Importance of the γ-Carboxyl Group of Glutamate-462 of the Large α-Subunit for the Catalytic Function and the Stability of the Multienzyme Complex of Fatty Acid Oxidation from *Escherichia coli*†

Xue-Ying He, Huinan Deng, and Song-Yu Yang*

Department of Pharmacology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York 10314

Received July 24, 1996; Revised Manuscript Received October 9, 1996⊗

ABSTRACT: His^{450} of the large α-subunit of the multienzyme complex of fatty acid oxidation from *Escherichia coli* was recently identified as an essential catalytic residue of L-3-hydroxyacyl-CoA dehydrogenase [He, X.-Y., & Yang, S.-Y. (1996) *Biochemistry 35*, 9625−9630]. To explore the roles of acidic residues in the dehydrogenase catalysis, every conserved acidic residue in the dehydrogenase functional domain except for those in the NAD-binding motif was replaced with alanine. The resulting mutant complexes were overproduced and characterized. Their component enzymes other than the dehydrogenase were affected very slightly. Removal of the β-carboxyl group of Asp^{524} and Asp^{542} caused only a 3- and 4-fold, respectively, decrease in the catalytic efficiency of the dehydrogenase, thereby showing that their involvement in the dehydrogenase catalysis was limited. In contrast, the α/Glu^{462} → Ala mutant complex showed a greater than 160-fold reduction in the k_{cat} of the dehydrogenase in the forward direction without a significant change of the K_m for the substrate. The catalytic properties of the α/Glu^{462} → Gln mutant complex were found to be similar to those of the α/Glu^{462} → Ala mutant complex except that the k_{cat} of the dehydrogenase in the backward direction was about 4-fold lower and the K_m for the substrate of the thiolase was 6-fold higher. It is concluded that the negative charge of the γ-carboxyl group of Glu^{462}, but not its ability to form a hydrogen bond, is critical for its interaction with His^{450}, thereby assisting in the catalysis of the dehydrogenase. The pK_a of His^{450} in the E·NADH binary complex was virtually unchanged by the replacement of Glu^{462} with Ala or Gln. It seems that the binding of substrate is necessary for forming a strong interaction between His^{450} and Glu^{462} with the result that the electroneutrality in the active site is maintained and the activation energy of the reaction is lowered. Additionally, the negative charge of Glu^{462} increases the thermostability of the multienzyme complex.

The multienzyme complex of fatty acid oxidation of *Escherichia coli*, consisting of two types of subunits (α, 79 kDa; β, 41 kDa), catalyzes five different reactions that are necessary for the degradation of saturated and unsaturated fatty acids (Binstock & Schulz, 1981). The small β-subunit is a 3-ketoacyl-CoA thiolase (EC 2.3.1.16) that catalyzes the last step of a β-oxidation cycle, whereas the large α-subunit is a multifunctional protein (Yang & Schulz, 1983; Yang et al., 1990, 1991). Enoyl-CoA hydratase (EC 4.2.1.17) and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8) are both located in the amino-terminal domain of the large α-subunit, and they share a substrate-binding site (Yang et al., 1995). The 3-hydroxyacyl-CoA epimerase activity of the complex (EC 5.1.2.3) is due to the ability of the enoyl-CoA hydratase to catalyze the reversible dehydrations of L- and D-3-hydroxyacyl-CoAs to produce 2-*trans*-enoyl-CoA (Yang & Elzinga, 1993; Yang et al., 1995). The carboxyl-terminal domain of the large α-subunit harbors L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), which catalyzes the third reaction of the β-oxidation cycle (Yange et al., 1991). The amino-terminal region of this dehydrogenase forms the Rossmann folds, serving as an NAD-binding site (Rossmann et al., 1974, 1975); the rest of the enzyme contains residues key to substrate binding and catalysis; e.g., histidine-450 has recently been identified as an essential catalytic residue of L-3-hydroxyacyl-CoA dehydrogenase (He & Yang, 1996).

For elucidating structure−function relationships in L-3-hydroxyacyl-CoA dehydrogenase, it is necessary to identify other residues important for the catalytic process. There are a total of three conserved acidic residues, not including those in the NAD-binding motif, in all nine known L-3-hydroxyacyl-CoA dehydrogenases (S.-Y. Yang, unpublished alignments). The corresponding acidic residues of the large α-subunit of the *E. coli* fatty acid oxidation complex were chosen for "alanine-scanning" mutagenesis to determine their importance for the dehydrogenase activity. The residue identified as the most important was substituted again with the amide of the corresponding residue by site-directed mutagenesis. The rationale is that an amide group at the terminal of a side chain could mimic the hydrogen bonding of the carboxyl group, whereas alanine would neither carry a negative charge nor permit formation of a hydrogen bond. A comparison of the catalytic properties of these mutant complexes would provide clues about the role such a conserved acidic residue(s) play(s) in the dehydrogenase catalysis. Also, it could reveal other major roles these

† This work was supported in part by U.S. Public Health Service Grant DK 47392 from the National Institute of Diabetes and Digestive and Kidney Diseases and by the New York State Office of Mental Retardation and Developmental Disabilities.

* To whom correspondence should be addressed. Telephone: (718) 494-5317. Fax: (718) 494-1080.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

S0006-2960(96)01841-7 CCC: $14.00

conserved acidic residues may play in the multienzyme complex.

In this paper, we show that a negative charge on the γ-carboxyl group of glutamate-462 of the large α-subunit is catalytically important to the L-3-hydroxyacyl-CoA dehydrogenase. The catalytic residue, histidine-450, of the dehydrogenase may interact with glutamate-462, and this electrostatic interaction was strengthened by the binding of substrate. Moreover, the negatively charged carboxyl group of glutamate-462 proved to be necessary to the thermal stability of the multienzyme complex.

MATERIALS AND METHODS

Materials. NAD^+, NADH, CoASH, pig heart L-3-hydroxyacyl-CoA dehydrogenase, and all other standard biochemicals were obtained from Sigma. 2-*trans*-Hexadecenoic acid was bought from ICN. 3-*cis*-Tetradecenoic acid (Luo et al., 1993), 2-hexadecynoic acid (Freund et al., 1985), and acetoacetyl-CoA (Seubert, 1960) were synthesized according to published procedures. The CoA derivatives of 2-*trans*-hexadecenoic acid, 2-hexadecynoic acid, and 3-*cis*-tetradecenoic acid were synthesized by the mixed anhydride procedure as detailed by Goldman and Vagelos (1961). L-3-Hydroxyhexadecanoyl-CoA and 3-ketohexadecanoyl-CoA were enzymatically prepared from 2-*trans*-hexadecenoyl-CoA (He et al., 1992) and 2-hexadecynoyl-CoA (Thorpe, 1986), respectively, as described previously. Bovine liver crotonase (Steinman & Hill, 1975) and pig heart 3-ketoacyl-CoA thiolase (Staack et al., 1978) 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 DNA ligase, and T4 DNA polymerase were supplied by Boehringer Mannheim. Sequenase Version 2.0 kit and radiochemicals were purchased from Amersham. Oligonucleotides were synthesized by Life Technologies, Inc.

Site-Directed Mutagenesis of the fadB Gene. The oligonucleotide-directed mutagenesis was carried out by an adaptation of the unique site elimination method (Deng & Nickoloff, 1992) as described previously (Yang & Elzinga, 1993). A synthetic oligonucleotide, 5′-CAGGCATG-C**A**C**G**C**G**TGGCGTAATC-3′, with a *Mlu*I site instead of a *Hin*dIII site was used as the selection primer. The following synthetic oligonucleotides were used as the mutagenic primers. The substituting nucleotide is underlined, and the mutant codon is in bold face type:

mutagenic primer	mutation of the large α-subunit
GCAAGATC**G**C**C**AAAGTGATG	Asp^{524}→Ala
ATCTGCTG**G**C**C**GTTGTGGG	Asp^{542}→Ala
GCCGTTGGTA**G**C**A**ATTATTCGCG	Glu^{462}→Ala
GCCGTTGGTA**C**A**A**ATTATTCGCG	Glu^{462}→Gln
ATATCTGCTG**A**A**C**GTTGTGG	Asp^{542}→Asn

The selection primer and a mutagenic primer were simultaneously annealed to the single-strand DNA 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. Suitable mutant clones were selected for the presence of the unique *Mlu*I site. Mutations were confirmed by dideoxy sequencing (Sanger et al., 1977). The mutant *fadB* gene containing the desired mutation was inserted into the *Bam*HI−*Sal*I site of the original expression plasmid pNDBA, which was previously used to produce the *E. coli* wild-type complex (Yang & Elzinga, 1993), to form a mutant expression plasmid. The mutant expression plasmids carrying the $\alpha/Asp^{524} \rightarrow$ Ala, $\alpha/Asp^{542} \rightarrow$ Ala, $\alpha/Glu^{462} \rightarrow$ Ala, $\alpha/Glu^{462} \rightarrow$ Gln, and $\alpha/Asp^{542} \rightarrow$ Asn mutations were designated as pNDBA-14, -15, -16, -17, and -18, respectively. The DNA sequence of plasmid pNDBA-17 around the mutant codon (**CAA**) was determined again by dideoxy sequencing to assure that the desired mutation is the only one in a 580-bp *Eco*RV−*Apa*I fragment. This fragment of DNA in the plasmid pNDBA-17 was replaced by the corresponding one from the plasmid pNDBA to reverse the $Glu^{462} \rightarrow$ Gln mutation; the resulting expression plasmid was designated pNDBA-17R.

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 isopropyl β-D-thiogalactoside (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 (Yang et al., 1988).

Protein and Enzyme Assays. Protein concentrations were determined by the method of Bradford (1976). Proteins were separated by SDS−PAGE on a 4−20% gradient gel at pH 8.3 as described previously (Blackshear, 1984). The enoyl-CoA hydratase activity was measured by the direct assay method at 280 nm with 2-*trans*-hexadecenoyl-CoA as substrate, and an extinction coefficient of 5100 M^{-1} cm^{-1} was used to calculate rates (He et al., 1992). The activity of L-3-hydroxyacyl-CoA dehydrogenase was determined with L-3-hydroxyhexadecanoyl-CoA and 3-ketohexadecanoyl-CoA as substrates by measuring the initial rates of the forward reaction (He et al., 1989) and the backward reaction (Binstock & Schulz, 1981), respectively, according to published procedures. Assays of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase with 3-*cis*-tetradecenoyl-CoA as substrate and 3-ketoacyl-CoA thiolase with 3-ketohexadecanoyl-CoA as substrate were also performed as described previously (Binstock & Schulz, 1981). Kinetic parameters of different component enzymes and pK_a values of L-3 hydroxyacyl-CoA dehydrogenase of the wild-type and mutant complexes were estimated by analysis of the kinetic data with the computer program LEONORA (Cornish-Bowden, 1995). 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 per minute.

Thermal Denaturation of Wild-Type and Mutant Fatty Acid Oxidation Complexes. The purified multienzyme complex (1 mg/ml) was incubated at 60 °C in 0.2 M potassium phosphate, pH 6.6, containing 10% (v/v) glycerol and 10

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactoside; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; 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

	specific activity[a] (units/mg of protein)						
enzyme[b]	pND-1 (no complex)	pNDBA (wild type)	pNDBA-14 (α/Asp^{524} → Ala)	pNDBA-15 (α/Asp^{542} → Ala)	pNDBA-16 (α/Glu^{462} → Ala)	pNDBA-17 (α/Glu^{462} → Gln)	pNDBA-17R (α/Gln^{462} → Glu)
L-3-hydroxyacyl-CoA dehydrogenase	0.04	25.5	5.12	2.11	0.30	0.21	26.9
enoyl-CoA hydratase	0.03	3.06	1.56	0.65	2.29	1.66	3.49
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	0.07	6.82	4.42	1.78	3.40	2.85	7.60
3-ketoacyl-CoA thiolase	0.02	9.92	4.44	2.17	4.35	1.47	8.95

[a] Values are averages of three determinations. [b] Enzyme activities were assayed with 14 μM long-chain (C_{16}) substrates except that a medium-chain (C_{14}) substrate was used for the isomerase assay.

mM mercaptoethanol. Aliquots from each sample were taken at different times, and assayed immediately for the activities of long-chain enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase as described previously (Binstock & Schulz, 1981; He et al., 1992).

RESULTS

Expression of the Mutant Complexes. The wild-type multienzyme complex of fatty acid oxidation encoded by the *E. coli fadBA* genes and different mutant complexes each bearing a point mutation in their large α-subunits as described under Materials and Methods were produced in BL21 (DE3) pLysS transformants containing the corresponding plasmids. Expression of these mutant complexes by IPTG induction was as effective as the production of the wild-type complex so that they made up about one-third of the total soluble proteins in *E. coli* cells (data not shown) (Yang & Elzinga, 1993). Four different enzyme activities were measured in cell extracts of the transformants containing plasmid pNDBA-14, -15, -16, -17, and -17R, respectively, and are listed in Table 1. These enzyme activities were hardly detectable in the cell extract of the transformant containing a blank vector, because the operator of the *fadBA* operon on the *E. coli* chromosome was blocked by a repressor that was not removed by IPTG (Nunn, 1986). In contrast, the activities of the β-oxidation enzymes in the cell extract containing the wild-type complex were very high; e.g., the long-chain 3-hydroxyacyl-CoA dehydrogenase activity was more than 600-fold higher than the activity in the vector control (Table 1). This system is suitable for studying the impact of mutations on the dehydrogenase and the other activities of the complex.

The long-chain enoyl-CoA hydratase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activities in cell extracts containing the mutant complexes were only slightly lower than the wild-type control levels. This indicates that replacement of any of these conserved acidic residues by alanine did not disrupt the structure of the large α-subunit. The long-chain 3-hydroxyacyl-CoA dehydrogenase activity in cell extracts containing α/Asp^{524} → Ala, α/Asp^{542} → Ala, and α/Glu^{462} → Ala mutant complexes was about 5-, 12-, and 85-fold lower, respectively, as compared with the wild-type control. Furthermore, the dehydrogenase activity in the cell extract containing the α/Glu^{462} → Gln mutant complex was 120-fold lower than the wild-type control (Table 1). Of these acidic residues investigated, glutamate-462 appears to be the most important one for dehydrogenase function. In addition, the amidation of glutamate-462 caused a 7-fold decrease in the long-chain 3-ketoacyl-CoA thiolase activity in the cell extract containing the α/Glu^{462} → Gln mutant complex. Since reversing the α/Glu^{462} → Gln mutation restored the thiolase activity to the wild-type control level as shown by the transformants containing plasmid pNDBA-17R (Table 1), the possibility of a harmful mutation present in the small β-subunit was excluded. When enoyl-CoA hydratase was inactivated by substituting glutamine for glutamate-139, the large α-subunit also lost a negative charge, but the thiolase activity was completely unchanged in that mutant complex (Yang et al., 1995). This difference implies that the carboxyl-terminal domain of the large α-subunit is perhaps in contact with the small β-subunit. If so, the spatial arrangement of the component enzymes in the multienzyme complex is in order of the β-oxidation reaction sequence (hydratase·dehydrogenase·thiolase) and thus might favor the β-oxidation intermediate channeling (Yang et al., 1985).

The Negative Charge on Glutamate-462 Is Important to the Catalytic Activity of L-*3-Hydroxyacyl-CoA Dehydrogenase*. The catalytic properties of the purified mutant complexes were characterized by steady-state kinetic measurements (Segel, 1975). The kinetic parameters of enoyl-CoA hydratase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase of the mutant complexes were similar to those of the wild-type complex except for a significant increase in the K_m value of the hydratase associated with the α/Asp^{542} → Ala mutant complex (Table 2). It is not known how this mutation could affect the substrate binding to the hydratase because information on the X-ray structure of the *E. coli* multifunctional fatty acid oxidation protein or other homologous multifunctional β-oxidation enzymes is not yet available. A 2−5-fold decrease of the K_m value for NADH was observed in those mutant dehydrogenases where a conserved acidic residue was substituted by alanine. The Asp^{524} → Ala and Asp^{542} → Ala mutations cause a 9-fold and a 17-fold reduction of the L-3-hydroxyacyl-CoA dehydrogenase catalytic rate constant (k_{cat}), respectively, whereas the Glu^{462} → Ala mutation leads to a 67-fold decrease in k_{cat} (see Table 2). These data indicate that the carboxyl groups of both Asp-524 and Asp-542 are far less important than that of Glu-462 for the dehydrogenase catalysis. With respect to the wild-type and mutant L-3−hydroxyacyl-CoA dehydrogenases characterized in this study, the value of the dissociation constant (K_{ia}) of NADH calculated from kinetic measurements (Dalziel, 1975) was found to be very close to the K_m value of the same enzyme

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

	wild type		α/Asp^{524} → Ala mutant		α/Asp^{542} → Ala mutant		α/Glu^{462} → Ala mutant		α/Glu^{462} → Gln mutant	
enzyme and substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
enoyl-CoA hydratase	29 ± 0.1		14 ± 0.4		27 ± 0.5		24 ± 0.5		18 ± 2.3	
2-*trans*-hexadecenoyl-CoA		4.6 ± 0.0[a]		4.9 ± 0.5		75 ± 1.8		4.6 ± 0.2		4.5 ± 1.6
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	62 ± 1.6		43 ± 3.1		20 ± 1.1		29 ± 0.9		23 ± 0.2	
3-*cis*-tetradecenoyl-CoA		5.8 ± 0.3		6.3 ± 1.0		9.4 ± 1.0		5.9 ± 0.5		5.2 ± 0.1
L-3-hydroxyacyl-CoA dehydrogenase	1066 ± 36		114 ± 10		64 ± 13		16 ± 1.0		4.5 ± 0.1	
3-ketohexadecanoyl-CoA		37 ± 1.8		22 ± 3.0		44 ± 12		64 ± 5.2		15 ± 0.4
NADH		32 ± 2.7		15 ± 3.4		13 ± 8.0		4.6 ± 2.7		53 ± 1.5
3-ketoacyl-CoA thiolase	275 ± 69		ND[b]		ND		128 ± 52		101 ± 37	
3-ketohexadecanoyl-CoA		26 ± 7.5		ND		ND		11 ± 6.1		60 ± 24
CoASH		115 ± 30		ND		ND		173 ± 76		220 ± 83

[a] Zero implies error of <0.05. [b] Not done.

Table 3: Comparison of the Catalytic Efficiencies of Wild-Type and Mutant L-3-Hydroxyacyl-CoA Dehydrogenases Associated with the *E. coli* Fatty Acid Oxidation Complex

	catalytic efficiency[a]	
mutation	actual ($s^{-1}\cdot\mu M^{-2}$)	relative (*x*-fold)
none	$(8.5 \times 10^{-1}) \pm (3.6 \times 10^{-2})$	1
Asp^{524} → Ala	$(2.6 \times 10^{-1}) \pm (2.9 \times 10^{-2})$	1/3
Asp^{542} → Ala	$(2.0 \times 10^{-1}) \pm (6.5 \times 10^{-2})$	1/4
Glu^{462} → Ala	$(6.1 \times 10^{-2}) \pm (9.2 \times 10^{-3})$	1/14
Asp^{542} → Asn	$(3.7 \times 10^{-2}) \pm (6.2 \times 10^{-3})$	1/23
Glu^{462} → Gln	$(6.3 \times 10^{-3}) \pm (1.2 \times 10^{-4})$	1/135

[a] The catalytic efficiency for the dehydrogenase is estimated by the ratio of the constants, $k_{cat}/K_{ia}K_{mB}$ (Lewendon & Shaw, 1993).

for NADH.[2] When the catalytic efficiencies, $k_{cat}/K_{ia}K_{mB}$ (Lewendon & Shaw, 1993), of the wild-type and mutant dehydrogenases were compared, it was found that removal of the β-carboxyl groups of Asp-524 and of Asp-542 caused only a 3-fold and a 4-fold decrease in the catalytic efficiency of the dehydrogenase, respectively. These effects were similar to the effects of the same mutations on the other component enzymes of the large α-subunit. However, it is noteworthy that the removal of the γ-carboxyl group of Glu-462 resulted in a 14-fold decrease in the catalytic efficiency of the dehydrogenase. Introduction of an amide group at Asp-542 caused the catalytic efficiency of the dehydrogenase to decrease by 23-fold, but this is much less than a 135-fold decrease resulting from the Glu^{462} → Gln mutation (Table 3). All the results showed that glutamate-462 of *E. coli* L-3-hydroxyacyl-CoA dehydrogenase plays a significant role in the catalytic process.

The impairment of the dehydrogenation reaction by the Glu^{462} → Ala and the Glu^{462} → Gln mutations was further investigated. The kinetic parameters of the forward reaction catalyzed by the wild-type and mutant dehydrogenases were determined by use of a coupled assay (He et al., 1989), and the results are listed in Table 4. The K_m of the dehydrogenase for L-3-hydroxyhexadecanoyl-CoA was virtually unchanged by either mutation, whereas the second-order rate constants of the mutant dehydrogenases were 2 orders of magnitude lower than that of the wild-type enzyme. Changing Glu-462 to Ala or to Gln in the *E. coli* multifuntional fatty acid oxidation protein reduced the overall catalytic efficiency of the dehydrogenase by 127- and 100-fold, respectively (Table 4). The γ-carboxyl group of Glu-462 was estimated to stabilize the transition state by 2.9 kcal/mol (Wilkinson et al., 1983) at pH 8.0. Since the dehydrogenase activity was seriously impaired by the substitution of Glu-462 with either Ala or Gln, the data suggest that the negative charge of this carboxyl group, but not its capacity to form a hydrogen bond, is important for the dehydrogenase catalysis.

pH Dependence of k_{cat}/K_m of the Wild-Type and Mutant L-3-Hydroxyacyl-CoA Dehydrogenases. The pH dependence of the kinetic parameters of the wild-type and mutant dehydrogenases was studied in the presence of saturating concentrations of NADH. The pK_a of the catalytic residue, His-450, of the wild-type dehydrogenase was calculated to be 7.3 ± 0.2 from pH-dependent data using the computer program LEONORA (Cornish-Bowden, 1995). Since NADH is acid-labile and measurements below pH 5.5 are unreliable owing to coenzyme instability (Clarke et al., 1988), it is not feasible to accurately determine the pK_a value of an acidic residue of the dehydrogenase in this study. Although the specificity constants (k_{cat}/K_m) of the mutant dehydrogenases were 2−3 orders of magnitude lower than that of the wild-type enzyme, the pK_a of His-450 in the E·NADH binary complex was virtually unchanged by the substitution of Ala or Gln for glutamate-462 (see Figure 1). This is similar to the situation of lactate dehydrogenase, where the pK_a of His-195 was not disturbed by the replacement of Asp-168 by either asparagine or alanine even though Asp^{168} and His^{195} are located close together in the active site and play a key role in the catalysis (Clarke et al., 1988). The observation of a similar lack of effect on the pK_a of His-450 in the mutant L-3-hydroxyacyl-CoA dehydrogenases suggests that the carboxyl group of Glu-462 and the imidazole of His-450 strongly interact only when both the coenzyme and substrate are bound to the enzyme. The binding of substrate would cause desolvation and structural rearrangement such that the imidazole−carboxyl interaction was strengthened. For members of a dehydrogenase family, functional amino acid residues usually retain the same spatial arrangement (Rossmann et al., 1974, 1975). The location of glutamate-462, which corresponds to Glu-175 of pig heart dehydrogenase (Bitar et al., 1980; Birktoft et al., 1987; Yang et al., 1991), is near the amino terminus of α-helix F in the topological diagram for the structure of the amino-terminal domain of the *E. coli* dehydrogenase (He & Yang, 1996). The 3-carbonyl group of the substrate sits between the nicotina-

[2] Although the definition of the dissociation constant (K_{ia}) is distinct from that of the Michaelis constant (K_m), K_{ia} is numerically equal to K_{mA} as long as the reciprocal plots of an ordered Bi-Bi reaction ($1/v$ vs 1/[B]) converge on the abscissa (Segel, 1975).

Table 4: Effects of the Glu[462] → Ala and Glu[462] → Gln Mutations on Kinetic Parameters of the *E. coli* L-3-Hydroxyacyl-CoA Dehydrogenase[a]

kinetic parameter	enzyme		
	wild type	Glu[462] → Ala mutant	Glu[462] → Gln mutant
k_{cat} (s^{-1})	113 ± 2.7	0.7 ± 0.1	1.4 ± 0.1
K_m(L-3-hydroxyhexadecanoyl-CoA) (μM)	3.6 ± 0.3	3.9 ± 1.1	4.7 ± 0.7
K_m(NAD^+) (μM)	95 ± 9.1	61 ± 28	123 ± 43
$k_{cat}/K_{ia}K_{mB}$[b] ($s^{-1}\cdot\mu M^{-2}$)	$(3.8 \times 10^{-1}) \pm (7.5 \times 10^{-2})$	$(3.0 \times 10^{-3}) \pm (1.3 \times 10^{-3})$	$(3.8 \times 10^{-3}) \pm (7.3 \times 10^{-4})$

[a] Determined at pH 8 in the forward direction (He et al., 1989). [b] Catalytic efficiency of the dehydrogenation reaction (Lewendon & Shaw, 1993).

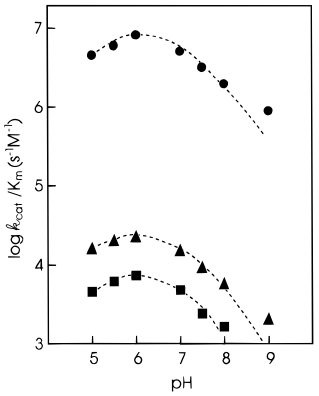

FIGURE 1: pH dependence of k_{cat}/K_m for the *E. coli* wild-type and mutant L-3-hydroxyacyl-CoA dehydrogenases. In order to determine the steady-state kinetic parameters of the dehydrogenase over a range of pH, the initial rate at different acetoacetyl-CoA concentrations was measured at 25 °C at the saturating level of NADH in 84 mM potassium phosphate buffer adjusted to the appropriate pH. Symbols: wild type (●), α/Glu[462] → Ala mutant (▲), and α/Glu[462] → Gln mutant (■) fatty acid oxidation complex. The data were fit with the computer program Leonora (Cornish-Bowden, 1995) to generate three dashed curves, and each of them has a pK_a value of 7.3 ± 0.2.

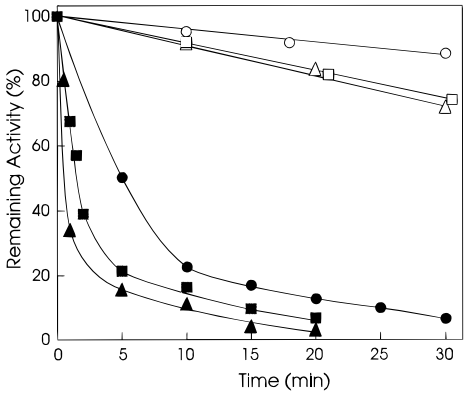

FIGURE 2: Thermal denaturation of the wild-type and the α/Glu[462] → Gln mutant multienzyme complex of fatty acid oxidation from *E. coli*. The remaining activities of β-oxidation enzymes of the wild-type complex (open symbols) and the mutant complex (solid symbols) were determined with long-chain (C16) substrates at different incubation time when these purified complexes were incubated at 60 °C under the conditions as described under Materials and Methods. Symbols: circles, L-3-hydroxyacyl-CoA dehydrogenase; squares, enoyl-CoA hydratase; triangles, 3-ketoacyl-CoA thiolase.

mide ring of NADH and the imidazole of His-450, whereas glutamate-462 lies on the other side of histidine-450. Since deletion or amidation of the γ-carboxyl group of Glu-462 has little effect on the substrate binding but greatly reduces the catalytic rate constant of the dehydrogenase (Tables 2 and 4), it is unavoidable to conclude that in a ternary complex the negative charge of glutamate-462 alters the environment of the catalytic residue, His-450, to facilitate the proton transfer to and from the imidazole of the histidine. The replacement of Glu-462 by glutamine resulted in a decrease in the specificity constant of the dehydrogenase even greater than was observed in the α/Glu[462] → Ala mutant complex (see Figure 1). Given that the amino group of $-CONH_2$ could act as a hydrogen-bond donor to form an alternative hydrogen bond (Fersht et al., 1987), this additional feature is likely to be a disadvantage to the catalysis of 3-ketoacyl-CoA reduction.

Multienzyme Complex of Fatty Acid Oxidation Stabilized by the γ-Carboxyl Group of Glutamate-462. When the *E. coli* fatty acid oxidation complex was incubated at 60 °C for 10 min, its enzyme activities decreased only a few percent. In contrast, half of the activities of thiolase, hydratase, and dehydrogenase were lost within less than 1, 2, and 5 min, respectively, when the *E. coli* α/Glu[462] → Gln mutant complex was incubated under the conditions described earlier (Figure 2). After incubation at 60 °C for half an hour, the wild-type complex retained nearly 90% of the dehydrogenase activity and 75% of the other two β-oxidation enzyme activities, whereas the mutant complex was almost inactive (Figure 2). Activity losses of the three β-oxidation enzymes of the wild-type and mutant complexes as a function of the incubation time were similar to one another although the thiolase and hydratase were slightly more sensitive to thermal denaturation than the dehydrogenase. Similarly, the α/Glu[462] → Ala mutant complex was rapidly denatured when it was tested under the same conditions (data not shown). It is clear that the *E. coli* fatty acid oxidation complex is remarkably stable but this multienzyme complex was severely destabilized when Glu[462] of the large α-subunit was replaced by Gln or Ala.

As mentioned previously, Glu[462] of the *E. coli* multifunctional fatty acid oxidation protein is located near the amino terminus of an α-helix in the dehydrogenase functional domain. It has been reported (Nicholson et al., 1988) that an acidic residue close to the N-terminus or a basic residue near the C-terminus of an α-helix can be a "dipole-compensating residue", and that such residues enhance protein thermostability by electrostatic interaction without hydrogen bonding. In keeping with this model, the negative charge of glutamate-462 may reduce the positive charge of the α-helix dipole and thus stabilize the α-helix and the whole protein structure.

DISCUSSION

A conserved histidine residue at position 450 of the *E. coli* multifunctional fatty acid oxidation protein was recently identified as the catalytic residue of L-3-hydroxyacyl-CoA dehydrogenase (He & Yang, 1996). It is of great interest to

know whether its catalytic function is influenced by an acidic residue, since a His-Asp pair was previously found in some NAD-dependent dehydrogenases, such as malate dehydrogenase and lactate dehydrogenase (Birktoft & Banaszak, 1983; Clarke et al., 1988). The substitution of such an aspartate, Asp-168 of lactate dehydrogenase, with asparagine or alanine greatly increased the K_m for pyruvate so that the catalytic efficiency of the mutant enzyme was significantly reduced (Clarke et al., 1988). To our knowledge, there is no precedent for a glutamate residue playing a crucial role in the catalysis of an NAD^+-dependent dehydrogenase. Recently, the conserved glutamate-264 of lactate dehydrogenase was reported to modulate the optimal pH, but this glutamate residue was not critical for enzyme catalysis (Kochhar et al., 1992). In the present study, the conserved glutamate-462 of the large α-subunit was shown to be catalytically important to L-3-hydroxyacyl-CoA dehydrogenase. Removal or amidation of its γ-carboxyl group caused a remarkable reduction of the catalytic rate constant of the dehydrogenase, but did not significantly affect the binding of substrate (Tables 2 and 4). Moreover, the α/Asp^{524} → Ala or α/Asp^{542} → Ala mutation and even the α/Asp^{542} → Asn mutation did much less damage to the overall catalytic efficiency of the dehydrogenase than the same types of mutations introduced at glutamate-462 (Table 3). The Asp-524, Asp-542, and His-450 of the *E. coli* multifunctional protein correspond to Asp-238, Asp-261, and His-165 of pig heart L-3-hydroxyacyl-CoA dehydrogenase, respectively, as shown by Figure 4 and by the previously reported sequence alignments (Yang et al., 1991; Yang, 1994). Based on the published X-ray structure of the pig heart dehydrogenase (Birktoft et al., 1987), the putative catalytic residue, His-165, is located in one lobe while both of the conserved aspartates are located in the other lobe, and these lobes are separated by a cleft accommodating the hydrocarbon chain of the fatty acid moiety. Since *E. coli* and pig heart L-3-hydroxyacyl-CoA dehydrogenases have similar tertiary structures (He & Yang, 1996), it is unlikely that either Asp-524 or Asp-542 could be coupled to histidine-450 to form a His-Asp pair in the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase. The adverse effects of the Glu^{462} → Ala and Glu^{462} → Gln mutations on the catalytic rate constant and the catalytic efficiency of the *E. coli* dehydrogenase resulted primarily from the loss of a negative charge on the γ-carboxyl group of glutamate-462, and this situation can be illustrated by a model of the active site as shown in Figure 3. The carboxyl group of Glu-462 interacts with the catalytic residue, histidine-450, to stabilize the transition state. In the process of the substrate dehydrogenation, a positive charge on the nicotinamide ring of NAD^+ flows down to the imidazole of His-450. The presence of a negative charge in the vicinity of the catalytic histidine residue not only would maintain the electroneutrality in the active site but also could reduce the free energy of activation of the reaction.

The binding of substrate apparently results in a structural rearrangement of the dehydrogenase. This is suggested by the impact of substrate binding on the conformation of the amino-terminal domain of L-3-hydroxyacyl-CoA dehydrogenase, which includes the catalytic residue histidine-450 and the well-conserved glutamate-462 besides the NAD-binding motif (He & Yang, 1996). When a short-chain substrate (acetoacetyl-CoA) was bound to the dehydrogenase, the K_m value for NADH was 2μM (Yang et al., 1995).

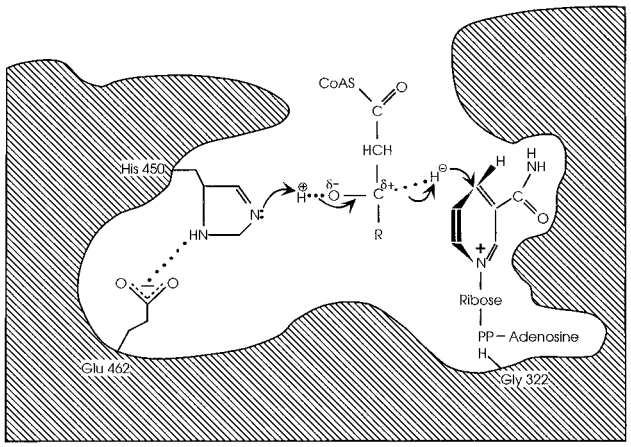

FIGURE 3: Schematic diagram of a model of the active site of *E. coli* L-3-hydroxyacyl-CoA dehydrogenase. Glutamate-462 is not directly involved in the binding of substrate or coenzyme. The negatively charged γ-carboxyl group of Glu^{462} significantly reduces the free energy of the transition state due to an electrostatic interaction with the catalytic residue, His^{450}, in the ternary complex and maintaining electroneutrality in the desolvated active center during catalysis. In the dehydrogenase·coenzyme·substrate ternary complex, His^{450} serves as a general catalytic base/acid to catalyze the carbonyl/alcohol interconversion of the substrates and the coupled redox reaction of nicotinamide dinucleotide (He & Yang, 1996). The substrate takes such an orientation that a large part of the CoA moiety extends into the solution (Hartmann et al., 1991), and the nicotinamide ring of the coenzyme has "B-side" specificity (You, 1992).

However, the K_m for NADH increased to 32 μM if a long-chain substrate (3-ketohexadecanoyl-CoA) was bound to the enzyme under the same experimental conditions (see Table 2). A 17-fold increase of the dissociation constant (K_{ia}) for NADH, calculated from kinetic measurements (Dalziel, 1975), was found as a result of the bound substrate having a longer hydrocarbon chain tail. This suggests that substrate binding causes a considerable change in the conformation of the dehydrogenase. Moreover, the binding of substrate would likely cause a marked desolvation at the active site to reduce the "apparent" dielectric constant, thereby increasing the strength of an imidazole−carboxyl interaction. The structural rearrangement and the desolvation arising from substrate binding may make possible the strong electrostatic interaction between glutamate-462 and histidine-450. This model would also explain why the pK_a of the dehydrogenase was virtually unchanged by substituting Glu-462 with Ala or Gln. Such phenomena are similar to the effects of pH on the wild-type and mutant lactate dehydrogenases, but in lactate dehydrogenase the acidic residue, Asp-168, is very close to the catalytic residue, His-195 (Clarke et al., 1988). Thus, formation of a dehydrogenase·coenzyzme·substrate ternary complex may be required for glutamate-462 to exert its influence on the catalytic residue, histidine-450 (see Figure 3). The catalytic properties of the other component enzymes of the *E. coli* multifunctional protein and the K_m values for substrates of the dehydrogenase were changed very slightly by substituting Ala or Gln for Glu-462 (see Table 2). These results indicate that the protein conformation of the large α-subunit of the *E. coli* fatty acid oxidation complex is not severely perturbed by such mutations. However, since the γ-carboxyl group of Glu-462 was found to be important for the thermostability of the multienzyme complex (see Figure 2), the possibility that mutations at Glu-462 might result in

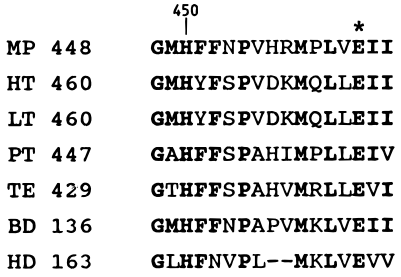

FIGURE 4: Comparison of the amino acid sequence around glutamate-462 and histidine-450, the catalytic residue of L-3-hydroxyacyl-CoA dehydrogenase (He & Yang, 1996), of the *E. coli* fatty acid oxidation multifunctional protein (MP) (Yang et al., 1991) with those of homologous regions of the large α-subunit of human mitochondrial trifunctional β-oxidation complex (HT) (Kamijo et al., 1994a), pig mitochondrial long-chain-specific bifunctional enzyme (LT) (Yang et al., 1994), plant glyoxysomal tetrafunctional protein (Preisig-Müller et al., 1994), rat peroxisomal trifunctional enzyme (TE) (Osumi et al., 1985), β-hydroxybutyryl-CoA dehydrogenase from *Crostridium acetobutylicum* (BD) (Youngleson et al., 1989), and pig L-3-hydroxyacyl-CoA dehydrogenase (HD) (Bitar et al., 1980). Standard one-letter amino acid abbreviations are used. The well-conserved glutamate residue at position 462 of MP is indicated by an asterisk.

a small but harmful structural alteration (for example, misalignment of the catalytic residue, His-450) cannot be excluded until high-resolution structural data of the wild-type and mutant complexes become available.

The large α-subunit of the *E. coli* fatty acid oxidation complex is the prototype of the extant multifunctional β-oxidation enzymes. The large α-subunit of the mitochondrial membrane-bound trifunctional β-oxidation complex is a long-chain enoyl-CoA hydratase:3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme, and is evolutionarily more closely related to the *E. coli* multifunctional fatty acid oxidation protein than to the mitochondrial matrix or peroxisomal β-oxidation enzymes (Yang, 1994). The role of glutamate-462 in the catalytic mechanism of L-3-hydroxyacyl-CoA dehydrogenase suggested by this study would probably be applicable to other members of the dehydrogenase family. The multiple sequence alignment in Figure 4 reveals that His-462 and Glu-474 of the large α-subunit of the mitochondrial trifunctional β-oxidation complex correspond to His-450 and Glu-462 of the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase, respectively. It is most likely that His-462 is the catalytic residue of the long-chain dehydrogenase and the function of Glu-474 is analogous to that of Glu-462, as described in this paper. The *E. coli* fatty acid oxidation complex is perhaps the best available model of the mitochondrial trifunctional β-oxidation complex (Yang, 1994). If so, the information on the structure−function relationship of the former complex is useful for understanding the molecular basis of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency, a human metabolic disease (Hale et al., 1990; Wanders et al., 1990). LCHAD deficiency patients have been assigned into two groups: a few cases with a total loss of mitochondrial trifunctional β-oxidation complex were classified as group I, and all other cases as group II, i.e., so-called "isolated LCHAD deficiency". It was reported (IJlst et al., 1994) that 24 of 26 group II patients were homozygous for a $Glu^{510} \rightarrow$ Gln mutation on the large α-subunit of the trifunctional β-oxidation complex. Recently, a careful investigation of this mutation at the genomic DNA level revealed that less than half of group II patients were homozygous for this mutation. The overestimation of incidence of such a mutation was due to the imperfect methodology used previously (Ding et al., 1996). Since the cDNA coding for the trifunctional β-oxidation complex of only a few patients has been sequenced, other mutations are expected to be found. Although the $Glu^{510} \rightarrow$ Gln mutation was suggested to be a disease-causing mutation, it was pointed out by Wanders and Hashimoto and their co-workers (IJlst et al., 1994) that definitive evidence that this mutation affects the dehydrogenase activity has to come from expression studies. Unfortunately, neither the wild-type nor the mutant human mitochondrial trifunctional β-oxidation complex has so far been successfully expressed and characterized. The results described here provide timely evidence that the long-chain dehydrogenase may be inactivated by amidation of the γ-carboxyl group of a conserved glutamate that corresponds to Glu-462 of the *E. coli* multifunctional protein (see Figure 4). Since the precursor of the large α-subunit of trifunctional β-oxidation complex has a leader peptide consisting of 36 residues, the aforementioned Glu-510 is actually identical to Glu-474, which is the counterpart of Glu-462 of the large α-subunit of the *E. coli* fatty acid oxidation complex (see Figure 4). More importantly, an understanding of the catalytic mechanism of L-3-hydroxyacyl-CoA dehydrogenase (see Figure 3) could explain why the replacement of Glu-510 by Gln would affect the dehydrogenase activity of the large α-subunit of the trifunctional β-oxidation complex.

In support of the concept of isolated LCHAD deficiency in group II patients, it was reported (IJlst et al., 1994) that: "In all these patients, there is a deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase with near normal activities of mitochondrial long-chain enoyl-CoA hydratase and long-chain 3-ketothiolase." However, since the long-chain thiolase activity of the *E. coli* $\alpha/Glu^{462} \rightarrow$ Gln mutant complex was found to be significantly lower than the wild-type level and this adverse effect resulted from the mutation on the large α-subunit (see Table 1), we re-evaluated the previously published LCHAD patients' data. In addition to a decrease of LCHAD activity by 75%, the recognition of an average loss of 41% of long-chain thiolase activity in patients' fibroblast homogenates[3] [see Table 1 reported by IJlst et al. (1994)] allowed us to conclude that the $Glu^{510} \rightarrow$ Gln mutation, like the $\alpha/Glu^{462} \rightarrow$ Gln mutation in the *E. coli* complex, could cause a decrease in the catalytic efficiency of the long-chain 3-ketoacyl-CoA thiolase. Moreover, it is likely that Glu-510, similar to its counterpart in the *E. coli* complex (Glu-462) (see Figure 2), is also important for the thermal stability of human mitochondrial trifunctional β-oxidation complex. This inference appears to receive support from a case report (Kamijo et al., 1994b) describing the degradation rate of the trifunctional β-oxidation complex in cells from a group II patient to be faster than that from control cells. Therefore, the notion (IJlst et al., 1994) that the $Glu^{510} \rightarrow$ Gln mutation would not affect the stability of the enzyme complex is probably incorrect. Demonstration that glutamate-462 of the large α-subunit plays a significant role in the catalytic functions and the

[3] The long-chain 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase activities measured in fibroblast homogenates reflect not only the activities of the mitochondrial membrane-bound trifunctional β-oxidation complex but also those of the mitochondrial matrix and peroxisomal β-oxidation enzyme systems.

stability of the *E. coli* fatty acid oxidation complex has shed new light on the pathogenesis of the $Glu^{510} \rightarrow Gln$ mutation of the large α-subunit of trifunctional *β*-oxidation complex in human disease.

ACKNOWLEDGMENT

We are grateful to Dr. Horst Schulz and Dr. Marshall Elzinga for critical reading of the manuscript.

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BI961841E