Histidine-450 Is the Catalytic Residue of L-3-Hydroxyacyl Coenzyme A Dehydrogenase Associated with the Large α-Subunit of 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 Gln or Ala substituting for His^{450} or with Ala in place of Gly^{322} in the large α-subunit have been purified and characterized. The α/Gly^{322} → Ala mutation did not significantly affect the catalytic efficiencies (k_{cat}/K_m) of different component enzymes except for a 6.1-fold decrease in the k_{cat}/K_m of L-3-hydroxyacyl-CoA dehydrogenase and a 10-fold increase in the K_m for NADH. This observation confirms the prediction [Yang, X.-Y. H., Schulz, H., Elzinga, M., & Yang, S.-Y. (1991) *Biochemistry 30,* 6788−6795] that the *E. coli* dehydrogenase has an NAD-binding site at its amino-terminal domain and structurally resembles the pig heart dehydrogenase. The pH dependence of the k_{cat}/K_m of the *E. coli* dehydrogenase suggested the catalytic involvement of an amino acid residue with a pK_a of 6, which is presumably a histidine residue as proposed previously on the basis of chemical modifications. Since His^{450} of the *E. coli* multifunctional protein is the only histidine conserved in all known L-3-hydroxyacyl-CoA dehydrogenases, and since its counterpart in pig heart enzyme appeared to be close to the 3-keto group of the fatty acyl moiety of the substrate, His^{450} was replaced by either Gln or Ala. The catalytic properties of 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase, and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase of the α/His^{450} → Gln mutant complex were virtually unchanged except for a small decrease in the k_{cat} values of the latter two enzymes. In contrast, the dehydrogenase of this mutant complex was almost inactive due to a greater than 3000-fold decrease in its k_{cat} and a 6-fold increase in the K_m for NADH. The α/His^{450} → Ala mutant complex showed similar catalytic behaviors. Taken together, several lines of evidence lead to the conclusion that His^{450} is the catalytic residue of L-3-hydroxyacyl-CoA dehydrogenase of the *E. coli* multifunctional fatty acid oxidation protein.

L-3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) catalyzes the third reaction of the fatty acid β-oxidation spiral, i.e., L-3-hydroxyacyl-CoA + NAD^+ ⇆ 3-ketoacyl-CoA + NADH + H^+ (Schulz, 1991). The kinetic properties of L-3-hydroxyacyl-CoA dehydrogenases from various sources were reported previously (Wakil et al., 1954; Bradshaw & Noyes, 1975; Osumi & Hashimoto, 1980; He et al., 1989). However, there is almost no information available about the amino acid residues that are key to the binding and catalysis of the dehydrogenase. Members of the L-3-hydroxyacyl-CoA dehydrogenase family include monofunctional and multifunctional β-oxidation enzymes. For instance, the large α-subunit of the multienzyme complex of fatty acid oxidation from *Escherichia coli* exhibits enoyl-CoA hydratase (EC 4.2.1.17), Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (EC 5.3.3.8), and L-3-hydroxyacyl-CoA dehydrogenase activities and is actually a prototype of the extant multifunctional β-oxidation enzymes (Yang & Schulz, 1983; Yang, 1994). The central region of this 79 kDa multifunctional fatty acid oxidation protein is clearly homologous to the monofunctional L-3-hydroxyacyl-CoA dehydrogenase and was recognized as a putative dehydrogenase functional domain (Yang et al., 1991). By introducing point mutations into the amino-terminal domain of this multifunctional protein, glutamate-139 has recently been identified as the catalytic base of enoyl-CoA hydratase, which catalyzes the dehydration of both D- and L-3-hydroxyacyl-CoA (Yang et al., 1995). Δ^3-*cis*-Δ^2-*trans*-Enoyl-CoA isomerase was also found to reside in the amino-terminal domain of the *E. coli* multifunctional protein (Yang & Elzinga, 1993) and to share a substrate-binding site with the hydratase (Yang et al., 1995). Since the catalytic function of L-3-hydroxyacyl-CoA dehydrogenase was virtually unaffected by these mutations except for a slight increase in the K_m values for substrates, the dehydrogenase activity is most likely associated with a distinct domain. It was proposed (Yang et al., 1991) that the dehydrogenase functional domain begins with an NAD-binding domain of which a putative ADP-binding βαβ-fold is present at the amino terminus. If this is verified by further experimental evidence, the location of the dehydrogenase functional domain will be established.

L-3-Hydroxyacyl-CoA dehydrogenases belong to class B of the NAD^+-dependent dehydrogenases, by which transfer of a hydride ion between substrate and C4 of the nicotinamide ring of the coenzyme NAD is B-side-specific (You, 1982). However, the central question of which amino acid residue plays a critical role in the L-3-hydroxyacyl-CoA

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dehydrogenase catalysis remains to be answered. It has been speculated for more than a decade that an imidazole group of a histidine residue is crucial to the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase activity (Yang & Schulz, 1983). The catalytic residue of some L-2-hydroxy acid dehydrogenases was a histidine, which serves as a general catalytic base/acid to transfer a proton between the 2-hydroxy group of the substrate and the imidazole ring. Although L-3-hydroxyacyl-CoA dehydrogenases are functioning in a metabolic pathway quite distinct from that of L-2-hydroxy acid dehydrogenases, the reactions they catalyze are quite similar in the carbonyl/alcohol interconversion. Hence, it seems reasonable to consider the possibility that a histidine residue is involved in the catalysis of L-3-hydroxyacyl-CoA dehydrogenase when the structure−function relationship of this important β-oxidation enzyme is explored.

In this report, we provide evidence that a histidine residue at position 450 of the *E. coli* multifunctional fatty acid oxidation protein is the catalytic residue of the L-3-hydroxyacyl-CoA dehydrogenase. In addition, we show that the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase has an NAD-binding site in the amino-terminal domain whose topological features resemble those of the monofunctional pig heart L-3-hydroxyacyl-CoA dehydrogenase.

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), 2-hexadecynoic acid (Freund et al., 1985), and acetoacetyl-CoA (Seubert, 1960) were synthesized according to published procedures. The CoA derivatives of 2-hexadecynoic acid and 3-*cis*-tetradecenoic acid were synthesized by the mixed anhydride procedure as detailed by Goldman and Vagelos (1961). 3-Ketohexadecanoyl-CoA was enzymatically prepared from 2-hexadecynoyl-CoA as described previously (Thorpe, 1986). 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. The 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 single-stranded DNA template for mutagenesis was prepared as described previously (Yang & Elzinga, 1993). The oligonucleotide-directed mutagenesis was carried out by an adaptation of the unique site elimination method (Deng & Nickoloff, 1992). A synthetic oligonucleotide 5′-CAGGCATGC**A**C**GC**G**T**GGCGTAATC-3′ with an *Mlu*I site instead of a *Hin*dIII site was used as the selection primer. Oligonucleotides 5′-CTGGGTGCAG**CG**ATTATGGG-3′, 5′-CGGGATGCA**A**TTCTTTAACC-3′, and 5′-CTGCGGGATG-**GC**CTTCTTTAACC-3′ containing codon changes from GGC (Gly) to GCG (Ala), from CAC (His) to CAA (Gln), and from CAC (His) to *GCC* (Ala), 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 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). 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 (Yang & Elzinga, 1993), to form a mutant expression plasmid. The mutant expression plasmid carrying the α/Gly^{322} → Ala mutation was designated as pNDBA-5, and those with the α/His^{450} → Gln and the α/His^{450} → Ala mutation (Figure 1) were designated as pNDBA-8 and pNDBA-9, respectively.

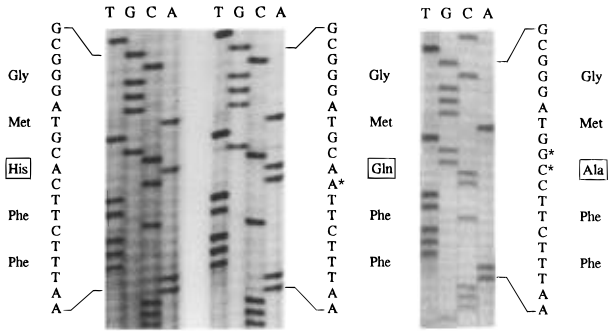

FIGURE 1: CAC (His) to CAA (Gln) and to GCC (Ala) mutations in the *fadB* gene shown by autoradiography of the sequencing gel of wild type and mutant *fadB*. Asterisks indicate the sites of the mutations.

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 for 4 h. Cells were harvested by centrifugation for 10 min at 3000*g* at 4 °C and washed twice with ice-cold 200 mM potassium phosphate buffer at 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). Proteins were separated by SDS−PAGE[1] on a 4 to 20% gradient gel at pH 8.3 as described previously (Blackshear, 1984). The activity of L-3-hydroxyacyl-CoA dehydrogenase was determined with acetoacetyl-CoA and 3-ketohexadecanoyl-CoA as substrates according to a published procedure (Binstock & Schulz, 1981). Assays of Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase with 3-*cis*-tetradecenoyl-CoA as substrate and enoyl-CoA hydratase with crotonyl-CoA as substrate were also performed as described previously (Binstock & Schulz, 1981). Kinetic parameters of the enzymes were estimated by a linear regression analysis of the double-

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

Table 1: Kinetic Parameters of Different Component Enzymes of the Wild Type and the Mutant Multienzyme Complex of Fatty Acid Oxidation from *E. coli*

	wild type		α/Gly^{322} → Ala mutant		α/His^{450} → Gln mutant		α/His^{450} → Ala 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)
enoyl-CoA hydratase	775		1316		349		489	
crotonyl-CoA		53		119		71		67
Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase	63		65		21		40	
3-*cis*-tetradecenoyl-CoA		5.8		4.2		6.3		15
L-3-hydroxyacyl-CoA dehydrogenase	382		175		0.12		0.22	
acetoacetyl-CoA		69		192		68		55
NADH		2.0		20		12		12
3-ketoacyl-CoA thiolase	117		164		135		101	
acetoacetyl-CoA		96		101		148		98
CoASH		102		103		101		121

reciprocal plot (Segel, 1975). 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 catalyzed the conversion of 1 μmol of substrate to product per minute.

RESULTS AND DISCUSSION

Location of a Peptide Fragment Folding into an ADP-Binding βαβ Motif. A putative ADP-binding βαβ-fold was proposed to be present in the central region of the *E. coli* multifunctional fatty acid oxidation protein on the basis of the results of sequence analysis (Yang et al., 1991). Since the peptide fragment (residues 315−343) has one residue different from the published fingerprint (Wierenga et al., 1986), further experimental evidence was needed to determine whether it is actually involved in the binding of NAD. By inspecting the X-ray structures of several different dehydrogenases Wierenga et al. (1985) predicted that steric hindrance with the bound dinucleotide would occur with a side chain at the second glycine of a glycine-rich turn (GXGXXG) of an ADP-binding βαβ-fold. On the basis of this theory, an α/Gly^{322} → Ala mutant fatty acid oxidation complex was produced by expression of the recombinant plasmid pNDBA-5 and then purified and characterized by procedures described in Materials and Methods. The kinetic parameters of the component enzymes of this mutant complex are compared with those of the wild type and listed in Table 1. In contrast to the α/Gly^{116} → Phe mutation that caused severe damage to enoyl-CoA hydratase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase but not to L-3-hydroxyacyl-CoA dehydrogenase (Yang & Elzinga, 1993), the catalytic efficiencies (k_{cat}/K_m) of the hydratase and the isomerase of the α/Gly^{322} → Ala mutant complex were found to be virtually identical with those of the wild type while that of the dehydrogenase of this mutant complex was reduced. The replacement of glycine-322 with alanine significantly increased the K_m value for NADH by a factor of 10. There was also a 2.8-fold increase in the K_m value for acetoacetyl-CoA and a 2.2-fold reduction in the k_{cat} value of the dehydrogenase. According to these data, this point mutation mainly affects the binding of NADH. It was found that the dissociation constant (K_{ia}) of the E·NADH complex calculated from the initial rate measurements (Dalziel, 1975) increased 10-fold due to the addition of a methyl group to the α-carbon of glycine-322. This corresponds to a loss of 1.4 kcal/mol in the free energy of binding (Wilkinson et al., 1983). The importance of the second conserved glycine in the -GXGXXG- consensus sequence to the coenzyme binding was thus demonstrated for the first time in a real catalytic process, and this result confirmed the existence of an ADP-binding βαβ-fold at the predicted location (Yang et al., 1991).

The establishment of this structural landmark enables us to generate an image of the tertiary structure of the L-3-hydroxyacyl-CoA dehydrogenase functional domain even though no X-ray structural data of any multifunctional β-oxidation enzyme are available yet. As is well known, the NAD-binding domains of different NAD^+-dependent dehydrogenases have very similar three-dimensional structures in spite of their completely different amino acid sequences (Rossmann et al., 1974). Under evolutionary pressures, the functional amino acids clearly retain a specific spatial arrangement if the function of a protein is to be conserved, and usually, the three-dimensional structure of the protein is also maintained (Rossmann et al., 1975). With respect to the *E. coli* and pig heart L-3-hydroxyacyl-CoA dehydrogenases, they are the same enzyme from different species. First, they catalyze the same reaction. Moreover, not only could the main peptide chain of the ADP-binding βαβ-fold of the *E. coli* dehydrogenase be superimposed on the corresponding βαβ motif of the pig enzyme (residues 17−45), but also the whole sequence of the latter enzyme showed clear homology with the dehydrogenase functional domain of the *E. coli* multifunctional protein (Bitar et al., 1980; Yang et al., 1991). Therefore, it is almost certain that these two L-3-hydroxyacyl-CoA dehydrogenases have very similar tertiary structures. Especially, one would expect a marked resemblance between the α-carbon backbones of their amino-terminal domains that contain the NAD-binding site. On the basis of the conformation of the polypeptide chain of the pig heart L-3-hydroxyacyl-CoA dehydrogenase determined by X-ray crystallography at 2.8 Å resolution (Birktoft et al., 1987), a hypothetical topological diagram of the amino-terminal domain of the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase, which consists of an eight-stranded β-sheet flanked by α-helices, was drawn (Figure 2). Glycine-322 is notably the first residue of α-helix A, which serves as a pyrophosphate binding helix (Wierenga et al., 1985). The small glycine residue at this position could allow the negatively charged pyrophosphate bridge of the NAD to approach the amino terminus of the α-helix, thereby forming a hydrogen bond and interacting with the dipole of the α-helix. When NAD binds to the dehydrogenase, it has such an extended conformation that its adenine ring is oriented

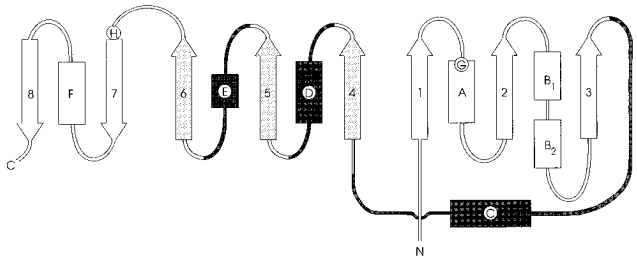

FIGURE 2: Topological diagram for the structure of the amino-terminal NAD-binding domain of the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase. This domain has the α/β twisted open-sheet structure, with an eight-stranded β-sheet in the middle surrounded by α-helices on both sides, similar to that of the amino-terminal domain of the pig heart L-3-hydroxyacyl-CoA dehydrogenase (Birktoft et al., 1987). Arrows denote strands of β-sheet. The β-strand order is reversed between strands 4 and 1. The first six strands are parallel to each other, and the last two strands run in the opposite direction. Rectangles denote α-helices; the white ones are above the β-sheet whereas the black ones stay underneath. The locations of glycine-322 and histidine-450 are indicated by circled letters G and H, respectively. The NAD-binding site is at the carboxy edge of the β-sheet, and at the amino terminus of the α-helices; the nicotinamide mononucleotide moiety sits across β-strands 4−6 (gray) and the adenosine monophosphate moiety across β-strands 1−3 (white) (Rossmann et al., 1974, 1975). The $\beta_1-\alpha A-\beta_2$ motif is the ADP-binding $\beta\alpha\beta$ structure, and the α-helix A is the pyrophosphate-binding helix (Wierenga et al., 1985).

toward the carboxyl terminus of β-strand 2 whereas the nicotinamide ring is close to that of β-strand 6, just as in the pig heart L-3-hydroxyacyl-CoA dehydrogenase (Birktoft et al., 1987) and other dehydrogenases (Rossmann et al., 1974, 1975).

Conservation of a Histidine Residue in All Known L-*3-Hydroxyacyl-CoA Dehydrogenases*. The effects of pH on kinetic parameters of the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase were studied over a broad range of pH values. A pK_a value of 6 was estimated from the dependence of k_{cat}/K_m of the dehydrogenase on the pH (data not shown). This value may reflect the involvement of an amino acid residue, whose side chain ionized at around pH 6, in the dehydrogenase catalysis. Of the various protic amino acid residues, only histidine would have such a pK_a value (Segel, 1975).

It was reported that *N*-ethylmaleimide inhibits 3-ketoacyl-CoA thiolase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase but not L-3-hydroxyacyl-CoA dehydrogenase of the *E. coli* fatty acid oxidation complex (Pawar & Schulz, 1981). In contrast to the very rapid inactivation of the thiolase of the complex, the inhibition of the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase by iodoacetamide proceeded rather slowly and could be prevented by saturating the enzyme with NAD and substrate (Yang & Schulz, 1983). It was observed that the activities of the dehydrogenase, the hydratase, and the isomerase of the *E. coli* fatty acid oxidation complex decreased by 50, 40, and 24%, respectively, when the dehydrogenase active site was labeled with iodo[1-^{14}C]-acetamide at a molar ratio of one bound inhibitor per two enzyme active sites (S.-Y. Yang and H. Schulz, unpublished data). Since the results of chemical modifications of the *E. coli* fatty acid oxidation complex also suggested that an imidazole group was critical to the *E. coli* dehydrogenase activity (Yang & Schulz, 1983), a histidine residue is very likely to be the catalytic residue. There are five histidine residues within the L-3-hydroxyacyl-CoA dehydrogenase functional domain of the large α-subunit of the *E. coli* fatty

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                    *
MP 442    RPENFCGMHFFNP
HT 454    RPEKVIGMHYFSP
LT 454    RPEKVIGMHYFSP
PT 442    R-DRIIGAHFFSP
TE 423    RPQLVIGTHFFSP
BD 130    RPDKVIGMHFFNP
HD 157    RQDRFAGLHFNVP
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FIGURE 3: Comparison of the amino acid sequence around histidine-450 of the *E. coli* multifunctional fatty acid oxidation protein (MP) (Yang et al., 1991) with those of homologous regions of the large α-subunit of human mitochondrial membrane-bound β-oxidation complex (HT) (Kamijo et al., 1994), pig mitochondrial long-chain-specific bifunctional enzyme (LT) (Yang et al., 1994), plant glyoxysomal tetrafunctional protein (PT) (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 heart mitochondrial L-3-hydroxyacyl-CoA dehydrogenase (HD) (Bitar et al., 1980). Standard one-letter amino acid abbreviations are used. The well-conserved histidine residue at position 450 is indicated by an asterisk.

acid oxidation complex (Yang et al., 1991). By multiple sequence alignment, one of these histidine residues was found to be conserved in all known L-3-hydroxyacyl-CoA dehydrogenases (Figure 3).

This well-conserved histidine residue at position 450 of the *E. coli* multifunctional fatty acid oxidation protein corresponds to histidine-165 of the pig heart L-3-hydroxyacyl-CoA dehydrogenase. By analogy with the pig heart dehydrogenase, it was predicted that histidine-450 of the *E. coli* multifunctional protein is located in a loop and near the amino terminus of β-strand 7 that is antiparallel to β-strand 6 (see Figure 2). The fact that the length of the acyl chain of the substrate has a significant effect on the turnover number of the pig heart L-3-hydroxyacyl-CoA dehydrogenase suggested that the fatty acid moiety instead of the major part of the CoA moiety of the substrate is in contact with the protein in the E·NAD·substrate ternary complex (He et al., 1989). A hypothetical model of the "more favorable orientation" of the substrate proposed by Birktoft et al. (1987) was supported by the results of a kinetic study (He et al., 1989). Inspection of a stereo diagram (Hartmann et al., 1991) showing the spatial arrangement of NAD and substrate bound to pig heart L-3-hydroxyacyl-CoA dehydrogenase suggested that the acetoacetyl moiety of the substrate fits into a cleft in such a way that its C3 carbon is near the nicotinamide ring with the C3 ketonic oxygen facing the carboxyl-terminal region of the loop that links β-strands 6 and 7. It is reasonable to expect that the substrate would bind to the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase in the same way as the pig heart dehydrogenase. Taken together, all information points to the conserved histidine-450 as the best candidate for the catalytic residue of the L-3-hydroxyacyl-CoA dehydrogenase.

Imidazole of Histidine-450 Is an Essential Group of the E. coli L-*3-Hydroxyacyl-CoA Dehydrogenase.* The *E. coli* fatty acid oxidation complexes carrying either an α/His^{450} → Gln or an α/His^{450} → Ala mutation were overproduced in *E. coli* cells by IPTG induction of the BL21 (DE3) plysS transformants containing the recombinant plasmids pNDBA-8 and pNDBA-9, respectively (Figure 4). The purified α/His^{450} → Gln and α/His^{450} → Ala mutant complexes have the same gross structure as the wild type complex (Pawar & Schulz, 1981). The kinetic parameters of the component

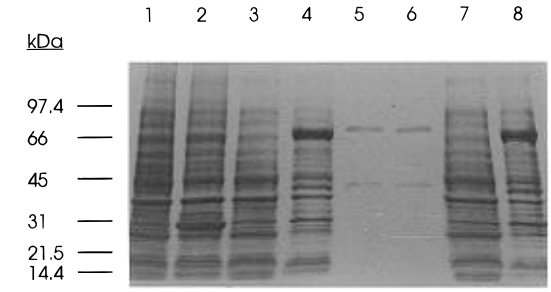

FIGURE 4: SDS−PAGE analysis of *E. coli* cell extracts and purified mutant multienzyme complexes: noninduced cell extracts of BL21 (DE3) pLysS transformants containing pND-1 (lane 1), pNDBA-8 (lane 3), and pNDBA-9 (lane 8); IPTG-induced cell extracts of BL21 (DE3) pLysS transformants containing pND-1 (lane 2), pNDBA-8 (lane 4), and pNDBA-9 (lane 7); and purified α/His^{450} → Gln mutant complex (lane 5) and α/His^{450} → Ala mutant complex (lane 6). Protein molecular mass standards were run on the same gel, and their positions are marked by the bar designated 97.4 kDa for phosphorylase B, 66 kDa for bovine serum albumin, 45 kDa for ovalbumin, 31 kDa for carbonic anhydrase, 21.5 kDa for trypsin inhibitor, and 14.4 kDa for lysozyme.

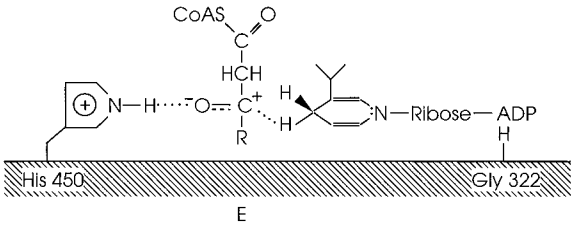

FIGURE 5: Schematic diagram of a dehydrogenase−NADH−3-ketoacyl-CoA ternary complex. The 3-keto group of the substrate lies between the imidazole ring of histidine-450 and the B-side of the nicotinamide ring of NADH. A partial negative charge developed on the C3 oxygen of the substrate during the transition state is stabilized by the positive charge of the protonated imidazole ring, and the electron-deficient C3 carbon is then prone to nucleophilic attack by a hydride ion donated by the dihydronicotinamide ring of NADH.

enzymes of the purified *E. coli* mutant complexes are compared with those of the wild type and listed in Table 1. The k_{cat} and K_m values of 3-ketoacyl-CoA thiolase from the wild type and the mutant complexes were found to be similar. The results suggest that these two histidine-450 mutations did not affect the small β-subunit (Yang et al., 1990) or the quaternary structure of the complex. The kinetic parameters of enoyl-CoA hydratase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase of the α/His^{450} → Gln mutant complex and the wild type complex were similar except for a slight decrease in the k_{cat} of the hydratase and isomerase from the mutant complex. The data suggest that this point mutation did not cause significant structural perturbation in the large α-subunit. The most striking change in the catalytic properties of the α/His^{450} → Gln mutant complex was a drastic drop of the L-3-hydroxyacyl-CoA dehydrogenase activity. The k_{cat} of the dehydrogenase from the mutant complex was more than 3000-fold lower than that of the wild type enzyme along with a 6-fold increase in the K_m value for NADH (see Table 1). A 6-fold increase of the dissociation constant (K_{ia}) for NADH, calculated from kinetic measurements (Dalziel, 1975), was also found in the mutant dehydrogenase. Changing histidine-450 to Gln in the *E. coli* multifunctional fatty acid oxidation protein reduced the overall catalytic efficiency, $k_{cat}/K_{ia}K_{mb}$ (Lewendon & Shaw, 1993), of the dehydrogenase by about 18000-fold. The dramatic decrease of the catalytic efficiency of the His^{450} → Gln mutant dehydrogenase resulted from a significant increase of the free energy of the transition state by 5.8 kcal/mol. When 3-ketohexadecanoyl-CoA was used as a substrate, the second-order rate constants of the dehydrogenases of the α/His^{450} → Gln mutant and the wild type complexes were determined to be 1290 s^{-1} and 0.41 s^{-1}, respectively. With the long-chain (C_{16}) substrate, the k_{cat} of the mutant dehydrogenase was also reduced more than 3000-fold with no significant change in the K_m value. Apparently, histidine-450 plays a key role in the catalytic mechanism of the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase.

The replacement of histidine-450 with Gln was a partially conservative substitution in that glutamine is similar in volume to histidine (Richards, 1977) and may mimic histidine in forming a hydrogen bond. On the other hand, the substitution of histidine-450 with Ala resulted in deletion of the imidazole ring. Changes in the catalytic properties of the component enzymes of the α/His^{450} → Ala mutant complex were found to be similar to those of the α/His^{450} → Gln mutant complex (Table 1). In particular, these two mutations have similar effects on raising the free energy of activation of the reaction catalyzed by L-3-hydroxyacyl-CoA dehydrogenase. The deletion of the imidazole by the His^{450} → Ala mutation resulted in loss of the hydrogen bond, but this would allow water to occupy the vacated space so that the transition state was solvated and the water could function in proton relay. In addition, the possibility that the residual activity of the His ^{450} → Ala mutant dehydrogenase resulted from a compensating role of another base in the neighborhood could not be ruled out since the detailed information on the three-dimensional structure of the mutant dehydrogenase remains to be obtained. Nevertheless, the results demonstrated that the imidazole group of histidine-450 is essential to the catalysis of the dehydrogenase.

A protonated histidine-450 is required for the formation of a reactive E·NADH·3-ketoacyl-CoA complex, as indicated by the effects of pH on the *E. coli* L-3-hydroxyacyl-CoA dehydrogenase (data not shown). The transition state of the ternary complex of L-3-hydroxyacyl-CoA dehydrogenase appears to be similar to those of L-2-hydroxy acid dehydrogenases (Birktoft & Banaszak, 1983). As shown in Figure 5, the partial negative charge on the C3 oxygen of the substrate in the transition state can be stabilized by the positive charge of the protonated imidazole ring of histidine-450. The interaction between the keto form of the substrate and the well-conserved histidine residue would also help in the polarization of the carbonyl moiety. The concomitant development of a partial positive charge at the C3 carbon of the substrate would facilitate the transfer of a hydride ion from NADH to the C3 carbon of 3-ketoacyl-CoA. Although it is not yet known whether the transfer of a proton from the imidazole ring of the conserved histidine and of the hydride ion from the B-side of the dihydronicotinamide ring of NADH to the substrate follows a sequential or concerted mechanism, the insight into the binding and catalysis of the L-3-hydroxyacyl-CoA dehydrogenase derived from this study is probably applicable to other multifunctional β-oxidation enzyme, and to monofunctional L-3-hydroxyacyl-CoA dehydrogenases as well.

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