Identification of the Active Site Catalytic Residue in Human Isovaleryl-CoA Dehydrogenase†

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ABSTRACT: Isovaleryl-CoA dehydrogenase (IVD) is a homotetrameric flavoenzyme which catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA. E376 of pig medium chain acyl-CoA dehydrogenase (MCAD), a homologous enzyme, has been identified as the active site catalytic residue. Amino acid sequence alignment shows that A375 is the corresponding residue in human IVD. Using the atomic coordinates determined for MCAD, molecular modeling suggests that E254 is the substituting catalytic residue in IVD. To substantiate the importance of this residue for enzyme function, cDNAs for the wild-type human IVD and E254G, E254D, E254Q, and E254G/A375E mutant IVDs were constructed and cloned into a prokaryotic expression vector. The proteins were synthesized in *Escherichia coli* and purified, and their properties were examined. The catalytic activity of the recombinant wild-type IVD was the highest in the presence of isovaleryl-CoA, and its UV/visible light spectrum in the presence of isovaleryl-CoA showed quenching of its characteristic absorption in the 445-nm region and appearance of absorption at 600 nm. The E254G and E254Q mutant IVDs had no detectable enzymatic activity, and isovaleryl-CoA did not induce quenching of the absorption in the 445-nm region or the appearance of absorption at 600 nm. The E254D mutant IVD had residual activity for isovaleryl-CoA, and its spectrum was altered compared to that of the wild type. The E254G/A375E mutant IVD exhibited catalytic activity toward isovaleryl-CoA, and its spectrum in the absence or presence of the substrate was similar to that of the wild-type IVD. These results suggest that E254 of IVD is in close proximity to the bound FAD and support the hypothesis that E254 is the active site catalytic residue.

Isovaleryl-CoA dehydrogenase (IVD;[1] EC 1.3.99.10) catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA in the leucine catabolism pathway. Deficiency of the enzyme in humans is responsible for isovaleric acidemia, a serious metabolic disorder (Tanaka et al., 1966, 1987; Tanaka & Isselbacher, 1967; Noda et al., 1980; Vockley et al., 1991, 1992). IVD shares sequence homology with other acyl-CoA dehydrogenases including short, medium, long, very long, and short/branched chain acyl-CoA dehydrogenases which catalyze similar α,β-dehydrogenation of their corresponding substrates (Ikeda et al., 1983, 1985a; Matsubara et al., 1989; Izai et al., 1992; Rozen et al., 1995). The reaction catalyzed by these enzymes has been postulated to start with the abstraction of the C-2 proton of the substrate by an active site base and then the transfer of the C-3 hydride to the FAD in the presence of the electron-transferring flavoprotein (ETF) which is the physiological electron acceptor (Ikeda et al., 1985b; Powell & Thorpe, 1988; Thorpe, 1991; Johnson et al., 1993). Formation of a covalent binary complex between pig medium chain acyl-CoA dehydrogenase (MCAD) and the inhibitor 2-octynoyl-CoA has suggested that the E376 carboxylate is the active site catalytic base responsible for abstracting the C-2 proton from the enzyme substrate (Powell & Thorpe 1988). This hypothesis has been confirmed by X-ray diffraction data of pig MCAD and octanoyl-CoA complex (Ghisla et al., 1992; Kim et al., 1993) and by results from site-directed mutagenesis of E376 of human MCAD (Bross et al., 1990). Alignment of the amino acid sequences of the various members of the acyl-CoA dehydrogenase family shows that E376 of MCAD is present within a highly conserved region. However, this residue is replaced by a glycine at the equivalent position in human long chain acyl-CoA dehydrogenase (LCAD) and rat IVD and by an alanine in human IVD (Matsubara et al., 1989, 1990). Using the atomic coordinates of the pig MCAD crystal structure determined by X-ray diffraction studies, molecular modeling has suggested that E261 of human LCAD and E254 of human IVD could be the substituting catalytic residues (Kim & Wang, 1992; Kim et al., 1994). This hypothetical role of LCAD E261 was supported by Djordjevic et al. (1994), who reported spectral evidence indicating that replacement of E261 with a glutamine in LCAD resulted in no detectable reduction of the flavin after prolonged incubation of the mutant protein and the enzyme substrate.

In this study, the active site catalytic residue of human IVD was identified by replacing the glutamate at position 254 with a glycine, a glutamine, and an aspartate, constructing a E254G/A375E double mutant IVD, and examining the catalytic and spectral characteristics of purified proteins. Preliminary accounts of results from this study have been

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[1] Abbreviations: DCIP, dichlorophenolindophenol; ETF, electron-transferring flavoprotein; FAD, flavin adenine dinucleotide; IPTG, isopropyl β-D-thiogalactoside; IVD, isovaleryl-CoA dehydrogenase; LCAD, long chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; SCAD, short chain acyl-CoA dehydrogenase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMS, phenazine methosulfate.

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presented (Mohsen & Vockley, 1993; Mohsen & Vockley, 1994).

MATERIALS AND METHODS

Materials. Acrylamide solution for sequencing gels was purchased from National Diagnostics Inc. (Nanville, NJ). Acrylamide for SDS−PAGE was from J. T. Baker Co. (Phillipsburg, NJ). Agarose, SDS−PAGE low molecular weight standards, and the Bio-Rad DC protein assay kit were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA). Butyryl-CoA, catalase, dichlorophenolindophenol (DCIP), glucose oxidase, hexanoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, valeryl-CoA, and phenazine methosulfate (PMS) were purchased from Sigma Chemical Company (St. Louis, MO). Isopropyl β-D-thiogalactoside (IPTG) was purchased from Jersey Lab and Glove Supply (Livingston, NJ). Deoxynucleotide triphosphates for PCR were purchased from Perkin Elmer (Oak Brook, IL). Diethylaminoethyl agarose (DEAE-Spharose Fast Flow), plasmid pKK223-3, and *Escherichia coli* K12 strain JM105 were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Ceramic hydroxyapatite (10 μm) was the gift of Bio-Rad Laboratories, Inc. (Hercules, CA). Pfu polymerase and its reaction buffer (buffer 1) for PCR, Bluescript II SK ± phagemid containing the *lacZ* gene for blue/white selection, and *E. coli* strain XL-Blue were purchased from Stratagene Cloning Systems (La Jolla, CA). ETF was purified from pig liver as described (Husain & Steepkamp, 1983). The 7-deaza-dGTP Sequenase sequencing kit was purchased from United States Biochemical (Cleveland, OH). Ampicillin, T4 ligase, and restriction enzymes were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). LB broth was from DIFCO Laboratories (Detroit, MI), and 2-YT medium was from GIBCO BRL, Life Technologies Inc. (Grand Island, NY). All other chemicals and reagents obtained commercially were reagent or molecular biology grade.

Oligonucleotides used for PCR were synthesized by the Molecular Biology core at the Mayo Clinic (Rochester, MN).

DNA Manipulations. (1) Construction of a High Level Expression Human IVD cDNA. Expression of wild-type human IVD cDNA (*HIVD*) coding for the mature IVD polypeptide was extremely low in *E. coli*. This *HIVD* cDNA was modified at its 5′-end coding region (m*HIVD*) to enhance expression. Details of this experimental procedure are reported elsewhere (Mohsen & Vockley, 1995). Briefly, this m*HIVD* cDNA was synthesized *via* PCR using the wild-type *HIVD* cDNA coding for the mature monomer form of IVD (1185 bp) as a template. A 5′-end primer was designed with specific modifications at the first 111 base pairs of the 5′-end region of the IVD cDNA to accommodate codon usage in *E. coli* and, in addition, contained the sequence for an *Eco*RI site. The 3′-end primer contained the last 20 coding bases, including the stop codon, followed by a sequence for a *Hin*dIII site. The PCR fragment was cloned into the pKK223-3 expression vector, giving the human IVD expression plasmid pKm*HIVD*.

(2) Construction of the Human Mutant IVDs. The wild-type *HIVD* cDNA coding for the mature IVD was used as template to synthesize insert fragments (235 bp) containing the desired codon modifications for the E254 amino acid replacements. The 5′-end primer used for the synthesis of the three single-mutant substitutions was 24 bases in length, and its sequence was complementary to the antisense strand of the IVD cDNA region containing the unique *Bgl*II restriction enzyme site (nucleotide 539−562). The 3′-end primers coding for the single mutations were 5′-^{792}AAG AG G CCC CCC GGC CAG CAC CAG CCG **XXX** CAG GTC CAG CC^{752}-3′, where the designated **X** positions were ATC, TTG, or TCC for the aspartate, glutamine, or glycine mutants, respectively. These primers contained the sequence for the unique *Dra*II restriction site. The synthesized PCR fragments were cloned into the *Bgl*II and *Dra*II sites within the coding region of the enhanced expression m*HIVD* cDNA subcloned in a Bluescript phagemid modified to eliminate the *Dra*II site in its multiple cloning site. The mutant IVD cDNAs were then inserted into the *Eco*RI and *Hin*dIII sites of the pKK223-3 expression vector.

The 5′-end primer used to introduce the A375E mutation was 5′-^{1076}AC TTT CCC ATG GGC CGC TTT CTT CGA GAT GCC AAG CTG TAT GAG ATA GGG **GAA** GGG ACC AGC GAG GTG A^{1144}-3′ containing an *Nco*I restriction site. The 3′-end primer was the last 20 coding bases including the stop codon followed by a sequence for a *Hin*dIII site. These primers were used to synthesize a DNA fragment *via* PCR. The resulting PCR product was digested with *Nco*I and *Hin*dIII and used to replace the corresponding region of the IVD cDNA containing the E254G mutation. (Note that the only other *Nco*I site, which was present at the 5′-end of the wild-type IVD cDNA, was eliminated in the 5′-end high level expression IVD cDNA.)

Bacterial Growth Conditions and Purification of IVD Proteins. The pKm*HIVD* plasmids harboring the wild-type or mutant IVD cDNAs were introduced into *E. coli* JM105 using a Cell-Porator (GIBCO BRL, Life Technologies Inc., Grand Island, NY). Transformed cells were grown in 2 L of medium in 4-L flasks. The medium consisted of 12.5 g each of LB and 2-YT media and 50 mg/L ampicillin in distilled water. IPTG was added to the culture at a final concentration of 0.5 mM when A_{550} was about 1, and the culture was incubated with shaking at 37 °C for 24 h. Preparation of cell-free extracts and purification of wild-type IVD was carried out as described (Mohsen & Vockley, 1995). Fractions with highest DCIP specific activity were pooled and concentrated using a Centriprep-30 concentrator (Amicon, Beverly, MA). Mutant IVD proteins were purified using the same purification procedure except that inactive mutant IVDs were monitored by their characteristic green color and SDS−PAGE. Protein determinations were carried out using the Bio-Rad DC protein assay.

Determination of Enzyme Purity and Molecular Mass. The purity of IVD was estimated visually from SDS−PAGE (10% T) after loading 20 μg of enzyme treated with SDS/β-mercaptoethanol buffer as described (Laemmli, 1970). The molecular mass of IVD was estimated from its migration distance using the midrange molecular weight SDS−PAGE SigmaMarker.

Enzyme Assays. IVD activity was measured using the ETF fluorescence or the DCIP dye reduction assays. The ETF assay was performed at 32 °C using a slight modification of the method described (Frerman & Goodman, 1985). The reaction mixture containing 50 mM Tris, pH 8.0, 0.5% glucose, and 0.05 mM acyl-CoA substrate in 760 μL was de-aerated by repeated vacuum and layering with oxygen-free argon in a tightly sealed cuvette. All assays were

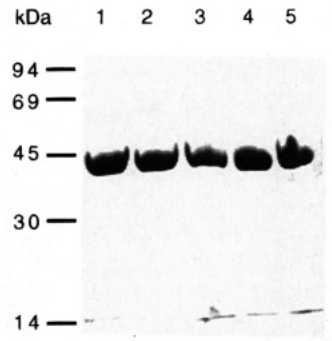

FIGURE 1: 0.1% SDS−12% PAGE showing purified human recombinant IVD proteins. Standards were phosphorylase b (94 kDa), bovine serum albumin (69 kDa), chicken egg ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and α-lactalbumin (14 kDa). Lanes 1−5: approximately 20 μg of wild-type IVD and E254G, E254Q, E254D, and E254G/A375E mutant IVD, respectively, were loaded in each lane. Conditions for 0.1% SDS−12% PAGE buffer system were as described (Laemmli, 1970).

conducted using freshly dissolved substrates in 2 mM sodium acetate, pH 5.0. Five microliters containing 40 units of glucose oxidase and 1 unit of catalase were added to remove any remaining dissolved oxygen, followed by 25 μL containing purified pig ETF to give a final concentration of 1 μM. The reaction was initiated by adding 10 μL of enzyme sample and enzyme activity was monitored by exciting the ETF flavin at 342 nm and monitoring quenching of fluorescence at 496 nm using the Luminescence spectrometer LS 50 B (Perkin Elmer, Oak Brook, IL); 1 unit is defined as the amount of enzyme needed to completely reduce 1 μmol of ETF per minute at 32 °C. The DCIP assay was performed as described using PMS as the intermediate electron acceptor (Ikeda et al., 1983). The decrease in absorption at 600 nm was monitored for 10 s using a DU 7400 spectrophotometer (Beckman, Arlington Heights, IL). The wild-type IVD activity was also measured at various isovaleryl-CoA concentrations as described above.

Spectral Analysis. Spectral scans were performed on purified protein samples dissolved in 0.1 M potassium phosphate, pH 7.8, at ambient temperature using a Beckman DU 7400 spectrophotometer. Wild-type and mutant IVD samples containing 2.3 nmol of protein were scanned in the presence of 23-fold excess isovaleryl-CoA under anaerobic conditions. Oxygen was removed from the substrate solution and from the samples in tightly sealed cuvettes using alternative cycles of vacuum and layering with oxygen-free argon. The substrate was then injected prior to the spectral analysis. Spectral analysis of wild-type IVD at various substrate concentrations was carried out using a sample containing 5 nmol of protein.

RESULTS

Purification and Characteristics of Wild-Type and Mutant IVDs. Expression levels of the mutant proteins using the high-level expression IVD cDNA (m*HIVD*) were as high as observed with wild-type IVD (Mohsen & Vockley, 1995). The high levels of expression facilitated the separation of mutant IVDs from *E. coli* proteins to near homogeneity (Figure 1).

Activity Measurements. The catalytic activity of wild-type IVD was apparent using the DICP/PMS assay. Using the ETF fluorescence assay, the wild-type IVD was most active when isovaleryl-CoA was used as the substrate; however, significant activity was detected when other acyl-CoA derivatives were included in the reaction mixture (Table 1). The double-reciprocal plot (v^{-1} versus [isovaleryl-CoA]$^{-1}$) was sigmoidal, suggesting that the enzyme exhibits a non-Michaelis−Menten behavior under the experimental conditions used. V_{max} was therefore extrapolated from the activity values at the highest substrate concentration and was 0.8 unit. This V_{max} value corresponds to a specific activity of 7.86 units mg^{-1}. The concentration of isovaleryl-CoA at 0.5V_{max} was extrapolated to be 1.0 μM.

Table 1: Substrate Specificity of Human Isovaleryl-CoA Dehydrogenase Expressed in *E. coli*[a]

substrate	amount of enzyme[b] (pmol)	sp act. (units/mg) ± SD	rel sp act.[c]
isovaleryl-CoA	0.29	8.10 ± 0.24	100
valeryl-CoA	0.58	3.71 ± 0.33	46
butyryl-CoA	1.45	1.71 ± 0.06	21
hexanoyl-CoA	1.45	1.19 ± 0.06	15
octanoyl-CoA	14.54	0.08 ± 0.01	1
isobutyryl-CoA	14.54	undetectable	

[a] The enzymatic activity was measured (n = 3) using the ETF fluorescence assay. [b] The amount of enzyme used was varied to acquire detectable activity. [c] The specific activity relative to the activity of the enzyme toward isovaleryl-CoA.

No activity could be detected for the E254G, E254D, and E254Q mutant IVDs using the DCIP/PMS assay and isovaleryl-CoA as the substrate. The E254G and E254Q mutant proteins had no detectable activity for isovaleryl-CoA using the ETF fluorescence assay, even when the amount of mutant protein used was increased 100-fold over that of the wild type. The E254D mutant IVD had barely detectable activity for isovaleryl-CoA (<0.1%). Enzymatic activity of these mutants toward other acyl-CoA substrates was not determined. Catalytic activity of the E254G/A375E double mutant IVD toward isovaleryl-CoA was detected using the DCIP/PMS assay. Catalytic activity of this mutant was also detected with various acyl-CoA substrates using the ETF fluorescence assay. With this assay, the activities of the double mutants for isovaleryl-CoA, valeryl-CoA, and hexanoyl-CoA were 4.0, 1.7, and 15%, respectively, of the activity of the wild type for each of the substrates.

Spectral Analysis of the Native IVD Proteins. The UV/visible spectra of the wild-type and mutant IVDs are shown in Figure 2, and the absorption maxima wavelength values and their ratios are listed in Table 2. The wild-type IVD shows absorption maxima at 269, 370, and 445 nm. In addition, there are shoulders at 278, 358, and 460 nm (Figure 2A). Similarly, the 267−278-nm region of the E254G, E254Q, and E254G/A375E IVD mutants shows an absorption maximum at 269 nm and a shoulder at 278 nm (Figure 2B). The E254D IVD mutant shows an alteration in this area where the 269- and 278-nm absorption maxima appear almost equivalent in magnitude.

The spectrum of the E254G mutant shows a shoulder in the 310-nm region and an absorption maximum at 358 nm compared to that at 370 nm in the wild type (Figure 2C). In the 370-nm region of the light spectrum, a 4-nm blue shift in absorption is observed with the E254Q mutant IVD. The spectral change of the E254D mutant is more dramatic near the 370-nm region. There are two unusual absorption maxima appearing at 339 and 357 nm. Absorption magni-

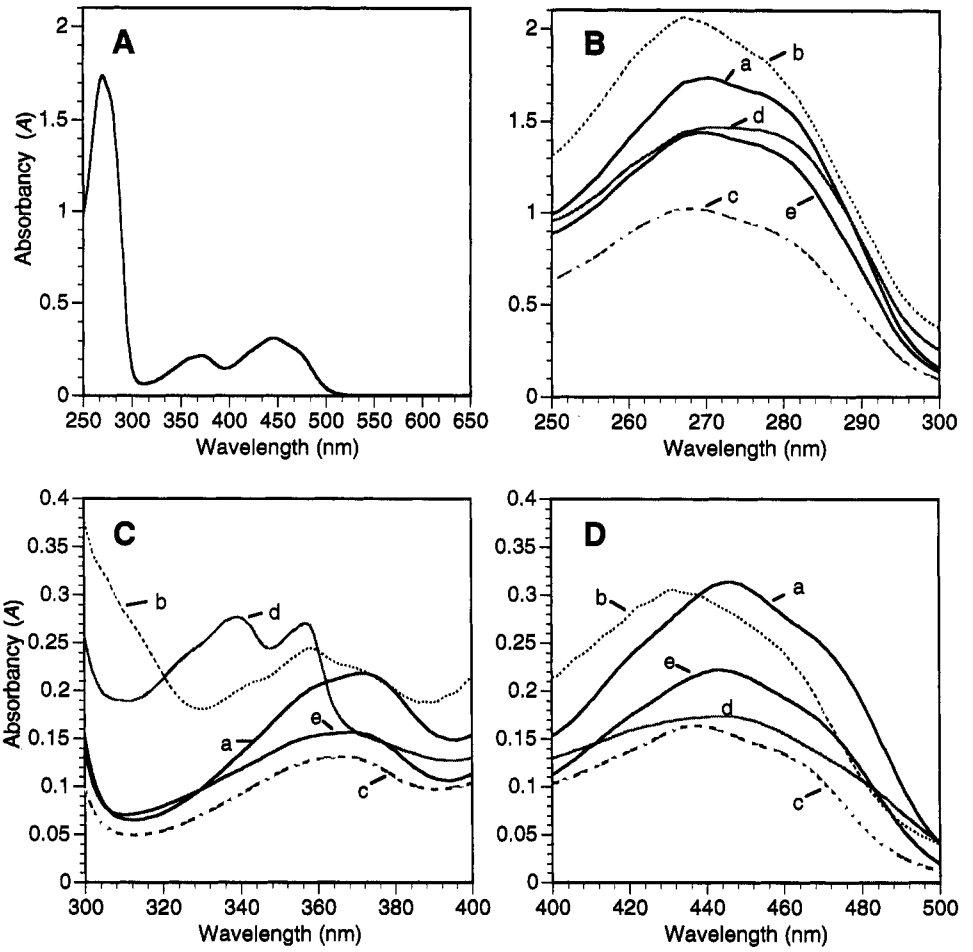

FIGURE 2: Ultraviolet/visible light absorption scans of IVD proteins at ambient temperature in 0.1 M potassium phosphate, pH 7.8. Scans of wild-type recombinant human IVD (7.2 μM) (A) and different regions of IVD protein spectra (B, C, and D): wild-type IVD (7.2 μM) (a) and the E254G (7.8 μM) (b), E254Q (4.4 μM) (c), E254D (3.6 μM) (d), and E254G/A375E (6.6 μM) (e) mutant IVD proteins.

Table 2: UV/Visible Absorption Maxima Values for Wild-Type and Mutant Isovaleryl-CoA Dehydrogenase

substrate	wavelength maxima[a] (nm)	absorption ratios
wild type	269, 370, 445	5.4:0.7:1
E254G	267, 358, 431	6.8:0.8:1
E254D	271, 339, 357, 442	8.7:1.6:1.6:1
E254Q	268, 366, 437	6.3:0.8:1
E254G/A375E	269, 369, 443	6.5:0.7:1
FAD[b]	265, 377, 449	3.3:0.8:1

[a] Values were determined using fresh protein samples and a Beckman DU 7400 spectrophotometer. [b] Dissolved in water.

tude at these wavelengths is twice that at 445 nm, and the visible color of a solution of this mutant protein is shifted from the wild-type IVD green to greenish yellow.

The absorption at 445 nm observed in the wild-type IVD appears shifted 14 nm toward a shorter wavelength in the E254G mutant (Figure 2D). The E254Q mutant had a similar (8 nm) blue shift in the same region. The absorption maxima of the E254G/A375E mutant IVD was similar to that of the wild-type IVD at the various regions of its spectrum (Figure 2B–D).

Effect of Isovaleryl-CoA on the Spectra of IVD Proteins. Spectral scans of the wild-type IVD at various concentrations of isovaleryl-CoA under anaerobic conditions showed quenching of the absorption characteristic of isoalloxazine at the 370- and 445-nm regions. In addition, a shoulder at 310 nm and a new absorption peak at 600 nm has appeared. The presence of the 310-nm shoulder was still evident in the wild-type IVD even after 24 h of incubation and complete quenching of absorption at 445 nm (data not shown).

Spectral scans of the E254G and E254Q mutant IVDs did not show quenching near the corresponding regions at 370 and 445 nm in the presence of a 23-fold molar excess of isovaleryl-CoA. Instead, 5- and 7-nm red shifts of the absorption in the equivalent 445-nm region were observed for the E254G and E254Q mutant IVDs, respectively. In contrast, with the E254G/A375E mutant IVD the response to the presence of isovaleryl-CoA was similar to that of the wild type, where absorption in the 370- and 445-nm regions was quenched.

DISCUSSION

Expression of IVD Proteins. Earlier reports describing purification of IVD from rat and human liver indicate substantial loss of FAD with perturbations in enzyme structure. This is apparent from the high 269/445-nm ratios and the reported enzyme-FAD spectra (Ikeda & Tanaka, 1983, 1988; Finocchiaro et al., 1988). Because we encountered similar problems attempting to purify the recombinant wild-type human IVD from *E. coli* due to very low expression of the pK*HIVD* plasmid, we have altered codon usage at the 5′-end of the wild-type cDNA to accommodate protein expression in *E. coli* and inserted this altered human IVD cDNA (m*HIVD*) into the expression plasmid. Expres-

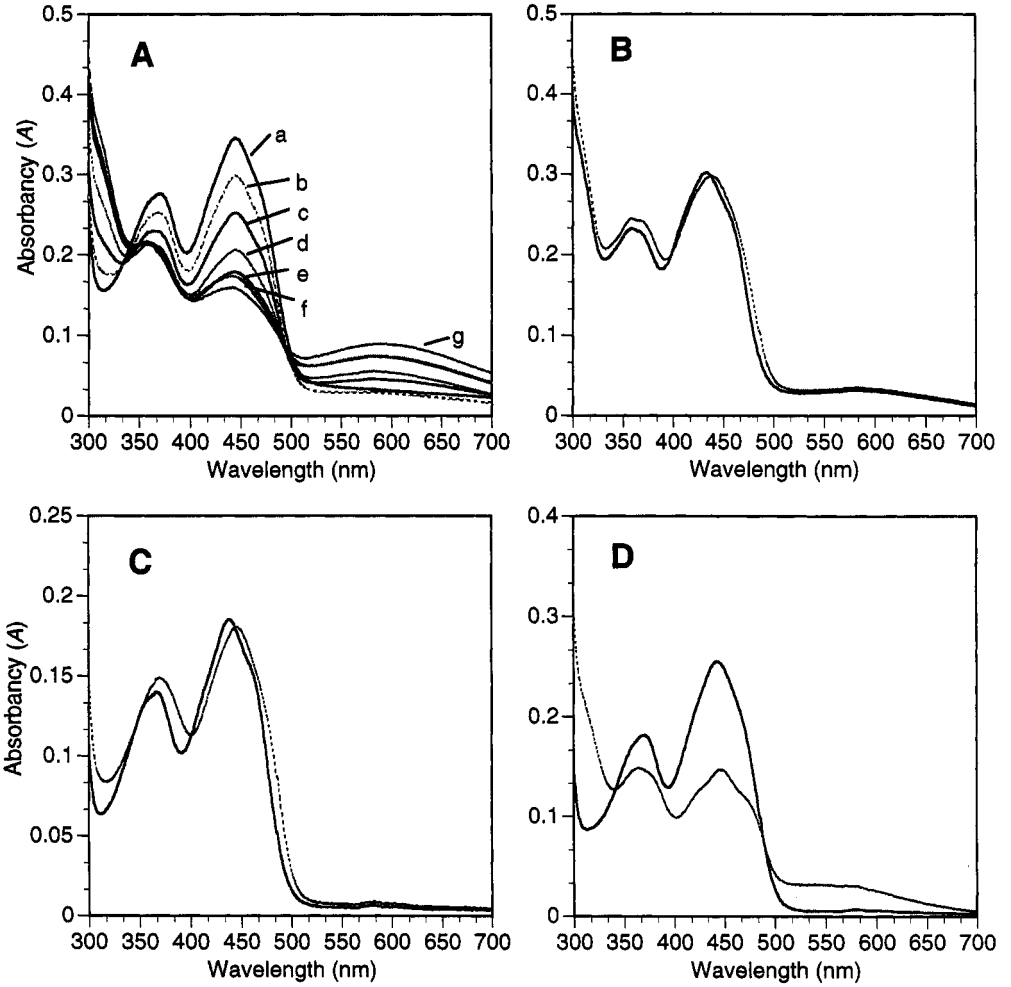

FIGURE 3: Ultraviolet/visible light absorption scans of IVD proteins in the presence of isovaleryl-CoA. (A) Five nanomoles of wild-type recombinant human IVD sample scanned in the presence of various amounts of substrate: 0 (a), 2.4 (b), 5.9 (c), 10.6 (d), 16.4 (e), 20.0 (f), and 32.9 (g) nmol of isovaleryl-CoA. (B–D) Scans of E254G (B), E254Q (C), and E254G/A375E (D) mutant IVD proteins in the absence (solid line) and presence (dotted line) of 14-, 24-, and 16-fold, respectively, molar excess of isovaleryl-CoA.

sion was substantially increased, and we were able to purify the enzyme to near homogeneity with negligible loss of FAD from the holoenzyme (Mohsen & Vockley, 1995). Expression of the E254G, E254D, and E254Q mutant and E254G/A375E double mutant IVD cDNAs, using the pK*HIVD* plasmid, was similarly very low, and we encountered extreme difficulty in purifying these proteins (unpublished data). Therefore, we introduced the appropriate nucleotide sequence mutations for these amino acid replacements in the high level expression m*HIVD* cDNA and thus were able to purify the mutant IVD recombinant proteins to near homogeneity (Figure 1).

Activity Studies. Results from testing the purified E254G, E254D, and E254Q mutants for catalytic activity illustrate the importance of the glutamate at position 254 for IVD catalytic activity. Loss of catalytic activity resulting from these replacements was first monitored using the DCIP/PMS assay. Since the sensitivity of this assay was a concern, we have employed the ETF fluorescence assay as well. The latter assay involves the interaction of a postulated IVD–substrate binary complex with ETF where a transfer of charge to ETF takes place with concomitant quenching of its fluorescence (Frerman & Goodman, 1985; Gorelick et al., 1985; Thorpe, 1991). It is an extremely sensitive assay, and activity could readily be measured using as little as a 100 fmol of purified wild-type IVD. Still, no catalytic activity could be detected with the E254G and E254Q mutants. Using the ETF fluorescence assay, however, activity could be detected for the E254D mutant. This was not surprising since the E → D replacement is a relatively conservative one compared to the E → G or Q replacement. The dramatic loss of activity with these mutations can thus be attributed to the loss of the carboxylate group of a glutamate at the 254 position. These results are consistent with earlier studies which have reported minimal residual activity resulting from a similar replacement of the active site catalytic glutamate in human MCAD and LCAD and the short chain acyl-CoA dehydrogenase (SCAD) from *Megasphaera elsdenii* (Bross et al., 1990; Becker et al., 1993; Djordjevic et al., 1994).

To further confirm the hypothetical identity of E254 as the catalytic residue, we constructed the E254G/A375E mutant IVD. This double mutation should place the α-proton-abstracting base in the active site in an orientation similar to that of MCAD. This has resulted in restoration of the catalytic activity for the E254G mutant and further emphasizes the importance of the glutamate at position 254 for the catalytic activity of IVD. It also confirms the similarity of the tertiary structure of IVD with MCAD and other members of the enzyme family as predicted by molecular modeling. It was surprising, however, to find that the percent of restored activity of IVD for hexanoyl-CoA was significantly higher than the percent restored activity for isovaleryl-CoA, while

that for valeryl-CoA was less. Although subtle differences in the structure of the acyl moiety binding pocket are likely to determine substrate specificity in the acyl-CoA dehydrogenase family of enzymes, this does not explain the altered activity profile of the double mutant as compared to the wild type. It is possible that substrate-induced conformational changes may play a role in determining substrate specificity.

Spectral Studies. The spectral scans of the wild-type and mutant IVDs show several important differences (Figure 2). The most apparent spectral differences between the E254G mutant and wild-type IVD include a pronounced shoulder in the 310-nm region, an absorption maximum at 358 nm, and a 14-nm blue shift of the absorption maximum to 431 nm. Spectral studies of oxidized flavin compounds indicate that significant shifts could occur when the polar environment of the isoalloxazine ring system is altered (Müller, 1991). Accordingly, the spectral changes observed with the E254G mutant IVD suggest that perturbations in the ionic or polar environment in the active site pocket near the isoalloxazine ring of the FAD were induced by this amino acid replacement.

The E254D mutant IVD exhibited the most dramatic changes observed among the mutant IVD proteins (Figure 2C). The profound alteration in the 330−370-nm region of the spectrum of this mutant confirms the unusual proximity of the carboxylate of the aspartate substituent to the isoalloxazine moiety of the FAD. It should be noted that the E254D mutant IVD was the most unstable among the mutant IVDs. It precipitated into insoluble white aggregates more readily compared to the glutamine and glycine mutants, suggesting loss of FAD, and its spectrum deteriorated upon storage (data not shown.)

Substantial differences in the spectral scans of the wild-type and E254G/A375E mutant IVDs could not be detected. Therefore, the observed changes in the spectrum of the E254G mutant IVD were apparently reversed by the additional replacement of A375 with a glutamate. This implies that although the position of the hypothetical catalytic glutamate has changed, the carboxylate of the glutamate at position 375 in the double mutant is in close enough proximity to the FAD in the active site to provide the proper ionic environment for the isoalloxazine to exhibit the same spectrum as the wild type.

Binding of isovaleryl-CoA to the wild-type IVD had a profound effect on its spectrum. Absorption of IVD in the 445-nm region was substantially reduced and accompanied by the appearance of an absorption maximum at 600 nm. Quenching of absorption at this region and appearance of an absorption peak at 600 nm has been reported in earlier studies with IVD purified from rat liver and other acyl-CoA dehydrogenases and has been widely accepted as evidence of reduction and charge transfer to the FAD prosthetic group (Ikeda & Tanaka, 1983). Another observation is the appearance of a shoulder at 310 nm in the presence of substrate. At saturating substrate concentrations, this shoulder was still present after incubation of the enzyme/substrate sample for 24 h (data not shown). This minor absorption in the 310-nm region appears similar to that observed by Ikeda and Tanaka (1983) who reported an absorption at 325 nm in the spectrum of rat IVD in the presence of isovaleryl-CoA.

With the E254G and E254Q mutant IVDs, isovaleryl-CoA did not induce quenching of the characteristic absorption of the oxidized isoalloxazine ring at the equivalent 445-nm region. However, it induced a minor red shift in the absorption in the same region. Djordjevic et al. (1994) suggested that a similar red shift in the E261Q mutant LCAD indicated that the LCAD mutant was still capable of binding the acyl-CoA substrate. In addition to restoring activity and spectral properties, replacement of the alanine at position 375 of the E254G mutant IVD with a glutamate has also restored the ability of the protein to transfer charge to the FAD. This is evident from the spectral scan of the E254G/A375E mutant IVD, which shows quenching of absorption at 370 and 445 nm as well as the appearance of an absorption maximum in the 600-nm region.

The loss of IVD enzymatic activity resulting from the replacement of E254 with G, Q, or D, restoration of activity of the E254G/A375E mutant IVD, evidence from the spectral data of the mutant IVDs in the presence and absence of substrate, and molecular modeling analysis strongly indicate that E254 is the catalytic residue of human IVD. The advantage of the catalytic group being in a different position in the active site in IVD and LCAD compared to all other acyl-CoA dehydrogenases (including the *Megasphaera elsdenii* butyryl-CoA dehydrogenase) is not understood. X-ray diffraction data of the substrate-bound MCAD has shown that the enzyme quaternary arrangement is a dimer of dimers and suggest that amino acid residues from one monomer participate in binding of the FAD and the substrate of the other monomer within the same dimer (Kim et al., 1993). This implies that the active sites could be in close enough proximity to interact during catalysis. Our findings raise the question of whether structural differences among the active sites of the various acyl-CoA dehydrogenases could affect enzyme activity and substrate specificity. X-ray data of IVD crystals may provide valuable information to compare the structural differences among the acyl-CoA dehydrogenases and their possible effect on reaction mechanism and substrate specificity.

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