Glutamate 170 of Human L-3-Hydroxyacyl-CoA Dehydrogenase Is Required for Proper Orientation of the Catalytic Histidine and Structural Integrity of the Enzyme*

Received for publication, May 26, 2001, and in revised form, July 11, 2001
Published, JBC Papers in Press, July 12, 2001, DOI 10.1074/jbc.M104839200

Joseph J. Barycki‡, Laurie K. O’Brien§, Arnold W. Strauss§, and Leonard J. Banaszak‡¶

From the ¶Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455 and the §Department of Pediatrics, Vanderbilt Medical Center, Nashville, Tennessee 37232

L-3-Hydroxyacyl-CoA dehydrogenase (HAD), the penultimate enzyme in the β-oxidation spiral, reversibly catalyzes the conversion of L-3-hydroxyacyl-CoA to the corresponding 3-ketoacyl-CoA. Similar to other dehydrogenases, HAD contains a general acid/base, His158, which is within hydrogen bond distance of a carboxylate, Glu170. To investigate its function in this catalytic dyad, Glu170 was replaced with glutamine (E170Q), and the mutant enzyme was characterized. Whereas substrate and cofactor binding were unaffected by the mutation, E170Q exhibited diminished catalytic activity. Protonation of the catalytic histidine did not restore wild-type activity, indicating that modulation of the pKa of His158 is not the sole function of Glu170. The pH profile of charge transfer complex formation, an independent indicator of active site integrity, was unaltered by the amino acid substitution, but the intensity of the charge transfer band was diminished. This observation, coupled with significantly reduced enzymatic stability of the E170Q mutant, implicates Glu170 in maintenance of active site architecture. Examination of the crystal structure of E170Q in complex with NAD+ and acetoacetyl-CoA (R = 21.9%, Rfree = 27.8%, 2.2 Å) reveals that Glu170 no longer hydrogen bonds to the side chain of His158. Instead, the imidazole ring is nearly perpendicular to its placement in the comparable native complex and no longer positioned for efficient catalysis.

L-3-Hydroxyacyl-CoA dehydrogenase (HAD); EC 1.1.1.35) is the penultimate enzyme in the β-oxidation spiral, reversibly catalyzing the oxidation of the hydroxyl group of L-3-hydroxyacyl-CoA to a keto group, concomitant with the reduction of NAD+ to NADH, as shown in Scheme 1.

The dimeric enzyme displays broad substrate specificity, utilizing substrates with 4–16 carbons in the acyl chain (1). Hydroxide transfer occurs at the pro-S position of the nicotinamide ring, making HAD a “B-side”-specific dehydrogenase (2). As illustrated, His158 is thought to serve as a general acid/base in the catalytic mechanism of HAD, with catalysis facilitated by the presence of a conserved glutamate residue, Glu170 (3–6). However, the precise role of Glu170 in catalysis has not been established.

Crystallographic studies of HAD have shown that Glu170 is located adjacent to His158 in the enzyme active site (5, 6). The backbone carboxyl and amide groups of Glu170 are positioned to form hydrogen bonds with the backbone amide and carbonyl groups of His158, respectively. In addition, a carbonylate oxygen of Glu170 is within hydrogen bond distance of ND1 of His158 as depicted in Scheme 1. However, Glu170 also forms contacts that may be important for dynamic enzyme movements. HAD exhibits a two-domain topology (7), with the N-terminal domain (residues 12–200) adopting a β-α-β fold similar to NAD(P)−-binding enzymes and the C-terminal domain (residues 201–302) consisting primarily of α-helices involved in subunit dimerization. Two distinct conformers of the enzyme structure have been identified (5, 6). In the open conformation, as described for apo and cofactor-bound enzyme, a large cleft is observed between the NAD−-binding and the C-terminal domains. The addition of substrate results in a conformational change in which the NAD−-binding domain rotates inward toward the dimer interface, sequestering the enzyme active site. This domain shift appears necessary for high affinity substrate binding and critical for effective catalysis. The linker region, composed of residues 201–207, relates the two domains and contains a consensus Pro203-Gly204-Phε205 sequence, which appears to be the pivot point for domain movement. Numerous interactions between Glu170 and residues within this region are observed, including a hydrogen bond with a conserved water molecule.

Charge transfer complex formation by HAD provides a spectroscopic assay for structural integrity that can be used to complement binding studies and kinetic analysis. As described previously, the abortive ternary complex composed of HAD, NADH−, and AACoA exhibits a broad absorbance band centered between 410 and 420 nm (6). AACoA, which is bound as an enolate in the abortive complex, acts as an electron donating species and the nicotinamide ring of NADH− serves as the electron acceptor. The intensity of the charge transfer band is pH-dependent, with the protonation of a single group resulting in decreased enolate and charge transfer complex formation. The spectroscopic properties of the charge transfer complex are sensitive to perturbations in the protein structure and can be used to probe active site integrity.

To evaluate the contribution of Glu170 to catalysis, this residue was substituted with glutamine by site-directed mutagenesis, and the resultant enzyme (E170Q) was analyzed by ki-
Importance of Glu$^{170}$ in L-3-Hydroxyacyl-CoA Dehydrogenase

Results

Protein Expression and Initial Characterization of E170Q—The E170Q mutant enzyme was overexpressed in E. coli and purified to homogeneity. E170Q protein yields were ~4-fold lower than wild-type enzyme, with 15–20 mg produced per liter of cell culture. In addition, E170Q appeared to be less stable than native enzyme. Visible precipitate was observed in the concentrated E170Q stock solution (~10 mg/ml) after 5–7 days when stored at 4°C; native enzyme was stable indefinitely under similar conditions. In addition, the specific activity of the E170Q protein decreased ~10% over this time period, while that of native enzyme remained unchanged. However, initial specific activity values for the E170Q enzyme could be restored by removal of the precipitated protein. As observed with wild-type protein, uncleaved and thrombin-cleaved E170Q exhibited similar levels of enzyme activity, cofactor affinity, and substrate binding (data not shown), indicating that the N-terminal histidine tag did not significantly alter enzyme structure or function.

A comparison of native and E170Q kinetic parameters is provided in Table I. The concentration of acetoacetyl-CoA was varied at a saturating NADH concentration (100 μM), with initial rates determined in triplicate. Data were then fit to the Michaelis-Menten equation to obtain $V_{\text{max}}$ and $K_m$ for NADH by the reciprocal experiments. However, as judged by fluorescence quenching experiments (5), E170Q bound reduced cofactor with equal or slightly greater affinity than native enzyme; $K_m$ values were typically less than 1 μM (data not shown). The E170Q mutation did not alter substrate binding as evidenced by comparable $K_m$ values for native and radiated produced by a Rigaku RU-200BH rotating anode fitted with an Osnic MaxFlux confocal multilayer fixed focus optical system and an R-AXIS IV++ image plate system. Crystals were maintained under cryogenic conditions by an X-stream cooling system, and data were analyzed with the Crystal Clear software package (10) (Molecular Structure Corp.). The structure of the E170Q abortive ternary complex was isomorphous with the corresponding wild-type complex (Protein Data Bank accession code 1F0Y).

Model Building and Refinement—The E170Q model was refined using the crystallography and NMR system CNS, version 0.3 (11) and rebuilt after each round of refinement using the program O (12), as described previously (5). Ambiguous regions of the electron density map were evaluated using 2$F_o$−$F_c$ simulated annealing omit maps, in which designated regions of the structure were omitted and the remaining model was subjected to simulated annealing prior to map calculation. Water molecules obeying proper hydrogen bonding constraints with electron densities greater than 1.0 σ on a 2$F_o$−$F_c$ map and 4.0 σ on an $F_o$−$F_c$ map were included as model refinement near completion. Bound substrate and cofactor were also added at this point using coordinates from the analogous wild-type structure. Note that the positions of O3 and C4 of acetoacetyl-CoA could not be assigned unambiguously in the electron density. The current models have O3 positioned within hydrogen bonding distance of Nε2 of His$^{156}$, the proposed catalytic base. Similarly, the orientations of His$^{156}$, Glu$^{170}$, and Asp$^{199}$ side chains cannot be determined unequivocally and are modeled such that potential hydrogen bond interactions are maximized. Coordinates and structure factors for the E170Q model have been deposited in the Protein Data Bank under the accession code 1IL0.

Table I

|                   | $V_{\text{max}}$ (μmol/min/mg) | $K_m$ (μM) |
|-------------------|-------------------------------|------------|
| Native            | 500                           | 14.8 ± 1.26 |
| E170Q             | 783                           | 16.9 ± 1.63 |

References

1. Cleland WW. Kinetic analysis of enzymes and enzyme mechanisms. 2nd ed. San Diego: Academic Press; 1997.

2. Noyes PM, Bradshaw J. L-3-Hydroxyacyl-CoA dehydrogenase. Methods Enzymol 1977;49:82–95.

3. Asp$^{199}$ side chains cannot be determined unequivocally and are modeled such that potential hydrogen bond interactions are maximized. Coordinates and structure factors for the E170Q model have been deposited in the Protein Data Bank under the accession code 1IL0.
**Importance of Glu\textsuperscript{170} in L-3-Hydroxyacyl-CoA Dehydrogenase**

Initial rates were measured at a saturating concentration of NADH cofactor (100 μM) with ACoA as the varied substrate. At an indicated pH, a substrate saturation curve was determined in triplicate, and the data were fit to the Michaelis-Menten equation to yield values for \(V_{\text{max}}\) and \(K_m\). Apparent pK\(_a\) values were obtained by fitting the appropriate equation to the observed data, as described under “Experimental Procedures,” and are summarized in Table II. A, \(\log(V_{\text{max}}/K_m)\) versus pH for native (■) and E170Q (●) HAD. B, \(\log(V_{\text{max}})\) versus pH for native (■) and E170Q (●) HAD. C and D illustrate the pH dependence of ACoA binding (log 1/K\(_m\)) for native and E170Q enzyme, respectively.

**pH Profiles of Kinetic Parameters**—By analogy to other dehydrogenases (13–15), a potential role of Glu\textsuperscript{170} is to elevate the apparent pK\(_a\) of His\textsuperscript{158}, rendering the enzyme-catalyzed reaction more reversible at neutral pH. The E170Q mutation would therefore be predicted to alter the pH dependence of a given kinetic parameter. \(V_{\text{max}}\) and \(K_m\) values were determined as described above for native and mutant enzymes over the pH range of 5.0–8.0 in 0.2 increments. Steady-state kinetic parameters are presented as log values versus pH (Fig. 1), with the data fit according to the methodology of Cleland (9) to obtain apparent pK\(_a\) values (Table II). Catalytic efficiency (\(V_{\text{max}}/K_m\)) was pH-independent for native enzyme in the neutral pH range (Fig. 1A), with decreased enzyme efficiency in the acidic region of the curve indicative of a single ionizable group with an apparent pK\(_a\) of 5.14 ± 0.53 (Table II). In contrast, the pH dependence of E170Q enzyme activity fit to a bell-shaped curve described by two ionization events. The first (pK\(_{a1}\) = 5.18 ± 0.57) was comparable with wild-type enzyme, and the second (pK\(_{a2}\) = 6.80 ± 0.45) accounted for the basic limb of the curve.

To assign the observed pK\(_a\) values to a binding event or to catalysis, individual kinetic parameters were examined. A plot of \(\log V_{\text{max}}\) versus pH (Fig. 1B) revealed that catalysis by E170Q was dependent on a single ionizable group with an apparent pK\(_a\) of 6.69 ± 0.12, comparable with the value of 6.8 obtained from the \(V_{\text{max}}/K_m\) analysis (Table II). In contrast, native enzyme turnover appeared to be essentially pH-independent. A slight decrease in \(V_{\text{max}}\) was seen in the basic pH range (>7.0), but this observation was attributable to underestimation of \(V_{\text{max}}\) due to a considerable product inhibition. This effect was not observed in the acidic range, reducing the calculated initial rates. Native and E170Q enzymes exhibited nearly identical pK\(_a\) values for the hinge region of the protein (Table II). These values are in agreement with those obtained from the \(V_{\text{max}}/K_m\) analysis.

**Comparison of Native HAD and E170Q Charge Transfer Complexes**—A charge transfer complex composed of HAD, NAD\(^+\), and ACoA has been described (6), and its spectroscopic properties were used to prove the integrity of the E170Q active site. Difference spectroscopy experiments were performed in which the sum of the individual spectra of the components was subtracted from the spectrum of the charge transfer complex. Both native and mutant enzymes displayed similar difference spectra with a sharp peak at 302 nm, corresponding to the enolate species of ACoA, and a broad peak centered between 410 and 420 nm, indicative of charge transfer (data not shown). The topologies of the two spectra were nearly identical, suggesting that key components involved in charge transfer complex formation were not dramatically perturbed by the E170Q mutation. However, the E170Q difference spectrum was uniformly reduced in magnitude (~3-fold relative to that of the native enzyme, indicating generally diminished enolate formation. This could result from lower affinity of the protein for ACoA or from altered positioning of key residues. The former was discounted by the observation that a 5-fold increase of ACoA concentration did not produce an increase in the intensity of the charge transfer band.

To further investigate the possible structural effects resulting from the E170Q mutation, the pH dependence of enolate formation by native and mutant HAD was assessed indirectly by measuring charge transfer complex formation as a function of pH (Fig. 2). As observed in the difference spectroscopy measurements, native and E170Q charge transfer bands exhibited an ~3-fold difference in magnitude at a given pH. However, a similar pH transition for charge transfer complex formation was observed for both native and mutant enzymes. Charge transfer band intensities were plotted as a function of pH and fit to a sigmoidal curve, yielding a single transition at pH 5.25 ± 0.02 and 5.33 ± 0.08 for native and E170Q enzymes, respectively. Values for this transition are, furthermore, in agreement with pK\(_a\) values for ACoA binding obtained by kinetic measurements.

**Assessment of Protein Stability**—Decreased stability of the E170Q mutant protein was suggested by the formation of precipitate upon storage of the enzyme, as described above. This observation, along with previous structural characterizations, suggested that Glu\textsuperscript{170} may function to maintain active site structure, prompting a detailed investigation of relative protein stability. An examination of tryptophan fluorescence as a function of guanidine hydrochloride concentration indicated a sharp transition at low denaturant concentration (~0.75 M) for native enzyme (data not shown). Significant stability differences between native and E170Q HAD could not be detected by this method, possibly because of the location of the single tryptophan residue of the enzyme; Trp\textsuperscript{295} is positioned at the interface of the NAD\(^+\)-binding and C-terminal domains near the hinge region of the protein. This region has been demonstrated to undergo significant conformational changes in response to substrate binding and may be more susceptible to

**Table II**

| pK\(_a\) | \(V_{\text{max}}/K_m\) | \(V_{\text{max}}\) pK\(_a\) | 1/K\(_m\) pK\(_a\) |
|---|---|---|---|
| Native | 5.14 ± 0.53 | — | — |
| E170Q | 5.18 ± 0.57 | 6.80 ± 0.45 | 6.69 ± 0.12 | 5.43 ± 0.29 |

*Native enzyme does not display a basic limb (Fig. 1A).*

The slight decrease in catalytic activity observed at pH values greater than 7 (Fig. 1B) is attributable to strong product inhibition and therefore underestimation of \(V_{\text{max}}\).
disruption by chemical denaturants. Hence, tryptophan quenching may not be a reliable probe for overall structural integrity. Therefore, an alternative measure of protein stability was developed. Native and E170Q proteins (1 mg/ml, pH 7.0) were incubated at 37 °C, and enzyme activity was monitored over time. As shown in Fig. 3, native enzyme activity (squares) was completely retained over the time course, whereas the E170Q activity (circles) declined with a half-life of ~2 h. Furthermore, loss of enzyme activity was accompanied by precipitation of the E170Q protein, indicating reduced structural stability of the mutant relative to native HAD.

X-ray Diffraction Studies and Model Refinement—Structural studies of the E170Q mutant were undertaken to aid in the interpretation of the biochemical observations and provide a molecular model of catalysis by the mutant enzyme. X-ray diffraction data and model refinement statistics are summarized in Table III. E170Q enzyme crystals belonged to space group P2₁2₁2₁ and were isomorphous with those of the previously determined HAD abortive ternary complex. A nearly complete data set was collected with diffraction data extending beyond 2.2 Å. To minimize model bias, a simulated annealing test set of 5% of the total reflections was used. An R_free test set of 5% of the total reflections was used.

Comparison of Native and E170Q L-3-Hydroxyacyl-CoA Dehydrogenase Structures—The E170Q enzyme structure was compared with the analogous wild-type HAD model to assess the effects of the mutation on protein structure. Overall, the E170Q and native models are quite similar with a root mean square deviation for α-carbons of 0.46 Å and overall B-factors of 29.9 Å² and 21.5 Å², respectively. However, subtle differences between the two models are observed throughout the active site region. This region of the final E170Q model (dark gray) and its corresponding 2 | Fobs | - | Fcal | omit map density is superimposed upon the analogous wild-type structure (light gray) in Fig. 4. As illustrated, the electron density map supports an E170Q model in which the imidazole ring of His158 has rotated ~90° relative to its placement in the native HAD structure. In addition, the loop containing the E170Q mutation has retreated slightly from the enzyme active site.

The position of the His158 side chain was the most dramatic difference observed between the native and E170Q structures. Shown in Fig. 5 are the native (Fig. 5a) and E170Q (Fig. 5b) enzyme active sites, with relevant interatomic distances of <3.5 Å illustrated as dashed lines; actual distances are summarized in Table IV. Instead of hydrogen bonding with the side chain of Glu170 in a fashion similar to wild-type protein, the imidazole ring of His158 is rotated ~90° in the E170Q model.

**Table III**

| Parameter | Value |
|-----------|-------|
| Space group | P2₁2₁2₁ |
| Cell dimensions | a (Å) 49.66, b (Å) 87.72, c (Å) 158.40 |
| Resolution (Å) | 30–2.2 |
| Percentage complete (highest shell) | 99.8% (99.3%) |
| Ramachandran geometry | Most favored 88.5%, Allowed 10.9%, Generously allowed 0.4%, Disallowed 0.2% |
| r.m.s. deviation bond lengths | 0.007 Å |
| r.m.s. deviation angles | 1.7° |
| r.m.s. deviation dihedral | 20.9° |
| Estimated coordinate error (Luzzatti) | 0.28 Å |
| Average B-factor | 29.9 Å² |
| R cryst (highest shell) | 21.9% (28.2%) |
| R free (highest shell) | 27.6% (34.0%) |

* The Ramachandran geometry was monitored using Procheck (16).
* R cryst = $\sum |F_o| - |F_c|/\sum |F_o|$.
* An R free test set of 5% of the total reflections was used.

**Fig. 3.** Assessment of native and mutant L-3-hydroxyacyl-CoA dehydrogenase stability. Native (■) and E170Q (○) enzymes (1 mg/ml) were incubated in citrate/phosphate buffer, pH 7.0, at 37 °C. Aliquots were removed at the indicated time points and assayed for activity as described under "Experimental Procedures." Stability is represented as the mean percentage of initial enzyme activity plotted as a function of time for three separate determinations. Native HAD retains complete enzymatic activity over the course of the assay, whereas E170Q HAD exhibits an apparent half-life of ~2 h.

**Fig. 2.** Effects of pH on charge transfer complex formation by native and E170Q L-3-hydroxyacyl-CoA dehydrogenases. Concentrated solutions containing the abortive ternary complex of HAD, NAD⁺, and ACoA were diluted into buffers of the indicated pH. The intensity of this charge transfer band for native (■) and E170Q (○) HAD was monitored at 412 nm and plotted as a function of pH (see "Experimental Procedures"). Measurements were made in triplicate, and the data were fit to a sigmoidal curve, yielding nearly identical pKₐ values of 5.25 ± 0.02 and 5.33 ± 0.08 for native and mutant enzymes, respectively.

![Image](http://www.jbc.org/Downloaded from http://www.jbc.org/)
New hydrogen bonds are suggested between ND1 of His$^{158}$ and the backbone carbonyl of Phe$^{159}$ as well as between NE2 of His$^{158}$ and the side chain amide of Asn$^{208}$. As a result, NE2 of His$^{158}$ is removed more than 0.7 Å from the 3-keto oxygen of AACoA (O3) and no longer positioned for optimal hydrogen bond formation. This is reflected by an increase in the average B-factor of this water molecule (subunits A and B) is 29.4 (Wat 809) as evidenced by its elevated B-factor. The average B-factor of O3, from 13.4 Å$^2$ in native enzyme to 50.0 Å$^2$ in the E170Q model, and is consistent with reduced catalytic efficiency of the mutant enzyme. Other than at the C5 carbonyl of AACoA, substrate binding appears to be unaffected by the E170Q mutation, with comparable protein/ligand interactions observed in native and mutant enzymes.

An examination of potential hydrogen bond interactions in the E170Q model provided additional insight into its altered active site geometry. The center of each active site contains a conserved water molecule, which has been observed in every HAD structure determined to date. This conserved water molecule may have a structural role, since it forms numerous hydrogen bonds with residues located in the hinge region of the protein. The water molecule in the E170Q model (Wat 984) as evidenced by its elevated B-factor. The average B-factor of this water molecule (subunits A and B) is 29.4 Å$^2$ for native enzyme over the pH range of 5.0–8.0 (Fig. 1B), indicating the importance of Glu$^{170}$ in the catalytic dyad. A potential function of Glu$^{170}$ is to elevate the apparent pH of the active site region may be responsible for the large decrease in E170Q enzyme stability.

**DISCUSSION**

Previous structural studies of HAD from this laboratory have suggested the importance of several enzyme active site residues in the reversible oxidation of 3-hydroxyacyl-CoA substrates (5, 6). Of particular interest is a histidine-glutamate dyad (His$^{158}$-Glu$^{170}$) thought to be essential for catalysis (3, 4). In the current report, Glu$^{170}$ was replaced with a glutamine residue by site-directed mutagenesis, and the mutant enzyme (E170Q) was characterized by kinetic, spectroscopic, and crystallographic analysis. These studies suggest that Glu$^{170}$ facilitates catalysis by orienting the imidazole ring of His$^{158}$ to optimize productive interaction with the substrate. Furthermore, Glu$^{170}$ appears to be critical for the structural stability of HAD.

Site-directed mutagenesis was employed to investigate the role of the His$^{158}$-Glu$^{170}$ dyad in catalysis by human HAD. Mutant proteins in which His$^{158}$ was replaced by either a leucine (H158L) or glutamine (H158Q) residue had greatly diminished enzymatic activity (data not shown). Attempts at quantitative characterization of these mutants were unsuccessful due to protein instability; both H158L and H158Q formed detectable precipitates moments after clarification. Therefore, efforts focused on the characterization of a mutant protein in which Glu$^{170}$ was replaced with a glutamine residue (E170Q). This mutation was selected in an effort to minimize disruption of the active site architecture; glutamine is isosteric with glutamate and capable of forming multiple hydrogen bonds. However, the substitution removes the negative charge located adjacent to the active site histidine, His$^{158}$. Kinetic values for the reduction of acetoacetyl-CoA (AACoA) by native HAD and E170Q are given in Table I. Whereas substrate affinity appears unaltered by the mutation, a >60-fold reduction in enzymatic activity is detected at pH 7.0.

E170Q consistently displays reduced enzyme activity as compared with wild-type protein over the pH range of 5.0–8.0 (Fig. 1B), indicating the importance of Glu$^{170}$ in the catalytic dyad.
the active site histidine, His^{158}, which is proposed to serve as a general acid in the reduction of AAcOa to L-3-hydroxyacyl-CoA (4). As such, enzyme activity was predicted to exhibit a pH dependence reflecting the protonation state of His^{158}. The enzymatic rate of the E170Q mutant displays a definitive pH dependence, indicating that catalysis is facilitated by the protonation of a single ionizable group with an apparent pK_a of 6.69 ± 0.12 (Table II). It is not unreasonable to assign the observed pK_a to His^{158}, considering its proposed role in catalysis, but the inability to generate a stable His^{158} mutant makes such an assignment difficult to confirm. Unfortunately, the apparent pK_a of His^{158} in wild-type HAD cannot be determined.

**TABLE IV**

Comparison of equivalent interatomic distances

Each line represents comparable interatomic distances in the native and E170Q models. Note that Wat 809 and Wat 984 each correspond to the conserved active site water.

| Native  | E170Q  |
|---------|--------|
| Atom 1  | Atom 2 | Distance (Å) | Distance (Å) | Atom 1  | Atom 2 |
| Wat 809 | G204·O | 2.66         | 2.55         | Wat 984 | G204·O |
| Wat 809 | N208·N | 2.83         | 3.44         | Wat 984 | N208·N |
| Wat 809 | N208·ND2 | 3.35       | 3.07         | Wat 984 | N208·ND2 |
| Wat 809 | E170·OE1 | 2.64       | 2.77         | Wat 984 | Q170·NE2 |
| E170·OE2 | I206·N | 3.06         | 3.50         | Q170·OE1 | I206·N |
| E170·OE2 | V207·N | 3.16         | 3.91         | Q170·OE1 | V207·N |
| E170·OE1 | H158·ND1 | 2.69       | 3.72         | Q170·NE2 | H158·ND1 |
| H158·ND1 | F159·O | 4.65         | 2.87         | H158·ND1 | F159·O |
| H158·NE2 | N208·ND2 | 3.54       | 3.00         | H158·NE2 | N208·ND2 |
| H158·NE2 | AAcOa·O3 | 2.62       | 3.37         | H158·NE2 | AAcOa·O3 |
| N161·N | AAcOa·O1 | 2.98       | 3.21         | N161·N | AAcOa·O1 |

**FIG. 5.** Comparison of the enzyme active sites of native and E170Q L-3-hydroxyacyl-CoA dehydrogenases. The stereo diagrams depict the enzyme active sites of native HAD (A) and E170Q HAD (B) in complex with NAD⁺ and AAcOa. Relevant interatomic distances within 3.5 Å are illustrated as dotted black lines, with oxygen atoms colored in red, nitrogen in blue, carbon in green, sulfur in yellow, and phosphorus in purple. The imidazole ring of His^{158} adopts a significantly different conformation in the E170Q mutant structure as compared with the native model. Additional differences are discussed under “Results.”
using the current methodology. Native enzyme activity is virtually pH-independent over the range tested (pH 5–8), suggesting that the effect of His\textsuperscript{158} protonation state on activity is probably masked by a rate-limiting step not associated with catalysis. Considerable product inhibition is observed for wild-type protein, indicating that product release may be the rate-determining step, but further kinetic studies will be needed to confirm such an assertion.

Nonetheless, the pH dependence of enzymatic turnover (Fig. 1B) does provide valuable information about the role of Glu\textsuperscript{170} in catalysis. If the sole purpose of Glu\textsuperscript{170} is to elevate the apparent pK\textsubscript{a} of His\textsuperscript{158}, wild-type enzyme activity should be restored to the E170Q mutant by reduction of pH. Assuming a pK\textsubscript{a} value of 6.7 (Table II), His\textsuperscript{158} would be fully protonated at pH 5.0 and capable of providing a proton to AACoA upon hydride transfer from NADH. However, E170Q HAD remains ~30-fold less active than native enzyme at this pH (Fig. 1B), suggesting that efficient catalysis requires more than just protonation of His\textsuperscript{158}. Clearly, Glu\textsuperscript{170} has additional contacts within the active site that impact enzyme function.

Active site integrity can be further assessed by the formation of a charge transfer complex composed of HAD, NAD\textsuperscript{+}, and AACoA. Within this abortive ternary complex, the electron-rich enolate form of AACoA is juxtaposed to the electron-deficient nicotinamide ring of NAD\textsuperscript{+}, giving rise to the charge transfer band centered between 410 and 420 nm (6). Enolate formation, as judged by absorbance at 302 nm, is reduced dramatically in the E170Q mutant (data not shown), resulting in a comparably diminished charge transfer absorbance at 412 nm (Fig. 2). The disparity between native and E170Q charge transfer complex formation is nearly constant over the pH range tested with the intensity of the native charge transfer band ~3-fold greater than that of the E170Q mutant. Although E170Q exhibits a reduced ability to promote enolate formation, probably as a result of improper positioning of the active site histidine (Fig. 4), the precise role of His\textsuperscript{158} in charge transfer complex formation is not known. It may facilitate preferential binding of the enolate species of AACoA by serving as a hydrogen bond donor or it may actively promote enolate formation by abstracting the proton from the enol form. The pK\textsubscript{a} of 6.7 attributed to His\textsuperscript{158} by kinetic analysis of the E170Q mutant would suggest the former; His\textsuperscript{158} would be primarily protonated over much of the pH range assayed for charge transfer complex formation and thus able to function as a hydrogen bond donor. However, that pK\textsubscript{a} assignment has not been confirmed, nor can it be assumed that the pK\textsubscript{a} of His\textsuperscript{158} in the charge transfer complex is the same as in the Michaelis complex.

To facilitate interpretation of the biochemical data, x-ray crystallographic studies were undertaken. The E170Q abortive ternary complex structure is quite similar to the corresponding native model and exhibits an overall conservation of active site architecture (Figs. 4 and 5). The glutamate to glutamine substitution does result in perturbations of side chain orientations, most notably the rotation of the imidazole ring of His\textsuperscript{158} by ~90° relative to native enzyme. Although glutamine can potentially form a hydrogen bond with NE2 of His\textsuperscript{158}, there is no evidence for such an interaction within the E170Q crystal structure. Instead, the imidazole ring of His\textsuperscript{158} forms hydrogen bonds between its ND1 and the backbone carbonyl of Phe\textsuperscript{159} as well as between its NE2 and the side chain amide of Asn\textsuperscript{208} (Table IV). Thus, it appears that His\textsuperscript{158} requires Glu\textsuperscript{170} to serve as a strong hydrogen bond acceptor and/or provide a negative charge for proper orientation of its imidazole ring.

Disruption of the hydrogen bond between the ND1 of His\textsuperscript{158} and the carboxylate oxygen of Glu\textsuperscript{170} alters the preferred orientation of the imidazole ring, resulting in an enzyme active site that is no longer optimized for efficient catalysis. The interatomic distance between NE2 of His\textsuperscript{158} to O3 of AACoA increases from 2.62 Å in the native structure to 3.37 Å in the E170Q model (Table IV). In such a conformation, it is unlikely that His\textsuperscript{158} would be able to effectively serve as a general acid/general base in the E170Q mutant. Instead, the imidazole ring of His\textsuperscript{158} would have to adopt an orientation similar to wild-type enzyme to form a favorable interaction with the substrate. Accordingly, a greater than 3-fold increase in the B-factor for O3 of AACoA is observed for the mutant structure relative to that of native enzyme. In addition to its contact with His\textsuperscript{158}, the enolate of AACoA is stabilized by a hydrogen bond between its thioester carbonyl (O1) and the backbone amide of Asn\textsuperscript{161}. However, comparable interatomic distances of 2.98 and 3.21 Å are observed for wild-type and mutant enzymes, respectively, indicating that this interaction is not significantly compromised by the E170Q mutation (Table IV). Thus, the reduction in E170Q enzyme activity, as well as the decrease in charge transfer complex formation, could be attributed, at least in part, to the rotation of the His\textsuperscript{158} side chain.

Another consequence of the E170Q mutation is the disruption of the hydrogen bond network in the hinge region of the enzyme (residues 201–207), which relates the NAD\textsuperscript{+}-binding domain to the C-terminal domain. In native protein, a carboxylate oxygen of Glu\textsuperscript{170} forms hydrogen bonds with the backbone amides of Ile\textsuperscript{206} and Val\textsuperscript{207}. In contrast, Glu\textsuperscript{170} forms only a weak hydrogen bond (~3.5 Å) with the amide of Ile\textsuperscript{206} (Fig. 5, Table IV). In addition, the backbone amide of Asn\textsuperscript{208} moves more than 0.6 Å away from the conserved water molecule located at the enzyme active site in the E170Q structure. The result is a 3-fold increase in the B-factor for the water molecule in the E170Q model as compared with native enzyme. A manifestation of these structural perturbations is reduced E170Q protein stability at 37 °C (Fig. 3). Over the course of 4 h, wild-type HAD retains complete enzyme activity as opposed to the ~4-fold reduction in E170Q catalysis. Reduced protein stability could also account for the diminished protein yield from overexpression and purification of E170Q. Furthermore, disruption of the hinge region may contribute to reduced catalytic activity. Rotation of the NAD\textsuperscript{+}-binding domain inward toward the dimer interface is required for efficient substrate binding and catalysis. The structural rearrangements in this region, although subtle, may impair the dynamic movement of the enzyme.

Several examples of catalytic dyads composed of a histidine and an aspartate residue have been extensively characterized. As discussed by Levy and co-workers (15), the aspartate residue of such active site pairs has been postulated to serve various functions. In the case of glucose 6-phosphate dehydrogenase (15) and mandelate racemase (14), it appears that the aspartate residue facilitates catalysis by altering the pK\textsubscript{a} of the histidine. In both cases, the position of the histidine residue is relatively undisturbed by replacement of aspartate with an asparagine residue. Similarly, the position of the active site histidine is unaffected by analogous aspartate substitutions in either trypsin (17) or ribonuclease A (18). In these examples, aspartate is proposed to stabilize the productive tautomer of the active site histidine. Subsequent NMR studies with ribonuclease A have demonstrated that aspartate has only a modest effect on the pK\textsubscript{a} of the active site histidine (19). A third predicted function of aspartate is orientation of the imidazole ring of the histidine for optimal catalysis. However, significant perturbations in positioning of the imidazole ring as a result of aspartate substitution by asparagine have not been reported. Comparative studies with histidine-glutamate pairs have not been as comprehensive. \textsuperscript{d}-Isomer-specific 2-hydroxyacid dehy-
Importance of Glu$^{170}$ in L-3-Hydroxyacyl-CoA Dehydrogenase

This study addresses the role of Glu$^{170}$ in L-3-hydroxyacyl-CoA dehydrogenase (HAD) structure and function. The kinetic and spectroscopic properties of native and E170Q enzymes provide valuable mechanistic information regarding substrate binding and charge transfer complex formation. In particular, the importance of an unidentified residue with an apparent p$K_a$ of 5.3 has been demonstrated. Native and E170Q HAD exhibit comparable $K_m$ values for AACoA at each pH tested (Fig. 1, C and D), consistent with structural studies, which indicate that Glu$^{170}$ does not directly participate in substrate binding (Fig. 5). Examination of the pH profile of either protein suggests that an ionizable group with an apparent p$K_a$ of $\sim$5.3 is associated with substrate binding (Table II). Similarly, the pH profiles of charge transfer complex formation by E170Q and HAD are nearly identical, each yielding an apparent p$K_a$ of 5.3 for enolate formation. It seems likely that the similar p$K_a$ values associated with AACoA binding and enolate formation are related.

One possibility is that the p$K_a$ of 5.3 reflects a group on the substrate. AACoA is presumed to occur primarily in its enol form in solution and, as such, has three ionizable groups. The amine group on the adenine ring and the ribose phosphate of coenzyme A have reported p$K_a$ values of 4.0 and 6.4, respectively (25), although these values may shift under the current assay conditions. It is unlikely, however, that the protonation of either of these groups would significantly affect binding. The adenosine diphosphate portion of coenzyme A is only weakly associated with the enzyme as indicated by its relatively high B-factors (6) and is probably not a major determinant in substrate binding (26).

The major contact sites between HAD and AACoA involve only the pantetheine and fatty acyl portions of the substrate. Thus, the only ionizable group in the solution form of AACoA likely to be relevant to binding is its enol group. However, the p$K_a$ of enolate formation in solution has been estimated to be greater than 8.5 (27), much larger than the p$K_a$ associated with substrate binding. Circumstantially, it appears that the relevant ionizable group resides within the enzyme-cofactor binary complex.

Decreased enolate formation could result from reduced affinity of the HAD-NAD$^+$ binary complex for AACoA. The kinetic studies indicate that at low pH, an unidentified group becomes protonated and increases the $K_m$ for AACoA. This decrease in substrate affinity correlates well with decreased charge transfer formation. However, the charge transfer studies were carried out at an AACoA concentration of 2 mM, nearly 30-fold higher than the highest determined $K_m$ of AACoA ($64.8 \pm 3.18 \mu M$). At pH 5.0, the intensity of the charge transfer band is already reduced by greater than 50%, although enzyme should be saturated with AACoA. Furthermore, a 5-fold increase in either AACoA or NAD$^+$ concentration did not increase the intensity of the charge transfer band (data not shown), indicating that reduced substrate binding cannot account for the decrease in charge transfer complex formation.

Examination of the E170Q kinetic and charge transfer studies does provide a possible explanation for the observed p$K_a$. The pH profile of the $K_m$ values suggests that substrate binding is reduced upon protonation of an ionizable group with an apparent p$K_a$ of 5.3 (Fig. 1, C and D). However, charge transfer complex formation, which exhibits the same apparent p$K_a$ as substrate binding (Fig. 2), cannot be increased by the addition of excess AACoA. HAD may still bind AACoA with reduced affinity when this group is protonated but may fail to adopt the conformation necessary for enolate and charge transfer complex formation. Structural studies of HAD have suggested that
high affinity substrate binding and effective catalysis require a large conformation change in which the NAD⁺-binding domain of the enzyme swings toward the C-terminal dimerization domain, sequestering the enzyme active site (6). The apparent pKₐ of 5.3 may therefore reflect the protonation state of a group involved in this domain shift.

The trigger responsible for the domain shift observed upon substrate binding has not yet been identified. Ser¹³⁷ undergoes the biggest conformational change of active site residues upon formation of the abortive ternary complex, as compared with the binary NAD⁻-binding complex. In the binary complex, the hydroxyl group of Ser¹³⁷ is found within hydrogen bond distance of NE2 of His¹⁵₈ (5). Upon abortive ternary complex formation, the backbone carbon of Ser¹³⁷ shifts greater than 1 Å. Its hydroxyl group is found equidistant to O3 of AACoA and its critical role in catalysis. In this report, we have demonstrated that Glu¹⁷⁰ of human HAD is required for positioning of the active site histidine and the structural stability of the enzyme. Despite its similar size and potential for hydrogen bond formation, a glutamine residue cannot fulfill this requirement, suggesting that the negative charge of Glu¹⁷⁰ and/or its ability to serve as a strong hydrogen bond acceptor is needed for optimal catalysis and maintenance of active site integrity. Characterization of native and E170Q enzymes has also led to the identification of an ionizable group with an apparent pKₐ of 5.3 as an important determinant in substrate binding and charge transfer complex formation. The identity of this residue has been tentatively assigned to a second conserved glutamate residue, Glu¹¹⁰ but further study will be required to confirm this assertion.

**Acknowledgments**—We recognize Ed Hoeffner for contributions through the maintenance the x-ray and computational resources at the University of Minnesota. We acknowledge the Minnesota Supercomputer Institute for use of computational resources and thank Dr. Melanie Simpson (University of Minnesota) for careful review of the manuscript.

**REFERENCES**

1. Kobayashi, A., Jiang, L. L., and Hashimoto, T. (1996) *J. Biochem. (Tokyo)* **119**, 775–782.
2. Neves, B. E., and Bradshaw, R. A. (1973) *J. Biol. Chem.* **248**, 3052–3059.
3. He, X. Y., and Yang, S. Y. (1996) *Biochemistry* **35**, 9625–9630.
4. He, X. Y., Deng, H., and Yang, S. Y. (1997) *Biochemistry* **36**, 261–268.
5. Barycki, J. J., O'Brien, L. K., Pratt, J. M., Zhang, R., Sanishvili, R., Strauss, A. W., and Banaszak, L. J. (1999) *Biochemistry* **38**, 5767–5776.
6. Barycki, J. J., O'Brien, L. K., Strauss, A. W., and Banaszak, L. J. (2000) *J. Biol. Chem.* **275**, 2716–2719.
7. Birkho, J. J., Holden, H. M., Hamlin, R., Xuong, N. H., and Banaszak, L. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 8262–8266.
8. Elving, P. J., Markowitz, J. M., and Rosenthal, I. (1956) *Anal. Chem.* **28**, 1179–1180.
9. Cleland, W. W. (1979) Methods Enzymol. **63**, 103–138.
10. Pfugrath, J. W. (1999) *Acta Crystallogr. Sec. D Biol. Crystallogr.* **55**, 1718–1725.
11. Brungger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kusswetter, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1996) *Acta Crystallogr. Sec. D Biol. Crystallogr.* **54**, 905–921.
12. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr.* Sec. A **47**, 110–119.
13. Clarke, A. R., Wilks, H. M., Barstow, D. A., Atkinson, T., Chia, W. N., and Holbrook, J. J. (1986) *Biochemistry* **25**, 1617–1622.
14. Schäfer, S. L., Barrett, W. C., Kallarakal, A. T., Mitra, B., Konzich, J. W., Gerli, J. A., Clifton, J. G., Petsko, G. A., and Kenyon, G. L. (1996) *Biochemistry* **35**, 5662–5669.
15. Cosgrove, M. S., Geyer, S., Naylor, C. E., Vandeputte-Rutten, L., Adams, M. J., and Levy, H. R. (2000) *Biochemistry* **39**, 15092–15101.
16. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) *J. Mol. Biol.* **231**, 1049–1067.
17. Sprang, S., Standing, T., Fletterick, R. J., Stroud, R. M., Eimer-Mooore, J., Xuong, N. H., Hamlin, R., Rutter, W. J., and Craik, C. S. (1987) *Science* **237**, 905–909.
18. Schulz, L. W., Quirk, D. J., and Rains, R. T. (1999) *Biochemistry* **38**, 8866–8889.
19. Quirk, D. J., and Rains, R. T. (1999) *Biophys. J.* **76**, 1571–1579.
20. Bateman, A., Birney, E., Durbin, R., Eddy, S. R., Finn, R. D., and Sonnhammer, E. L. (1999) *Nucleic Acids Res.* **27**, 360–362.
21. Kutzanenko, A. S., Lavinin, V. S., and Popov, V. O. (1998) *FEBS Lett.* **423**, 105–109.
22. Tishkov, V. I., Matorin, A. D., Rojkova, A. M., Fedorchuk, V. V., Savitsky, P. A., Gerlt, J. A., Clifton, J. G., Petsko, G. A., and Kenyon, G. L. (1996) *Biochemistry* **35**, 5662–5669.
23. Cosgrove, M. S., Geyer, S., Naylor, C. E., Vandeputte-Rutten, L., Adams, M. J., and Levy, H. R. (2000) *Biochemistry* **39**, 15092–15101.
24. Bernard, N., Johnsen, K., Gelpi, J. L., Alvarez, J. A., Ferrant, T., Garmyn, D., Hols, P., Cortes, A., Clarke, A. R., Holbrook, J. J., and Delcour, J. (1997) *Eur. J. Biochem.* **244**, 213–219.
25. Dawson, R. M. C., Elliot, D. C., Elliot, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*, 3rd Ed., Oxford University Press, New York.
26. Hartmann, D., Philipp, R., Schmadel, K., Birktoft, J. J., Banaszak, L. J., and Broommer, W. E. (1991) *Biochemistry* **30**, 2792–2798.
27. Aufer, H. E., and Freeman, F. E. (1980) *J. Biol. Chem.* **255**, 8157–8163.
28. Yang, S. Y. (1994) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **109**, 557–566.
29. Roe, C. R., and Coates, P. M. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th Ed., (Scriber, C. R., Beaudet, A. L., Sly, W. E., and Valle, D., eds) pp 1501–1533, McGraw-Hill Inc., New York.
30. Sims, H. F., Brackett, J. C., Powell, C. K., Treem, W. R., Hale, D. E., Bennett, M. J., Gibson, B. Shapleigh, S., and Strauss, A. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 841–845.
31. Ljist, L., Ruiter, J. P., Hoovers, J. M., Jakobs, M. E., and Wanders, R. J. (1996) *J. Clin. Invest.* **98**, 1028–1033.
32. Strauss, A. W., Bennett, M. J., Rinaldo, P., Sims, H. F., O'Brien, L. K., Zhao, Y., Gibson, B., and Ibdah, J. (1999) *Semin. Perinatol.* **23**, 100–112.
Glutamate 170 of Human l-3-Hydroxyacyl-CoA Dehydrogenase Is Required for Proper Orientation of the Catalytic Histidine and Structural Integrity of the Enzyme
Joseph J. Barycki, Laurie K. O'Brien, Arnold W. Strauss and Leonard J. Banaszak

J. Biol. Chem. 2001, 276:36718-36726.
doi: 10.1074/jbc.M104839200 originally published online July 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104839200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 7 of which can be accessed free at http://www.jbc.org/content/276/39/36718.full.html#ref-list-1