalytic efficiency of the hydratase by 100-fold at pH 8.0 but also alters the pH profile. The hydratase of the α/Glu^{119} → Gln mutant complex has only a single pK_a value of 9.5 ($\pm$0.09) (see Figure 2), while the pK_a value of 5.9, attributed to glutamate-119, is abolished. The mutant hydratase retains a pK_a of 9.5 because of the presence of glutamate-139, which participates in the acid−base catalysis. However, the amidation of glutamate-119 abolished the

[2] The numbering of the hydratase reported previously reflected the precursor of rat liver mitochondrial enoyl-CoA hydratase, which has a leader peptide consisting of 29 residues (*43*). However, the mature hydratase was actually subjected to the investigation and thus was used for alignment in Figure 1.

Table 2: Kinetic Parameters of Different Component Enzymes of the Wild-Type and Mutant Multienzyme Complex of Fatty Acid Oxidation from *E. coli*

enzyme and substrate	wild type		α/Asp^{69} → Asn mutant		α/Glu^{119} → Gln mutant	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
enoyl-CoA hydratase	825 ± 43		108 ± 6		9.4 ± 0.2	
crotonyl-CoA		64 ± 5		83 ± 6		73 ± 2
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	62 ± 2		51 ± 7		32 ± 5	
3-*cis*-tetradecenoyl-CoA		5.8 ± 0.3		34 ± 6		373 ± 55
L-3-hydroxyacyl-CoA dehydrogenase	383 ± 3		284 ± 2		277 ± 6	
acetoacetyl-CoA		70 ± 1		112 ± 1		183 ± 5
NADH		1.8 ± 0.2		2.2 ± 0.1		8.2 ± 0.8
3-ketoacyl-CoA thiolase	95 ± 5		91 ± 24		57 ± 11	
acetoacetyl-CoA		82 ± 5		129 ± 39		87 ± 22
CoASH		89 ± 4		77 ± 21		133 ± 28

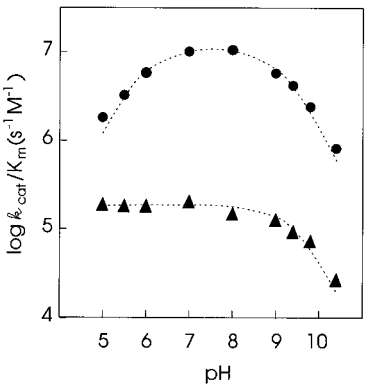

FIGURE 2: pH dependence of k_{cat}/K_m for the *E. coli* wild-type and α/Glu^{119} → Gln mutant enoyl-CoA hydratases. To determine the steady-state kinetic parameters of the hydratase over a range of pH, the initial rate at different crotonyl-CoA concentrations was measured at 25 °C in a buffer containing 200 mM potassium phosphate and bovine serum albumin (0.2 mg/mL), adjusted to the appropriate pH. Symbols: wild-type (●) and α/Glu^{119} → Gln mutant (▲) fatty acid oxidation complex. The data were fit with the computer program Leonora (*31*) to generate two dashed curves: the upper one shows pK_a values of 5.9 and 9.2, while the lower one has a single pK_a of 9.5.

ability of its γ-carboxyl group to transfer a proton in the physiological pH range. A simple explanation for the difference between the pH profiles of the wild-type and the Glu^{119}→ Gln mutant hydratases is that glutamate-119 of the large α-subunit is involved in the acid−base catalysis of the wild-type hydratase. The results suggest that a deprotonated glutamate-119 and a protonated glutamate-139 should synergetically participate in the hydration of 2-*trans*-enoyl-CoA catalyzed by the large α-subunit of the *E. coli* fatty acid oxidation complex.

Catalytic Mechanism of Enoyl-CoA Hydratase. On the basis of the kinetic data and pH dependence of the wild-type and the Glu^{119} → Gln mutant hydratases, we propose a molecular mechanism of the hydratase catalysis (Figure 3). Since the catalytic efficiency of the Glu^{119} → Gln mutant hydratase was about 100-fold lower than that of the wild-type hydratase (Table 2), the amidation of the γ-carboxyl group of glutamate-119 increased the free energy of the transition state by 2.7 kcal/mol. It was reported (*8*) that the Glu^{139} → Gln mutation of the large α-subunit resulted in a 2400-fold decrease in the catalytic efficiency of enoyl-CoA hydratase. Accordingly, the two ionizable carboxyl groups of glutamate-119 and glutamate-139 together would cause an estimated reduction of the activation energy of the hydration reaction by 7.3 kcal/mol, and the high catalytic efficiency of the hydratase is therefore attributed primarily to these two general acid−base functional groups. When the pH dependence of the wild-type and mutant hydratases also are taken into account, the following scenario of the hydratase catalysis shown in Figure 3 emerges. The protonated glutamate-139 transfers a proton to the α-carbon of the substrate (*8*), whereas a deprotonated glutamate-119 accepts a proton from water to form hydroxide anion, which adds to the β-carbon of the substrate. Other factors that could stabilize the transition state play a part in the catalytic process as well. For example, a proton donor adjacent to the carbonyl oxygen of the substrate may be involved in forming a strong hydrogen bond thereby reducing the $\Delta G^{\ddagger}$ value of the reaction (*17*, *35*), and a partial positive charge may be induced at the β carbon of the substrate due to an electronic rearrangement, which was detected in the acryloyl portion of substrate analogs upon binding to bovine liver crotonase (*19*). These factors probably make some contributions to the high catalytic efficiency of enoyl-CoA hydratase by reducing the pK_a of the α-proton (*17*, *35*) or by enhancing the electrophilicity of the β carbon of the substrate (*19*). However, the recognition of such factors should no longer be considered evidence for the theory (*17*, *19*) that a single functional group is responsible for the hydratase catalysis. In fact, the available evidence supports a new theory according to which two general acid−base groups function as shown in Figure 3.

During preparation of this paper, an article describing the NMR structure of 2,4-dienoyl-CoA, a poor substrate (*36*) bound to rat liver enoyl-CoA hydratase, appeared (*37*). It was reported (*37*) that the carbonyl oxygen was 2.7 Å from the backbone nitrogen of glycine-141 and that the β-carbon and α-carbon of the substrate were 4.1 and 3.6 Å, respectively, from the γ-carboxyl groups of glutamate-144 and glutamate-164, which were positioned on the same side of the substrate. The crystal structure of the hydratase complexed with an inhibitor, acetoacetyl-CoA, has recently been determined at 2.5 Å resolution (*38*). It was found (*38*) that a carboxyl oxygen of glutamate-164 was about 3.9 Å from the α carbon of the acetoacetyl moiety, while the carboxyl group of glutamate-144 was hydrogen-bonded to the carbonyl oxygen on the β carbon of the inhibitor. Furthermore, there is a bound water molecule in the unliganded active site and this water is 2.8 and 3.6 Å from the carboxyl oxygens of glutamate-144 and glutamate-164, respectively. Superposition of the unliganded active site on the liganded one revealed that this water is very close to the β carbon of the acetoacetyl moiety, at a distance of only 1 Å, so it may serve for hydrating the true substrate (*38*). Notably, both studies (*37*,

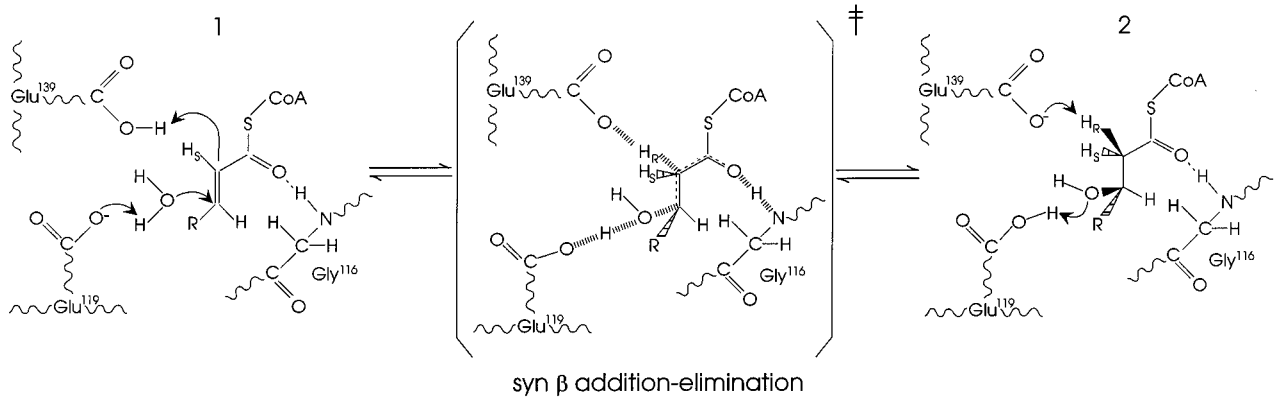

FIGURE 3: Schematic diagram of the hydration of 2-*trans*-enoyl-CoA catalyzed by the *E. coli* enoyl-CoA hydratase. Compounds 1 and 2 are 2-*trans*-enoyl-CoA and L-3-hydroxyacyl-CoA, respectively. The protonated Glu^{139} transfers a proton to the α-carbon of the substrate on the *re* face, whereas the deprotonated Glu^{119} attracts a proton from water whose oxygen makes a nucleophilic attack on the β-carbon of the substrate. The imino group of Gly^{116} in the peptide backbone acts as a hydrogen donor to form a hydrogen bond with the carbonyl oxygen so that an electronic rearrangement occurs in the acryloyl portion of the substrate. The transition state is shown in the square brackets. The product, L-3-hydroxyacyl-CoA, can then leave the active site to finish this reaction, which is the second step of the fatty acid β-oxidation pathway. Two general acid−base functional groups, the γ-carboxyl groups of Glu^{139} and Glu^{119}, play a major part in the hydratase catalysis. The dehydration of L-3-hydroxyacyl-CoA is a *syn* β-elimination reaction, which proceeds via the microscopic reverse process of the addition reaction. R represents a methyl or other odd-numbered alkyl group.

38) showed that the carboxyl group of glutamate-144 was in the proximity of the β carbon but not the α carbon of the fatty acyl moiety. Since enoyl-CoA hydratases from various sources catalyze the same reaction and since the amino acid sequences of their central region are highly homologous to one another (see Figure 1), their functional residues, such as glutamate-139 and glutamate-119 of the *E. coli* enoyl-CoA hydratase and the corresponding glutamate residues in the hydratases from other species, are most likely to retain a specific spatial arrangement under evolutionary pressure (*39*), as was the situation reported for the members of the L-3-hydroxyacyl-CoA dehydrogenase family (*5*). If so, the γ-carboxyl groups of glutamate-119 and glutamate-139 of the large α-subunit are expected to be very close to the β- and α-carbon of the substrate, respectively, as seen with their corresponding amino acids, glutamate-144 and glutamate-164, in rat liver enoyl-CoA hydratase.[2] The structural arrangement in the active site of the hydratase indicates that glutamate-119 probably does not play an active part in protonating the α carbon of the substrate. Moreover, the carbonyl oxygen of the substrate is near the backbone nitrogen of glycine-116, which is equivalent to glycine-141 of the rat hydratase,[2] such that the peptide nitrogen can serve as a hydrogen donor to form a hydrogen bond with the carbonyl oxygen of the substrate (see Figure 3). The catalytic mechanism of enoyl-CoA hydratase we propose on the basis of results of site-directed mutagenesis studies is consistent with the preliminary structural information on the active site of the hydratase (*37*, *38*). Several lines of evidence lead to the conclusion that the γ-carboxyl group of glutamate-119 serves as the second general acid−base functional group participating in the enoyl-CoA hydratase-catalyzed *syn* β addition−elimination.

Attempts to dissect the hydration/dehydration reaction catalyzed by the hydratase into two partial reactions have not been successful, but it has been done in the case of enolase (*40*). The γ-carboxyl group of glutamate-164 of rat liver enoyl-CoA hydratase plays a key role in abstraction of the *pro*-2*R* proton for catalyzing the hydration/dehydration reaction (*9*, *10*), and it can also catalyze the stereospecific exchange of the *pro*-2*S* proton of butyryl-CoA with solvent (*34*). However, there is no evidence for proton abstraction without elimination in the hydratase equilibrations with 3(*S*)-hydroxybutyryl-CoA, 3-hydroxypropionyl-CoA, or 3-chloropropionyl-CoA (*34*). Theoretically the possibility that some deprotonated glutamate-164 may be somehow involved in activating a water molecule cannot be absolutely excluded, though the "activated" water in the active site was found to be farther from the carboxyl oxygen of glutamate-164 than from that of glutamate-144 (*38*). However, this situation has little impact, if any, on the actual reaction because glutamate-139 of the *E. coli* enoyl-CoA hydratase, which corresponds to glutamate-164 of rat liver enoyl-CoA hydratase, was found to have a very high pK_a (see Figure 2) that is nearly 2 units above the physiological pH. Evidently, the catalytic mechanism of enoyl-CoA hydratase that we are proposing (see Figure 3) is applicable to the hydratases of other multifunctional β-oxidation enzymes (*41*, *42*) and to monofunctional enoyl-CoA hydratases (*43*, *44*) regardless of their substrate chain length specificities (*14*, *20*). It is, therefore, predicted that if the presence of a long-chain enoyl-CoA hydratase in *Clostridium acetobutylicum* (*45*) and a medium-chain enoyl-CoA hydratase in the mitochondrial matrix (*46*) are eventually confirmed by purification, their catalytic mechanisms will be found to be very similar to what we have presented here. The understanding of the catalytic mechanism of enoyl-CoA hydratase can also provide the best explanation why some proteins with a region homologous to the hydratase sequence, e.g., an AU-specific RNA binding protein (*47*), would have intrinsic enoyl-CoA hydratase activity. Finally, the finding of two conserved glutamate residues playing a major part in the hydratase catalysis has perhaps laid the foundation for ultimately solving the long debate whether the β addition−elimination reaction catalyzed by bovine liver crotonase proceeds via a concerted mechanism (*18*) or a stepwise mechanism (*17*).

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