Glutamate 139 of the Large α-Subunit Is the Catalytic Base in the Dehydration of Both D- and L-3-Hydroxyacyl-Coenzyme A But Not in the Isomerization of Δ^3,Δ^2-Enoyl-Coenzyme A Catalyzed by the Multienzyme Complex of Fatty Acid Oxidation from *Escherichia coli*†

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ABSTRACT: Multienzyme complexes of fatty acid oxidation from *Escherichia coli* with either an α/Glu^{139} → Gln or an α/Arg^{134} → Gln mutation in the large α-subunit have been overproduced and characterized. The catalytic properties of the five different component enzymes of the α/Arg^{134} → Gln mutant complex showed no significant changes as compared with those of the wild type complex. In contrast, the 3-hydroxyacyl-coenzyme A (CoA) epimerase activity of the α/Glu^{139} → Gln mutant complex was not detected, and this mutant complex has lost almost all of the enoyl-CoA hydratase activity due to a greater than 3000-fold decrease in the k_{cat} of the enoyl-CoA hydratase without a significant change in the K_m value. The catalytic properties of 3-ketoacyl-CoA thiolase and L-3-hydroxyacyl-CoA dehydrogenase were virtually unaffected by the mutation. Together, these observations lead to the conclusion that the γ-carboxylic group of Glu^{139} functions as a catalytic base in the dehydration of both D- and L-3-hydroxyacyl-CoA. These findings also support a dehydration/hydration mechanism for 3-hydroxyacyl-CoA epimerase but do not agree with an epimerase activity independent of enoyl-CoA hydratase as proposed for the glyoxysomal tetrafunctional protein [Preisig-Müller, R., Gühnemann-Schäfer, K., & Kindl, H. (1994) *J. Biol. Chem. 269*, 20475−20481]. Since this mutation caused the k_{cat} of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase to decrease by only 60%, even though the K_m value was significantly increased, it seems that Glu^{139} of the *E. coli* multifunctional protein does not function as a catalytic residue in the isomerization reaction. However, the *E. coli* enoyl-CoA hydratase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase may share a substrate-binding site because 3-*cis*-octenoyl-CoA, a substrate of the isomerase, was found to be a strong competitive inhibitor of the *E. coli* enoyl-CoA hydratase with a K_i of 3 μM.

The large α-subunit of the multienzyme complex of fatty acid oxidation of *Escherichia coli* is a 79-kDa multifunctional protein exhibiting four different enzymatic activities (Yang & Schulz, 1983; Yang et al., 1988, 1991). Enoyl-CoA hydratase (EC 4.2.1.17) and L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) catalyze the second and third reactions of the β-oxidation spiral, respectively, whereas Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) function in the degradation of unsaturated fatty acids (Schulz, 1991). The peroxisomal trifunctional β-oxidation enzyme from rat liver (Osumi & Hashimoto, 1979; Osumi et al., 1985) and the large subunit of the mitochondrial membrane-bound β-oxidation complex from rat liver (Uchida et al., 1992; Kamijo et al., 1993) and pig heart (Luo et al., 1993; Yang et al., 1994) are homologous to the large α-subunit of the *E. coli* fatty acid oxidation complex (Binstock et al., 1977; Yang et al., 1991), which is in fact a prototype of the extant multifunctional β-oxidation enzymes (Yang, 1994).

The amino-terminal region of the *E. coli* multifunctional fatty acid oxidation protein is involved in the hydratase, the epimerase, and the isomerase functions, while the dehydrogenase activity is associated with a functional domain in the central region of the polypeptide (Yang & Elzinga, 1993). On the basis of a study of the Gly^{116} → Phe mutation in the large α-subunit of the *E. coli* fatty acid oxidation complex, one of us has suggested that enoyl-CoA hydratase and 3-hydroxyacyl-CoA epimerase are associated with a common active site and that the key residue(s) required for catalyzing the isomerization reaction is distinct from the residue essential for crotonase activity (Yang & Elzinga, 1993). In contrast, Müller-Newen and Stoffel (1993) suggested that a single residue, homologous to the active site residue of rat mitochondrial Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase, is responsible for both the isomerase and hydratase activities associated with the rat peroxisomal trifunctional enzyme and the large α-subunit of the *E. coli* complex. Contrasting with this view is a recent claim that the isomerase and the hydratase of the large α-subunit of the *E. coli* complex and the plant glyoxysomal tetrafunctional protein are associated with two distinct regions of the amino-terminal half of these

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multifunctional proteins [Preisig-Müller et al. (1994) Figure 8]. Furthermore, while the plant glyoxysomal tetrafunctional protein is clearly homologous to the *E. coli* multifunctional fatty acid oxidation protein, it was reported (Preisig-Müller et al., 1994) that the amino-terminal fragment of this plant multifunctional protein harbors a 3-hydroxyacyl-CoA epimerase, which can convert D-3-hydroxydecanoyl-CoA to its L-isomers in the absence of crotonase activity. The conflicting views about the structure−function relationship of these multifunctional proteins must be clarified. To this end, several questions need to be answered. Is there a catalytic residue essential for both the hydration and epimerization functions of the *E. coli* multifunctional protein? If so, where is this residue located? How does it function in catalysis? Is the isomerization reaction also catalyzed by this active site residue of the *E. coli* crotonase? Does the isomerase and the hydratase of the *E. coli* multifunctional protein share a common binding site for their substrates?

Although it remains contested whether the β-elimination reaction catalyzed by bovine liver crotonase proceeds via a concerted mechanism (Bahnson & Anderson, 1991) or a stepwise mechanism (Gerlt & Gassman, 1992), it is certain that a protic residue is involved in the deprotonation and protonation at the α-carbon of CoA thiolesters. In order to identify the catalytic residue, some protic amino acid residues in a region which is well conserved in the enoyl-CoA hydratase family (Minami-Ishii et al., 1989; Yang, 1994) were chosen for site-directed mutagenesis studies.

In this report, we provide evidence that the γ-carboxylic group of Glu^{139} of the large α-subunit of the *E. coli* fatty acid oxidation complex is essential for the dehydration of both D- and L-3-hydroxyacyl-CoA. Since this residue functions in the reactions catalyzed by enoyl-CoA hydratase and 3-hydroxyacyl-CoA epimerase, it is unlikely for the multifunctional protein to have a crotonase-independent epimerase. On the basis of structural information and kinetic data of the wild type and mutant complexes, we propose a mechanism for the epimerization of D-3-hydroxyacyl-CoA. Since the substrate of the Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase was found to be a strong competitive inhibitor of the *E. coli* enoyl-CoA hydratase, it appears that the hydratase and the isomerase of the *E. coli* multifunctional protein share a substrate-binding site without Glu^{139} being an essential catalytic residue in the isomerization reaction.

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 (Luo et al., 1993), 3-*cis*-octenoic acid (Stoffel & Ecker, 1969), crotonyl-CoA (Weeks & Wakil, 1968), and acetoacetyl-CoA (Seubert, 1960) were synthesized according to published procedures. The CoA derivatives of 3-*cis*-tetradecenoic acid, 3-*cis*-octenoic acid, and D,L-3-hydroxyoctanoic acid were synthesized by the mixed anhydride procedure as detailed by Goldman and Vagelos (1961). Bovine liver crotonase (Steinman & Hill, 1975) and pig heart 3-ketoacyl-CoA thiolase (Staack et al., 1978) were purified according to published methods. The *E. coli* fatty acid oxidation complex and the pNDBA expression plasmid (Yang & Elzinga, 1993) were prepared as described previously. *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. Sequenase Version 2.0 kit was purchased from United States Biochemical Corp. Oligonucleotides were synthesized with an Applied Biosystems, Inc., 380B DNA synthesizer (Caruthers, 1985).

Site-Directed Mutagenesis of the fadB Gene. The single-stranded DNA template for mutagenesis was prepared as described previously (Yang & Elzinga, 1993). The oligonucleotide-directed mutagenesis was carried out by an adaption of the unique site elimination method (Deng & Nickoloff, 1992). A synthetic oligonucleotide, 5′-CAGGCATGCA**C**GC**G**TGGCGTAATC-3′, with a *Mlu*I site instead of a *Hin*dIII site was used as the selection primer. Two other synthetic oligonucleotides, 5′-CCGGATCTG-**CAA**ATCGGTCTGC-3′ and 5′-CGGTCTGCCG**C**AACCAAACT-3′ containing codon changes from CGC (Arg) to CAA (Gln) and from GAA (Glu) to CAA (Gln), respectively, were used as the mutagenic primers. The selection primer and either mutagenic primer 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 double-stranded DNA was introduced into *E. coli* BMH 71-18 *mutS*, the replicative form of M13 was isolated from the transformants and digested by *Hin*dIII. The 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 (Sanger et al., 1977). A fragment of 465-bp DNA at the 5′-end of the mutant *fadB* gene containing the desired mutation was reconstructed back into the *Bam*HI-*Sna*BI site of the original expression plasmid pNDBA, which could produce the *E. coli* wild type complex (Yang & Elzinga, 1993), to form a mutant expression plasmid. The mutant expression plasmids carrying the $\alpha/Arg^{134} \rightarrow$ Gln and $\alpha/Glu^{139} \rightarrow$ Gln mutations were designated as pNDBA-3 and pNDBA-4, 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. (1989). 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 two times with ice-cold 200 mM KP_i, pH 8.0. The preparation of cell extracts and the purification of the fatty acid oxidation complex were performed as described previously (Yang et al., 1988).

Protein Analysis and Enzyme Assays. Protein concentrations were determined by the method of Bradford (1976). Protein were separated by SDS−PAGE on a 10% gel at pH 8.3 as described previously (Laemmli, 1970). The enoyl-CoA hydratase activity was measured by the direct assay method (Binstock & Schulz, 1981) with crotonyl-CoA as substrate in the absence and presence of HPLC-purified 3-*cis*-octenoyl-CoA. L-3-Hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities were determined with acetoacetyl-CoA as substrate by published procedures (Binstock & Schulz, 1981). Assays of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase with 3-*cis*-tetradecenoyl-CoA as substrate

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

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                          116                 134
                           |                   |        *
MP 108    AVNGYALGGGCECVLATDYRLATPDLR--IGLPETKLGIMPG
TE 92     AIQGVALGGGLELALGCHYRIANAKAR--VGLPEVTLGILPG
PT 108    AIDGLALGGGLEVAMACHARISTPTAQ--LGLPELQLGIIPG
LT 104    AINGTCLGGGLELAISCQYRIATKDKKTVLGAPEVLLGILPG
EI 104    AINGASPAGGCLMALTCDYRIMADNSKYTIGLNESLLGIVAP
EH 104    AVNGYALGGGCELAMMCDIIYAG--EKAQFGQPEILLGTIPG
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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) (Yang et al., 1991) with those of homologous regions of rat peroxisomal trifunctional enzyme (TE) (Osumi et al., 1985), plant glyoxysomal tetrafunctional protein (PT) (Preisig-Müller et al., 1994), pig mitochondrial long-chain-specific bifunctional enzyme (LT) (Yang et al., 1994), rat mitochondrial Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (EI) (Palosaari et al., 1991), and enoyl-CoA hydratase (EH) (Minami-Ishii et al., 1989). 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.

and 3-hydroxyacyl-CoA epimerase with D-3-hydroxyoctanoyl-CoA as substrate, in the absence and presence of 2.5 μg of bovine liver crotonase, were also performed as described previously (Binstock & Schulz, 1981). All enzymes were assayed at 25 °C on a Gilford recording spectrophotometer (model 2600). One unit of activity is defined as the amount of enzyme that catalyzed the conversion of 1 μmol of substrate to product per minute.

RESULTS AND DISCUSSION

Overproduction of the α/Arg^{134} → Gln and α/Glu^{139} → Gln Mutant Complexes. A region near the center of the amino-terminal half of the *E. coli* multifunctional fatty acid oxidation protein is well conserved throughout the enoyl-CoA hydratase family (Yang et al., 1991). Studies with the *E. coli* fatty acid oxidation complex carrying the α/Gly^{116} → Phe mutation revealed that this region plays an important role in the catalytic functions of enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase, and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (Yang & Elzinga, 1993). An arginine residue at position 134 appeared to be highly homologous to a lysine at corresponding locations of other multifunctional and monofunctional proteins exhibiting either enoyl-CoA hydratase or Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activity or both of these activities. An exception is the glutamine residue at the corresponding position of the plant glyoxysomal tetrafunctional protein whose sequence was published only recently. Therefore, we focus on this basic residue (Arg^{134}) and a well-conserved acidic residue (Glu^{139}) both located in this region as the best candidates for catalytic residues of these enzymes (Figure 1). For this study, the *E. coli* fatty acid oxidation complexes bearing either an α/Glu^{139} → Gln or an α/Arg^{134} → Gln mutation were overproduced in *E. coli* cells as a result of the IPTG induction of the BL21 (DE3) pLysS transformants containing the recombinant plasmids pNDBA-3 and pNDBA-4, respectively (Figure 2). By measuring the enzyme activities of the cell extracts, it was found that the cell extract containing the overproduced α/Arg^{134} → Gln mutant complex exhibited all five component enzyme activities at levels comparable to those of the wild type control, whereas the cell extract containing the overproduced α/Glu^{139} → Gln mutant complex was devoid or nearly so of enoyl-CoA hydratase and 3-hydroxyacyl-CoA epimerase activities (Table 1). Since the IPTG induced transformant containing the blank vector (pND-1) without the *fadBA* insert showed almost no activities of these β-oxidation enzymes that are associated with the fatty acid oxidation complex (Yang et al., 1988), the enzyme activities detected in the cell extracts obviously represented those of the wild type or mutant complexes. Since the apparent K_m values of the component enzymes of the α/Arg^{134} → Gln mutant complex were also found to be similar to those of the wild type (data not shown), it is concluded that the substitution of Arg^{134} by Gln is a silent mutation. In contrast, the replacement of the nearby acidic residue Glu^{139} by Gln resulted in the inactivation of enoyl-CoA hydratase and 3-hydroxyacyl-CoA epimerase and a partial inactivation of the third enzyme, Δ^3-*cis*-Δ^2-enoyl-CoA isomerase. It should be noted that the loss of 3-hydroxyacyl-CoA epimerase activity was not due to a hydratase deficiency because an adequate amount of bovine liver crotonase was included in the epimerase assay. The findings prompted us to explore in more detail what roles glutamate 139 plays in the catalytic functions of these enzymes.

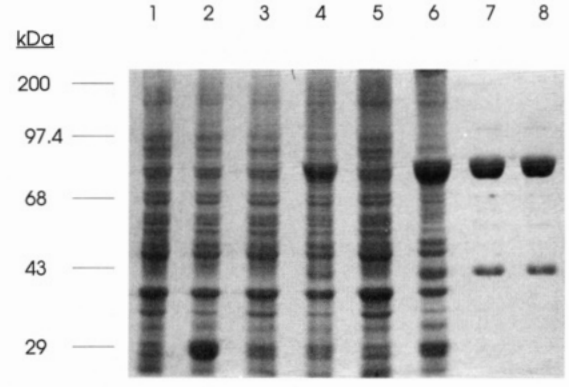

FIGURE 2: SDS−PAGE analysis of *E. coli* cell extracts and purified mutant and wild type multienzyme complexes. Noninduced cell extracts of BL21 (DE3) pLysS transformants containing pND-1 (lane 1), pNDBA-3 (lane 3), and pNDBA-4 (lane 5). IPTG-induced cell extracts of BL21 (DE3) pLysS transformants containing pND-1 (lane 2), pNDBA-3 (lane 4), and pNDBA-4 (lane 6). Purified α/Glu^{139}→Gln mutant complex (lane 7) and wild type complex (lane 8). Protein molecular weight standards were run on the same gel, and their positions are marked by bar designated 200 kDa for myosin (H-chain), 97.4 kDa for phosphorylase b, 68 kDa for bovine serum albumin, 43 kDa for ovalbumin, and 29 kDa for carbonic anhydrase.

Characterization of the Purified α/Glu^{139} → Gln Mutant Multienzyme Complex. The kinetic parameters of the five different component enzymes of the *E. coli* wild type and

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] (U/mg of protein)			
	pND-1 (no complex)	pNDBA (wild type)	pNDBA-3 (α/Arg^{134} → Gln)	pNDBA-4 (α/Glu^{139} → Gln)
enoyl-CoA hydratase	0.08	42.7	26.6	0.04
L-3-hydroxyacyl-CoA dehydrogenase	0.02	8.70	9.20	9.96
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	0[b]	2.87	4.45	0.94
3-hydroxyacyl-CoA epimerase	0[b,c]	1.23	1.50	0[b,c]
3-ketoacyl-CoA thiolase	0.01	2.39	2.73	3.45

[a] Values are averages of three determinations. [b] Zero implies activity of <0.005 U/mg. [c] Activities determined in the absence and presence of 2.5 μg of bovine liver crotonase.

Table 2: Kinetic Parameters of Different Component Enzymes of the Wild Type and the α/Glu^{139}→Gln Mutant Multienzyme Complex of Fatty Acid Oxidation from *E. coli*

enzyme and substrate	wild type		α/Glu^{139} → Gln mutant	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
enoyl-CoA hydratase	775		0.25	
crotonyl-CoA		53		41
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	63		25	
3-*cis*-tetradecenoyl-CoA		6		89
3-hydroxyacyl-CoA epimerase	13		0[a,b]	
D-3-hydroxyoctanoyl-CoA		17		–
L-3-Hydroxyacyl-CoA dehydrogenase	382		464	
acetoacetyl-CoA		69		185
NADH		2		3
3-ketoacyl-CoA thiolase	85		79	
acetoacetyl-CoA		96		69
CoASH		102		111

[a] Zero implies <0.005. [b] Activities determined in the absence and presence of 2.5 μg of bovine liver crotonase.

α/Glu^{139} → Gln mutant complexes were determined according to Lineweaver and Burk (Segel, 1975) and are listed in Table 2. The kinetic analysis showed that 3-ketoacyl-CoA thiolase of the mutant complex catalyzes the thiolysis reaction by a ping-pong mechanism in the same way as the wild type. Moreover, the k_{cat} and K_m values of 3-ketoacyl-CoA thiolase from the mutant and wild type complexes were found to be similar (see Table 2). This result suggests that the α/Glu^{139} → Gln mutation does not affect the small β-subunit (Yang et al., 1990) nor the quaternary structure of the complex. A comparison of the kinetic parameters of L-3-hydroxyacyl-CoA dehydrogenase from the mutant and wild type complexes revealed that the catalytic function of the dehydrogenase remained virtually unaffected by the α/Glu^{139} → Gln mutation except for a slight increase in the K_m values for substrates. The data suggest that this point mutation did not cause a long-range structural perturbation in the large α-subunit. The most striking changes in the catalytic properties of the α/Glu^{139} → Gln mutant complex is a dramatic decline of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA epimerase activities. The k_{cat} of the enoyl-CoA hydratase from the mutant complex was more than 3000-fold lower than that of the wild type enzyme, whereas the K_m value for crotonyl-CoA was virtually unchanged (see Table 2). Since the α/Glu^{139} → Gln substitution affected the catalytic activity but not substrate binding, it seems that glutamate 139 plays a key role in the catalytic mechanism of the *E. coli* enoyl-CoA hydratase. In addition, no epimerase activity was detected in the α/Glu^{139} → Gln mutant complex even in the presence of bovine liver crotonase. The simultaneous elimination of the 3-hydroxyacyl-CoA epimerase and enoyl-CoA hydratase activities corroborates the conclusion that both enzymes share the same active site (Yang & Elzinga, 1993) and further demonstrates that glutamate 139 is essential for both catalytic functions. In view of the fact that glycine has no side chain, the reported results of the α/Gly^{116} → Phe mutation (Yang & Elzinga, 1993) presumably reflect the local perturbation of the orientation of the key residues at the active site as a consequence of an increase of the volume occupied by residue 116 from about 66 to 203 Å (Richards, 1977). In contrast, the replacement of Glu^{139} by Gln does not cause a significant increase in the volume occupied by residue 139. Since the major effect of the amidation of the γ-carboxylic group of Glu^{139} was found to be on the k_{cat} rather than K_m value of the *E. coli* enoyl-CoA hydratase, it is reasonable to attribute the effect of such a mutation to the loss of the ability to transfer a proton from a neighboring group to another or to the solvent.

It was found that the k_{cat} of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase of the α/Glu^{139} → Gln mutant complex was about 40% of the k_{cat} of the wild type isomerase (see Table 2). This observation suggests that Glu^{139} is not directly involved in the catalysis of the isomerization of Δ^3,Δ^2-enoyl-CoA. Therefore, the proposal that Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase and enoyl-CoA hydratase of the large α-subunit of the *E. coli* fatty acid oxidation complex and of other multifunctional proteins share a catalytic residue (Müller-Newen & Stoffel, 1993) is not tenable. Since the α/Glu^{139} → Gln substitution resulted in a significant increase of the K_m value for the substrate of the isomerase, it seems that this mutation mainly affects the substrate ground state binding rather than the transition state stabilization by the isomerase. The experimental results favor the hypothesis that *E. coli* crotonase and isomerase share a CoA-binding site even though the key residues responsible for catalyzing the hydration and isomerization reactions are not the same (Yang & Elzinga, 1993).

Roles of Glutamate 139 in the Dehydration of D- *and* L-*3-Hydroxyacyl-CoAs Catalyzed by the Multifunctional Protein.* The findings of this study strongly support a reaction mechanism proposed for the epimerization of D-3-hydroxyacyl-CoAs catalyzed by the 3-hydroxyacyl-CoA epimerase of the *E. coli* multifunctional fatty acid oxidation protein. This process consists of two consecutive reactions: the dehydration of D-3-hydroxyacyl-CoA to yield 2-*trans*-enoyl-CoA followed by the hydration of the enoyl-CoA to L-3-hydroxyacyl-CoA (Smeland et al., 1991). The γ-carboxylic group of the glutamate 139 residue may function as a catalytic base to abstract the α-proton from the substrate.

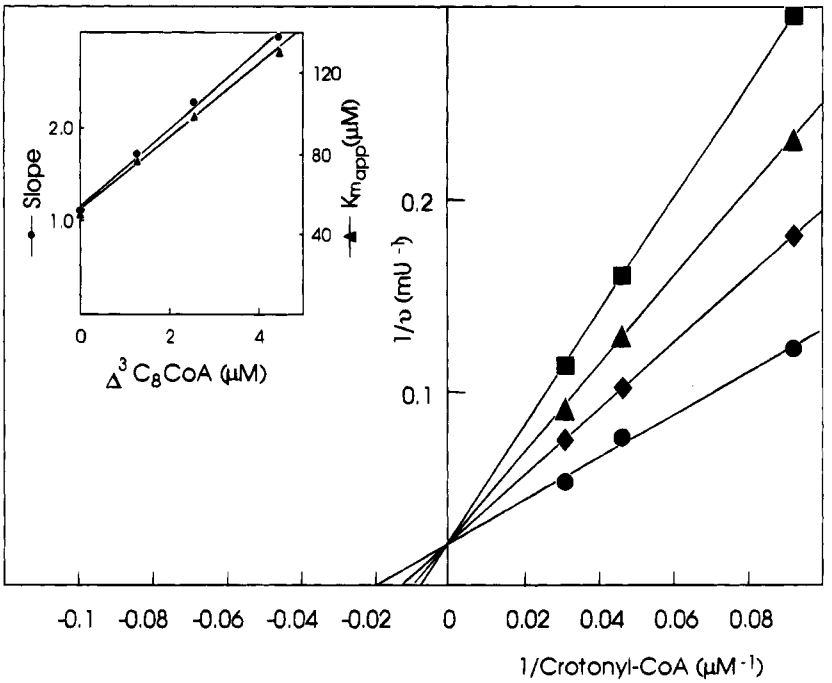

FIGURE 3: Activities of crotonase from the *E. coli* fatty acid oxidation complex as a function of the substrate concentration in the absence and presence of 3-*cis*-octenoyl-CoA. The fixed 3-*cis*-octenoyl-CoA (Δ^3C_8CoA) concentrations were 0 (●), 1.28 (♦), 2.56 (▲), and 4.46 (■) μM. The inset shows replots of slopes (●) and apparent K_m values (▲) versus the 3-*cis*-octenoyl-CoA concentrations.

Willadsen and Eggerer (1975) have reported that the *pro-R* α-proton was removed in the dehydration of 3-hydroxybutyryl-CoA catalyzed by bovine liver crotonase. However, it remains to be determined whether the same proton is removed in the dehydration reaction catalyzed by the *E. coli* multifunctional fatty acid oxidation protein. If the *pro-R* α-proton is removed, the stereochemical course of the epimerization postulated previously (Yang & Elzinga, 1993) would be confirmed. The residual hydratase activity of the α/Glu^{139} → Gln mutant complex (see Table 2) may reflect the inherent activity of the mutant complex rather than the presence of a small amount of revertant complex even though the possibility of the latter situation cannot be completely excluded. It is conceivable that another unidentified residue(s), such as a proton donor adjacent to the carbonyl oxygen of the substrate, may be involved in forming a hydrogen bond thereby reducing the $\Delta G^{\ddagger}$ value of the reaction (Gerlt & Gassman, 1992, 1993). In addition, some evidence has recently been presented for an electronic rearrangement in the acryloyl portion of substrate analogs upon binding to bovine liver crotonase (D'Ordine et al., 1994). However, the Glu^{139} residue of the *E. coli* crotonase does not seem to contribute significantly to such electronic rearrangement, as the K_m value of the enoyl-CoA hydratase of the α/Glu^{139} → Gln mutant complex was found to be very close to that of the wild type (see Table 2). Obviously, the substrate activation in this regard should be attributed to other structural elements at the enzyme active site.

The 3-hydroxyacyl-CoA epimerase activity of other prokaryotic multifunctional proteins, such as the α-subunit of the multienzyme complex from *Pseudomonas fragi* (Sato et al., 1992), presumably follows a similar reaction mechanism as described here. However, it was recently reported (Preisig-Müller et al., 1994) that the amino-terminal region of plant glyoxysomal tetrafunctional protein harbors a 3-hydroxyacyl-CoA epimerase that is independent of enoyl-CoA hydratase. Amino-terminal fragments of this multifunctional protein were overproduced in *E. coli* cells by use of 3′-end-truncated cDNAs. Since such protein fragments lost enoyl-CoA hydratase activity but retained very low activities of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase, it was concluded that the epimerization of D-3-hydroxyacyl-CoAs by the epimerase of the multifunctional protein did not proceed via a dehydration/hydration mechanism (Preisig-Müller et al., 1994). It remains to be established whether the conflicting conclusions could be attributed to differences in species and/or methodology used in experiments. However the following points are worth mentioning: (a) the sequence of the glyoxysomal tetrafunctional protein is clearly homologous to that of the *E. coli* multifunctional protein and also retains a conserved glutamate residue corresponding to Glu^{139} of the large α-subunit of the *E. coli* fatty acid oxidation complex (see Figure 1); (b) the removal of the carboxyl-terminal half of the protein and the addition of a 12-residue extension including six histidines at the amino-terminus may have severely disturbed the conformation of the amino-terminal domain of this multifunctional protein such that the associated enzyme activities were too low to be determined accurately; (c) the activities of the component enzymes of the wild type multifunctional protein made by overexpression of the recombinant plasmid were significantly different from those of the multifunctional protein purified from the plant, e.g., the reported ratio between the epimerase and the hydratase for the former protein is at least 13-fold lower than that of the latter. Obviously, the identification of Glu^{139} as the key residue at the active site of both the D- and L-dehydratases on the *E. coli* fatty acid oxidation protein has provided a sound basis for studying this problem with the glyoxysomal tetrafunctional protein.

Evidence for Sharing a Substrate-Binding Site between Enoyl-CoA Hydratase and Δ^3-cis-Δ^2-trans-Enoyl-CoA Isomerase of the Multifunctional Protein. It was interesting to find that the α/Glu^{139} → Gln mutant complex could

catalyze the isomerization reaction at a substantial rate (see Table 2). The results of this study do not agree with the recent prediction (Müller-Newen & Stoffel, 1993) that the hydratase and the isomerase of the *E. coli* multifunctional protein share a single catalytic residue because they belong to the hydratase/isomerase superfamily. The mechanism of the Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase reaction has not been well characterized even though a glutamate residue was recently identified as the catalytic residue of rat mitochondrial monofunctional isomerase (Müller-Newen & Stoffel, 1993). Glutamate 139 is not essential to the catalytic function of the isomerase of the *E. coli* multifunctional protein. However, the significant increase of the K_m value for the substrate of the isomerase suggests that the α/Glu^{139} → Gln mutation affects the binding of substrate to the active site of the isomerase.

In order to determine whether the active sites of the crotonase and isomerase overlap, initial rates of the enoyl-CoA hydratase of the *E. coli* fatty acid oxidation complex were measured, in the absence and presence of 3-*cis*-octenoyl-CoA, as a function of the crotonyl-CoA concentration by the direct assay method (Binstock & Schulz, 1981). It was found that the inhibition of *E. coli* enoyl-CoA hydratase by 3-*cis*-octenoyl-CoA, a substrate of the isomerase, was increasingly relieved with increasing concentration of the hydratase substrate in such a way that the V_{max} of the hydratase was not changed in the absence or presence of the isomerase substrate. The reciprocal plots shown in Figure 3 demonstrate that 3-*cis*-octenoyl-CoA is a strong competitive inhibitor of the *E. coli* enoyl-CoA hydratase. Its K_i value was estimated to be 3 μM by replots of the slopes and the apparent K_m values versus the concentrations of 3-*cis*-octenoyl-CoA (Figure 3, inset). This result agrees with a model of an active site shared in part by the isomerase and crotonase of the *E. coli* multifunctional protein. This conclusion probably holds true for other members of the multifunctional protein family, including rat peroxisomal trifunctional β-oxidation enzyme (Palosaari & Hiltunen, 1990), because of their evolutionary relationships. Considering the size of the amino-terminal half of the latter enzyme, it was proposed that this region of the peroxisomal trifunctional enzyme has only one CoA-binding site (Palosaari et al., 1991). However, their attempts to substantiate this proposal have not yet succeeded because the comparison of the amino acid sequence of rat peroxisomal trifunctional enzyme with those of rat peroxisomal 3-ketoacyl-CoA thiolase (Hijikata et al., 1987), human medium-chain acyl-CoA dehydrogenase (Kelley et al., 1987), and other enzymes, which use CoA derivatives as substrates, failed to find a consensus sequence, or a "signature", for the CoA-binding site (Palosaari et al., 1991).

Since the results of the present study contradict the recent proposal that the isomerase and the hydratase are associated with two distinct regions of the amino-terminal domains of the large α-subunit of the *E. coli* fatty acid oxidation complex and other multifunctional proteins [Preisig-Müller et al. (1994) Figure 8], we conclude that the amino-terminal domain of the *E. coli* multifunctional protein possesses a common CoA-binding site for different substrates which interact at the active site with the key residues for either the D- and L-dehydration of 3-hydroxyacyl-CoA or the enoyl-CoA isomerization (Figure 4). Since the consecutive reactions involve the same substrate-binding site, intermediates do not have to diffuse away, and this situation may be the underlying cause of intermediate channeling (Yang et al., 1985). In order to explore further details of the active site, the identification of the catalytic residue(s) of the isomerase of the *E. coli* multifunctional fatty acid oxidation protein is currently underway in our laboratory.

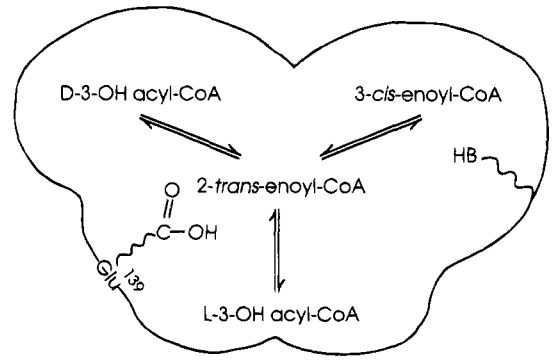

FIGURE 4: Reactions catalyzed by the amino-terminal domain of the *E. coli* multifunctional fatty acid oxidation protein. Glutamate 139 is catalyzing the epimerization of D-3-hydroxyacyl-CoA to its L-isomer, and 2-*trans*-enoyl-CoA is an intermediate. Alternatively, the isomerization of 3-*cis*-enoyl-CoA to its 2-*trans*-isomer can be catalyzed by an unidentified residue(s) (BH). All CoA thiolesters are competing with each other for binding to a common CoA-binding site of this functional domain.

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