THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 26, Issue of June 30, pp. 15545–15550, 1995
Printed in U.S.A.


# Spectroscopic Studies of the Characterization of Recombinant Human Dihydrolipoamide Dehydrogenase and Its Site-directed Mutants*

(Received for publication, April 25, 1995)

**Te-Chung Liu‡, Lioubov G. Korotchkina§¶, Susannah L. Hyatt§, Nataraj N. Vettakkorumakankav¶, and Mulchand S. Patel‡§¶‖**

*From the Departments of ‡Nutrition and §Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106 and the ¶Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York, Buffalo, New York 14214*

**In this paper, we report the overexpression and single-step purification of recombinant wild-type and site-directed mutants of human dihydrolipoamide dehydrogenase in *Escherichia coli* and detailed spectroscopic studies aimed at understanding the catalytic mechanism of this enzyme. One mutation (K37E) has been identified in a patient lacking dihydrolipoamide dehydrogenase activity and has been reported previously (Liu, T.-C., Kim, H., Arizmendi, C., Kitano, A., and Patel, M. S. (1993) *Proc. Natl. Acad. Sci. USA.* 90, 5186–5190), while the other two mutations were previously generated specifically to address the role of the active-site base (His-452) and its ion pair (Glu-457). Circular dichroic and fluorescence spectroscopic data illustrate the role of these amino acids in maintaining the structure and function of human dihydrolipoamide dehydrogenase. While mutant H452Q is severely crippled in catalysis of the physiological reaction, the reverse reaction is affected in the E457Q mutant. The K37E mutant shows very little deviation from the wild-type enzyme.**

Dihydrolipoamide dehydrogenase (*E*3[1]; EC 1.8.1.4) is a flavoprotein disulfide oxidoreductase that catalyzes the essential reoxidation of covalently linked dihydrolipoic moiety as part of the reactions catalyzed by the α-ketoacid dehydrogenase multienzyme complexes (for reviews, see Refs. 1 and 2). It is also a component of the glycine cleavage system referred to as the L-protein (3). The enzyme is a homodimer with a subunit size of ~50 kDa. Each monomer contains a tightly but noncovalently attached FAD molecule, a redox disulfide, and one $NAD^+$-binding site (1). Upon reoxidation of the dihydrolipoyl moiety, electrons are transferred from the dihydrolipoyl moiety to the redox disulfide, then to the FAD cofactor, and finally to the $NAD^+$ cofactor (1).

The reaction catalyzed by *E*3 can be divided into two half-reactions: (i) the reduction of the enzyme to the catalytically important 2-electron reduced form (*E*H2) and (ii) the reoxidation of this 2-electron reduced form to the oxidized enzyme (*E*) upon transfer of the electrons (4, 5). The catalysis also entails the participation of a histidine residue (His-452 in human *E*3), which is now believed to act as an active-site base, that is stabilized by a glutamic acid residue (Glu-457 in human *E*3) in the active site (6). The three-dimensional structures of *E*3 from *Azotobacter vinelandii* and *Pseudomonas putida* have been solved to high resolution, and these structures indicate the close proximity of these two important residues to the redox disulfide and the role played by a proline residue (Pro-453 in human *E*3) in positioning the histidine residue close to the redox disulfide (7, 8). The roles of the histidine and glutamic acid residues have been reiterated by site-directed mutagenesis of these residues in *E*3 from *Escherichia coli* and in the structurally homologous enzyme glutathione reductase from *E. coli* (9–11).

The primary structure of human *E*3 has been derived from the nucleotide sequence of the corresponding cDNA (12, 13). The amino acid sequence shows extensive similarities to *E*3 from other sources (14). Overexpression of human *E*3 in *E. coli* has also been achieved (15) and so has the site-directed modification of His-452 and Glu-457 (16). The His-452 mutant had ~0.2% of the wild-type specific activity, while the Glu-457 mutant had ~28% specific activity compared with the wild-type enzyme (16). In addition to these site-specifically altered mutants of human *E*3, two substitution mutations (P453L and K37E) have also been identified in our laboratory from an *E*3-deficient patient (17). The three-dimensional structure of *A. vinelandii E*3 indicates that Lys-37 is located in the FAD-binding site, with its main chain nitrogen atom expected to form a hydrogen bond with the adenine moiety of FAD (7). We now report the overexpression of the wild-type and three mutants of human *E*3 in high quantities in *E. coli* and the spectroscopic characterization of these proteins in order to gain more insight into the role played by these amino acids in the structure and function of *E*3.

## EXPERIMENTAL PROCEDURES

*Construction of Expression Vectors*—To achieve overexpression of human *E*3 in *E. coli*, an expression system (18) was used that contained the following: (i) *E. coli* M15 cells, (ii) a plasmid (pDMI.1) that produced the *lac* repressor protein, and (iii) the plasmid (pQE-9) containing the human *E*3 cDNA enabling a polyhistidine extension at the N-terminus of the protein. To construct the expression vector for the expression of wild-type human *E*3 in *E. coli*, the human *E*3 cDNA from the plasmid pBS-E3 was digested with *Xho*I, and the *E*3 cDNA sequence was amplified by polymerase chain reaction (PCR) using primers L1 and L2 (sense L1, 5′-GCG CGC GGA TCC GCA GAT CAG CCG ATT-3′; and antisense L2, 5′-GCG CGC GGA TCC TCA AAA GTT GAT TGA TTT GCC-3′) containing *Bam*HI sites prior the first codon at the 5′-end and subsequent to the stop codon at the 3′-end. The amplified DNA was then digested with *Bam*HI and ligated with pQE-9 that was previously digested with *Bam*HI and dephosphorylated (19). The ligation product, named pQE-9-E3, was used to transform *E. coli* M15 cells containing

* This work was supported by United States Public Health Service Grant DK42885. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Biochemistry, State University of New York, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214. Tel.: 716-829-2727; Fax: 716-829-2725.

[1] The abbreviations used are: *E*3, dihydrolipoamide dehydrogenase; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; L-protein, lipoamide dehydrogenase-protein.

TABLE I
*Purification of wild-type and mutant E3 enzymes*

Protein was calculated for a 1-liter preparation.

| Fraction | Wild-type *E3* | | K37E | | H452Q | | E457Q | |
|---|---|---|---|---|---|---|---|---|
| | Protein | Specific activity | Protein | Specific activity | Protein | Specific activity | Protein | Specific activity |
| | *mg* | *units/mg* | *mg* | *units/mg* | *mg* | *units/mg* | *mg* | *units/mg* |
| Supernatant | 380 | 34.6 | 376 | 15.7 | 715 | 1.4 | 515 | 3.4 |
| Flow-through | 248 | 3.5 | 224 | 3.4 | 583 | 1.2 | 345 | 1.6 |
| Pool 1 | 14 | 776 | 7.2 | 81 | Combined with pool 2 | | Combined with pool 2 | |
| Pool 2 | Combined with pool 1 | | 11 | 377 | 44 | 0.3 | 33 | 2.3 |

pDMI.1. The correct orientation and the sequence of *E3* in the constructed vector were confirmed by restriction analysis and dideoxynucleotide sequencing (20).

The construction and expression of the *E3* H452Q and E457Q mutants using the expression vector pOTSV have been described previously (16). To express these mutant proteins using the expression vector pQE-9, the mutant *E3* cDNA was amplified using PCR and the two primers (with *Bam*HI sites) used for the amplification of wild-type *E3*. The PCR products were then subcloned as described above. A two-step PCR strategy was used for the construction of the *E3* K37E mutant for expression using the pQE-9 plasmid. Briefly, the first step involved PCR with the mutagenic primer (5′-TGC ATT GAG GAA AAT GAA ACA) and the C-terminal primer L2. The mutant fragment generated served as a primer along with the N-terminal primer L1 to amplify the full-length mutant (K37E) *E3* cDNA. This product was digested with *Bam*HI, and the product was cloned into the expression vector pQE-9 as described above. The mutation and orientation were confirmed by dideoxy sequence analysis.

*Overexpression and Purification of Native and Mutant E3 Enzymes*—A single colony containing the plasmid pDMI.1 and the expression vector with the appropriate insert was used to inoculate 10 ml of LB medium containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). This overnight culture was used to inoculate a liter of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Cells were grown to an absorbance of 0.7 and induced with isopropyl-β-D-thiogalactopyranoside (200 μg/ml) at 30 °C overnight. The cells were harvested by centrifugation at 4000 × *g* for 20 min. The cell pellets were resuspended in 50 mM sodium phosphate (pH 8.0) containing 0.25 mM EDTA. At this stage, lysozyme was added to a final concentration of 1 mg/ml, and the suspension was stirred at 0 °C for 30 min. The cells were then lysed by French press treatment at 20,000 pounds/square inch. The unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 30 min, and the pellet was discarded. NaCl was added to a final concentration of 100 mM to the supernatant, and this was applied to a nickel-nitrilotriacetic-agarose column. The column was washed with 50 mM sodium phosphate (pH 8.0) containing 0.25 mM EDTA and 100 mM NaCl to elute unbound proteins. The adsorbed proteins were eluted by a linear 0–100 mM imidazole gradient in 50 mM sodium phosphate (pH 8.0) containing 0.25 mM EDTA and 100 mM NaCl. The recovered fractions were assayed for *E3* activity and pooled.

*Enzymatic Assays*—*E3* activity was assayed spectrophotometrically by following the reduction of $NAD^+$ at 340 nm at 37 °C in a Shimadzu UV160U recording spectrophotometer equipped with thermocontrollers as described before (16). DL-Dihydrolipoamide was synthesized according to Reed *et al.* (21). For determination of kinetic parameters, the steady-state activities were determined at seven concentrations of both dihydrolipoamide and $NAD^+$ (0.1, 0.15, 0.2, 0.3, 1.0, 3.0, and 6.0 mM), and the data were analyzed using the Graphic Fit program (Erithacus Software Ltd., Staines, United Kingdom).

*Estimation of the Molecular Size of the Proteins by High Performance Liquid Chromatography (HPLC)*—The objective of this experiment was to determine the effect of the mutation in question on the ability of the enzyme to form the dimeric structure that is required for activity. The proteins were chromatographed on a TSK SW3000G column using a Shimadzu Model L600 HPLC system interfaced to a C-R5A Chromatopac microprocessor/integrator. The mobile phase used for this procedure was 50 mM potassium phosphate (pH 8.0) containing 0.25 mM EDTA and 0.5% trifluoroacetic acid. The elution of the protein was detected by monitoring the absorbance of the effluent at 220 nm using a UV-visible spectrophotometric detector.

*Determination of the FAD Content of Wild-type and Mutant E3 Enzymes*—The protein (0.5–1 mg/ml) was denatured by boiling in a water bath for 5 min. The denatured proteins were removed by centrifugation, and the supernatants were scanned from 220 to 500 nm using a Shimadzu UV160U UV-visible recording spectrophotometer. The absorbance at 448 nm was used for quantitation of FAD using an extinction coefficient of 11,300 $M^{-1}$ $cm^{-1}$. The standard curve using known concentrations of FAD was obtained after its boiling for 5 min.

TABLE II
*Steady-state kinetic parameters of wild-type E3 and K37E*

| Protein | $K_{cat}$ | $K_m$ | |
|---|---|---|---|
| | | $NAD^+$ | DHL[a] |
| | $s^{-1}$ | *mM* | |
| *E3*[b] | 421 | 0.32 | 0.69 |
| H452Q[b] | 0.77 | 0.38 | 43.6 |
| E457Q[b] | 118 | 0.32 | 2.9 |
| K37E | 315 | 0.25 | 0.76 |

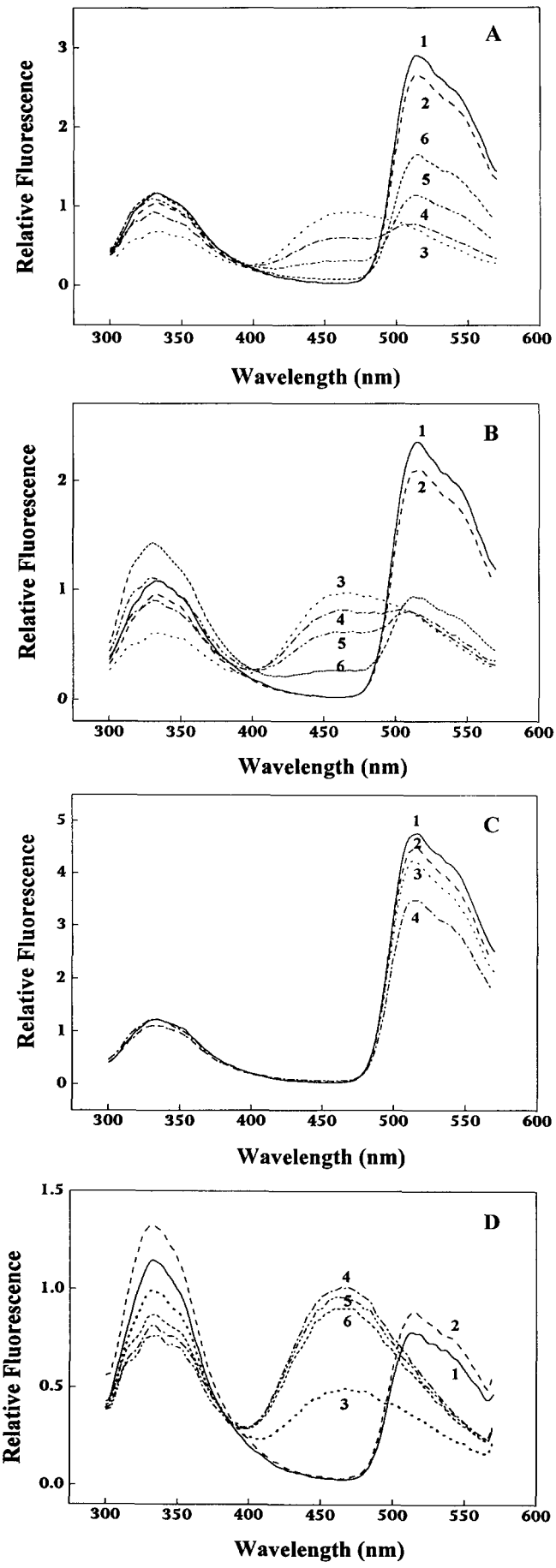
[a] DHL, DL-dihydrolipoamide.
[b] From Kim and Patel (16).

*Fluorescence Analysis of Wild-type and Mutant E3 Enzymes*—The fluorescence spectra were recorded using a Photon Technology International, Inc. spectrofluorometer. The protein solution (1 μM in 50 mM sodium phosphate) was excited at 296 nm, and the emission pattern was recorded from 300 to 560 nm. The influence of $NAD^+$ on the fluorescence spectrum was monitored by the addition of $NAD^+$ to a final concentration of 50 μM and subsequent incubation for 5 min. The effect of dihydrolipoamide on the above was monitored after the addition of this substrate to a final concentration of 250 μM at regular time intervals (0, 5, 10, and 30 min). The data were transferred to an ASCII file using the PTI program, and the spectra were drawn using the original program (Photon Technology International, South Brunswick, NJ).

*Circular Dichroism*—The CD spectra of native and mutant *E3* enzymes were determined in a Jasco J-600 spectropolarimeter. The protein solutions (1 μM) were scanned from 190 to 250 nm and from 300 to 600 nm. CD spectra were also generated in the 300–600 nm region after reduction of the enzyme with dihydrolipoamide (final concentration of 5 mM) or with NADH (final concentration of 0.5 mM). The reoxidation of the NADH-reduced enzyme with lipoamide (final concentration of 5 mM) and of the dihydrolipoamide-reduced enzyme with $NAD^+$ (final concentration of 0.5 mM) was also studied in the 300–600 nm range. The data were transferred to an ASCII file using the software supplied by Jasco, and the spectra were generated using the original program (MicroCal Software Inc., Northampton, MA).

## RESULTS

*Overexpression of E3 and Its Mutants*—The purification of overexpressed human *E3* was a one-step procedure that involved chromatography on a nickel-nitrilotriacetic-agarose column. This is a significant improvement over the previous purification strategy, which involved multiple chromatographic and heat treatment steps. The purification data obtained from typical purifications from 1-liter bacterial cell cultures for *E3*, K37E, H452Q, and E457Q are shown in Table I. It is apparent that this particular expression vector expressed a significant amount of protein. It was possible to obtain 14 mg of human recombinant *E3* from a 1-liter preparation with >98% purity (based on densitometry analyses of SDS-polyacrylamide gel electrophoresis) (data not shown). Similar purity and yields




were achieved for H452Q and E457Q (data not shown). The specific activity of the preparation is considerably increased from our previous overexpression and purification (15, 16). For example, the specific activity of wild-type *E*3 using the current protocol is 776 units/mg of protein and there is a 80% recovery of enzymatic activity compared with a specific activity of 407 units/mg and a yield of 36% (15). The overexpression and purification of the *E*3 K37E mutant has not been described previously. The kinetic parameters for wild-type *E*3 and K37E are illustrated in Table II. The values obtained for wild-type *E*3 are comparable to values previously determined (15). The kinetic parameters for the *E*3 H452Q and E457Q mutants have been reported elsewhere (16). Wild-type human *E*3 and all the mutant proteins showed a dimeric structure during gel filtration using HPLC (data not shown).

*FAD Content of Mutant E3 Enzymes*—To measure the FAD content, the protein solutions were boiled and clarified as described above. The UV-visible absorption spectra of the supernatants were determined in the 220–500 nm range, and the absorption at 448 nm was used to quantify the FAD content of the enzyme. The molar ratios of FAD to *E*3 were as follows: wild-type *E*3 = 1; K37E = 0.76; H452Q = 0.94; and E457Q = 0.9. It is evident that the substitutions affecting H452Q or E457Q had very little effect on the FAD content of the enzyme, while the K37E mutation affected FAD binding significantly.

*Fluorescence Analysis*—Excitation at 296 nm excites tryptophan residue(s) in *E*3, which emits the absorbed energy at 330 nm. This emitted energy is transferred to the FAD cofactor, which in turn emits at 510 nm. When wild-type *E*3 is reduced with dihydrolipoamide, there is no effect on the 330 nm emission, while the 510 nm emission is reduced as the FAD molecules become reduced. When $NAD^+$ is added to *E*3, the peak at 510 nm is decreased slightly, and upon the addition of dihydrolipoamide, the 510 nm emission is decreased significantly as the flavin is reduced, with the concomitant appearance of a 460 nm emission. This emission represents the formation of NADH as a result of oxidation of dihydrolipoamide by *E*3. The 460 nm emission disappears with time and is completely gone by 30 min, and the FAD fluorescence is restored concomitantly, although not to the original intensity seen for oxidized *E*3 at the beginning of the experiment. The disappearance of the NADH emission probably reflects the dissociation of NADH from *E*3. The fact that the FAD fluorescence is not restored to the original intensity indicates that the enzyme can be re-reduced by the NADH so generated and the establishment of an equilibrium between the oxidized enzyme and re-reduced enzyme species. The fluorescence changes in wild-type human *E*3 upon the addition of $NAD^+$ and dihydrolipoamide are illustrated in Fig. 1*A*. Similar experiments with the *E*3 K37E mutant (Fig. 1*B*) illustrate that the environment around the FAD cofactor is altered. This is evident from the fact that the intensity of the flavin fluorescence is only ~80% of wild-type *E*3. Apart from this difference, the fluorescence changes for this mutant are similar to those for wild-type *E*3, indicating that this mutation did not affect the catalytic mechanism of the enzyme.

The fluorescence emission spectrum of the *E*3 H452Q mutant is shown in Fig. 1*C*. The spectra generated indicate that

FIG. 1. **Fluorescence spectral changes of wild-type human *E*3 (*A*), K37E (*B*), H452Q (*C*), and E457Q (*D*) upon reduction with dihydrolipoamide in the presence of $NAD^+$.** Treatments were as follows: *trace 1*, none; *trace 2*, *E*3 plus 50 μM $NAD^+$; *trace 3*, 50 μM $NAD^+$ and 250 μM dihydrolipoamide; *trace 4*, 50 μM $NAD^+$ and 250 μM dihydrolipoamide after 5 min; *trace 5*, 50 μM $NAD^+$ and 250 μM dihydrolipoamide after 10 min; *trace 6*, 50 μM $NAD^+$ and 250 μM dihydrolipoamide after 30 min.

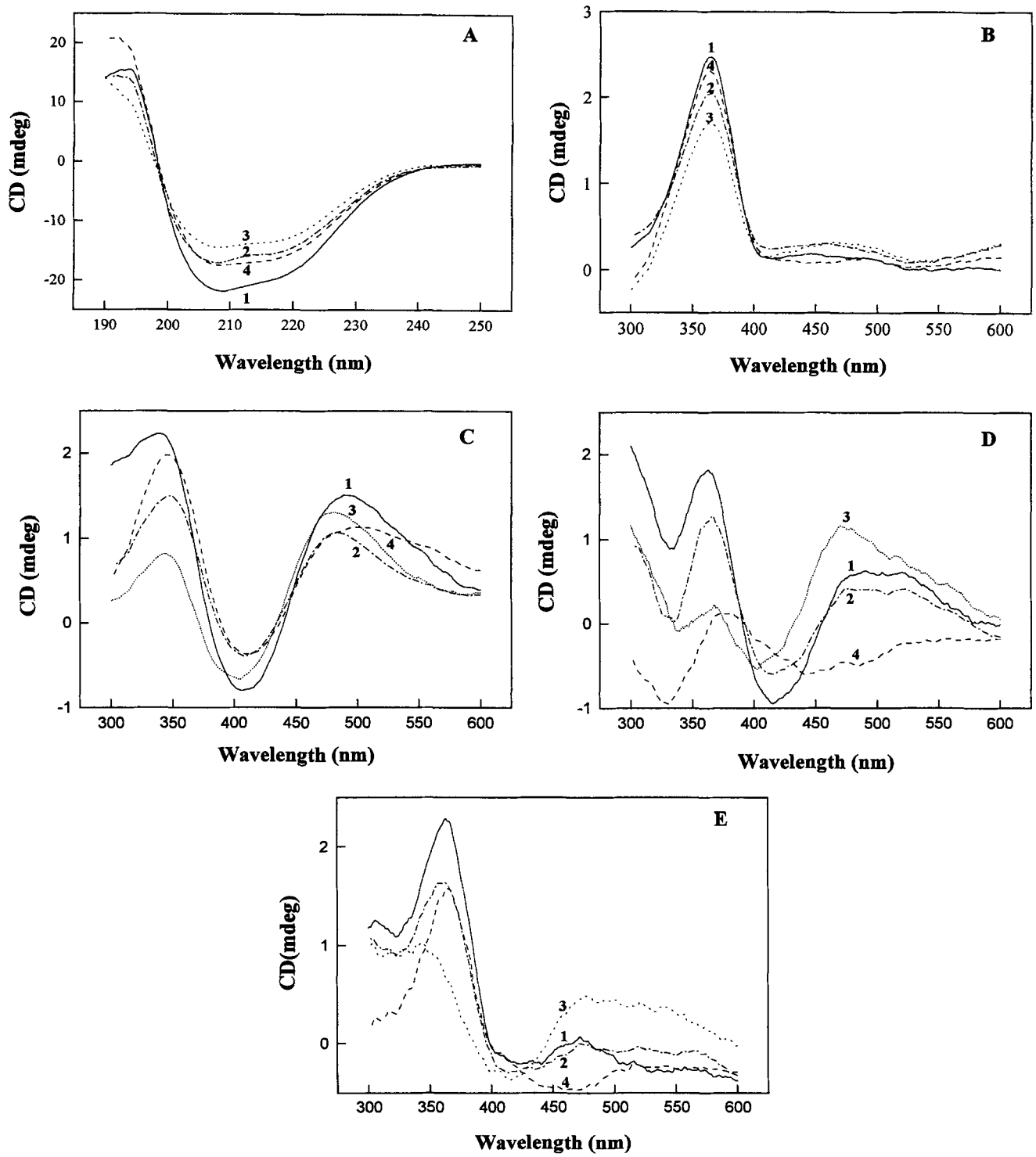



FIG. 2. **Circular dichroism spectra of wild-type and mutant *E*3 enzymes in the presence or absence of dihydrolipoamide or NADH in the 190–250 nm range (*A*), 300–600 nm range (*B*), 300–600 nm range upon the addition of dihydrolipoamide (*C*), 300–600 nm range upon the addition of NADH (*D*), and 300–600 nm range upon the addition of NADH and lipoamide (*E*) for wild-type human *E*3 (*trace 1*), K37E (*trace 2*), E457Q (*trace 3*), and H452Q (*trace 4*).** *mdeg*, millidegrees.

when this mutant *E*3 was reduced by dihydrolipoamide in the presence of $NAD^+$, there was no production of NADH as indicated by the absence of a peak at 460 nm. There was a slow reduction of the FAD peak at 510 nm. These results indicate the necessity of His-452 for the redox cycle catalyzed by this enzyme. For E457Q, the FAD fluorescence was markedly reduced (Fig. 1*D*), while the NADH formation proceeded without any significant change. However, the disappearance of the NADH peak was significantly slower than for the wild-type enzyme. These results suggest that the removal of Glu-457 affects the environment surrounding FAD, as evidenced by the decreased FAD fluorescence, and alters the affinity for $NAD^+$/NADH, as evidenced by the very slow disappearance of the NADH fluorescence.

*Circular Dichroism Spectra*—CD spectra were generated for all the proteins in the 190 to 250 nm and the 300 to 600 nm

ranges. The region of 190 to 250 nm is indicative of the secondary structure of the proteins, while the 300 to 600 nm range is indicative of the protein-FAD interactions. A comparison of the near- and far-UV CD spectra of the four different proteins is illustrated in Fig. 2. The 190 to 250 nm region (Fig. 2*A*) indicates varying degrees of structural perturbations depending on the mutation in question. The CD spectra in the 300 to 600 nm region (Fig. 2*B*) were quite similar for all the proteins. However, varying degrees of changes in the protein-FAD interactions can be observed for these mutants as evidenced by the intensities of the peak at 350 nm. The CD spectra of the *E*3 E457Q and K37E mutants are consistent with fluorescence data, indicating that these substitutions alter the environment of the FAD molecule.

In the CD spectra, the peak at 370 nm corresponds to the second absorption band of FAD, while the bands at 465 and 490 nm arise from vibronic transitions of the first absorption band (22). Upon the addition of dihydrolipoamide to the enzyme solutions, few changes were observed in the features of the CD spectra for the mutant proteins when compared with the wild-type enzyme (Fig. 2*C*). However, when the proteins were reduced with NADH, significant changes were observed (Fig. 2*D*). The CD spectra for the wild-type enzyme and the *E*3 K37E mutant are nearly identical, reiterating the similarity of these two proteins. For the *E*3 H452Q mutant, the vibronic transitions at the 465 and 490 nm regions disappeared almost completely, and the intensity of the 370 nm band was markedly reduced. This may reflect the reduction of the FAD molecule with greater ease by NADH as a result of the removal of the histidine base that would normally have stabilized the charge transfer interaction between the thiolate anion and the FAD cofactor (1). This modification probably results in a change of the redox potential of the FAD moiety of this protein. For the *E*3 E457Q mutant, the intensities of the peaks at 465 and 490 nm in the CD spectra are increased when compared with the other proteins. The spectra generated upon the addition of lipoamide to the NADH-reduced enzymes are illustrated in Fig. 2*E*. For the wild-type and *E*3 K37E mutant enzymes, the addition of lipoamide to the NADH-reduced enzyme restores the spectra of the oxidized enzyme, suggesting that catalysis proceeds normally. Virtually no changes are observed for the *E*3 H452Q mutant compared with the spectra of the NADH-reduced form of this protein. This illustrates the importance of this residue for the proper transfer of electrons from the reduced FAD molecule to the oxidized lipoamide. For the *E*3 E457Q mutant, the peaks at 465 and 490 nm decreased in intensity, suggesting a decrease in efficiency of electron transfer from the reduced flavin to the oxidized substrate.

## DISCUSSION

We have investigated the role of two catalytically important amino acids in human *E*3 and studied the effects of one substitution mutation found in a patient with *E*3 deficiency on the structure and function of this flavoenzyme. We have used molecular sieving and various spectroscopic techniques and estimated the FAD content of these proteins in order to obtain information on the effect of these mutations on *E*3 structure and function. These studies extend the information previously obtained on two active-site mutations, H452Q and E457Q (16). We have also improved the expression and purification of human *E*3 enzymes manufactured in *E. coli*, and the purification process now involves a single chromatographic step as opposed to the multiple purification steps required previously. The yield of purified *E*3 is very high compared with that obtained previously (15). We have also overexpressed and studied mutant K37E. This represents one of the two mutations identified in a patient lacking *E*3 activity. Mechanistic studies were also carried out on this mutant protein.

The K37E mutation causes few changes in the structure and function of *E*3, although it does affect the amount of FAD associated with the protein. Lys-37 is involved in a hydrogen bonding interaction with the adenine moiety of the FAD cofactor (7), and therefore, it is plausible that a mutation affecting this interaction would decrease the affinity for FAD. This substitution mutation is unique in that this has not been described previously in *E*3 from any other source.

Mutations affecting the catalytically important histidine and its charge transfer partner glutamate have been described before (23), and the conclusions are quite clear. His-452 is important for the stabilization of the thiolate anion involved in the charge transfer complex and is also important for the abstraction of a proton from the dihydrolipoamide substrate, thus activating the same for a nucleophilic attack on the redox disulfide of the enzyme (1). Fluorescence studies described in this study with the *E*3 H452Q mutant indicate that the transfer of electrons from the substrate dihydrolipoamide to the nicotinamide cofactor $NAD^+$ is extremely slow. This observation supports the role of His-452 in the stabilization of the thiolate-FAD charge transfer complex, which is important for the transfer of electrons. Also, the extent of reduction of the FAD cofactor is markedly reduced in this mutant, indicating the necessity of His-452 in the initial abstraction of a proton from the substrate to facilitate the formation of the charge transfer complex. In the predicted structural model of human *E*3, His-452 is in a position to interact electrostatically with Glu-457 (24). Substitution of this residue with glutamine affects the environment of the FAD cofactor as evidenced by the decreased fluorescence of the cofactor. The rate of formation of NADH as observed by fluorescence spectroscopy was not significantly altered. However, the eventual disappearance of this NADH was significantly slower than that for the wild-type enzyme. Thus, the substitution of Gln for either His-452 or Glu-457 alters the affinity of the enzyme for NADH. It is also possible that the reverse reaction, *i.e.* the reduction of the FAD/redox disulfide in this mutant, is inhibited because of the absence of the stabilizing interaction between His-452 and Glu-457. Our CD results provide further evidence supporting this observation. Determination of rates for individual reductive and oxidative half-reactions will prove useful in extending the present studies on the effect of these mutations on *E*3 and in supporting existing roles for these amino acids in *E*3 from bacterial systems.

*Acknowledgments*—We thank Dr. Joyce Jentoft (Case Western Reserve University) for critical reading of this manuscript. We are grateful to Dr. Karl A. Koehler (Departments of Biochemistry and Surgery, Case Western Reserve University) for providing a fluorometer and software for analysis.

## REFERENCES

1. Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) Vol. III, pp. 121–211, CRC Press, Inc., Boca Raton, FL
2. Patel, M. S., and Roche, T. E. (1990) *FASEB J.* **4,** 3224–3233
3. Kikuchi, G., and Hiraga, K. (1982) *Mol. Cell. Biochem.* **45,** 137–149
4. Massey, V., Gibson, Q. H., and Veeger, C. (1960) *Biochem. J.* **77,** 341–351
5. Massey, V., and Veeger, C. (1961) *Biochim. Biophys. Acta* **48,** 33–47
6. Thieme, R., Pai, E. F., Schirmer, R. H., and Schulz, G. E. (1981) *J. Mol. Biol.* **152,** 763–782
7. Mattevi, A., Schierbeek, A. J., and Hol, W. G. J. (1991) *J. Mol. Biol.* **220,** 975–994
8. Mattevi, A., Obmolova, G., Sokatch, J. R., Betzel, C., and Hol, W. G. J. (1992) *Proteins Struct. Funct. Genet.* **13,** 336–351
9. Allison, N., Williams, C. H., Jr., and Guest, J. R. (1988) *Biochem. J.* **256,** 741–749
10. Berry, A., Scrutton, N. S., and Perham, R. N. (1989) *Biochemistry* **28,** 1264–1269
11. Deonarain, M. P., Berry, A., Scrutton, N. S., and Perham, R. N. (1989) *Biochemistry* **28,** 9602–9607
12. Pons, G., Raefsky-Estrin, C., Carothers, D. J., Pepin, R. A., Javed, A. A., Jesse, B. W., Ganapathy, M. K., Samols, D., and Patel, M. S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1422–1426

13. Otulakowski, G., and Robinson, B. H. (1987) *J. Biol. Chem.* **262,** 17313–17318
14. Carothers, D. J., Pons, G., and Patel, M. S. (1989) *Arch. Biochem. Biophys.* **268,** 409–425
15. Kim, H., Liu, T.-C., and Patel, M. S. (1991) *J. Biol. Chem.* **266,** 9367–9373
16. Kim, H., and Patel, M. S. (1992) *J. Biol. Chem.* **267,** 5128–5132
17. Liu, T.-C., Kim, H., Arizmendi, C., Kitano, A., and Patel, M. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5186–5190
18. LeGrice, S. F. J., and Gueninger-Leitch, F. (1990) *Eur. J. Biochem.* **187,** 307–314
19. Sambrook, J., Maniatis, T., and Fritsch, E. F. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
21. Reed, L. J., Koike, M., Levitch, M. E., and Leach, F. R. (1958) *J. Biol. Chem.* **232,** 143–158
22. Benen, J., Berkel, W. V., Zak, Z., Visser, T., Veeger, C., and deKok, A. (1991) *Eur. J. Biochem.* **202,** 863–872
23. Benen, J., Berkel, W. V., Dieteren, N., Arscott, D., Williams, C. H., Jr., Veeger, C., and deKok, A. (1992) *Eur. J. Biochem.* **207,** 487–497
24. Jentoft, J. E., Shoham, M., Hurst, D., and Patel, M. S. (1992) *Proteins Struct. Funct. Genet.* **14,** 88–101