Human liver alcohol dehydrogenase isoenzymes \( \beta_1 \beta_1 \) and \( \beta_2 \beta_2 \), in which position 47 in the coenzyme binding domain is an arginine or histidine, respectively, differ remarkably in steady-state kinetics. To understand which catalytic steps affect these kinetics, apparent coenzyme dissociation and association rate constants, and apparent 4-trans-(N,N-dimethylamino)cinnamaldehyde (DACA) hydride transfer rate constants were obtained with stopped-flow kinetics. Enzymes containing site-specific mutations of Arg-47 in \( \beta_1 \beta_1 \) (847R) to His (847H or 847K), Lys (847K), or Gln (847Q) were studied. Apparent coenzyme dissociation rate constants are greatly affected by substitutions at position 47, in which mutant enzymes with a weak base or a neutral residue at this position (847H and 847Q) exhibit faster rate constants than 847R and 847K. Substitutions at position 47 have less effect on apparent coenzyme association rate constants. The kinetics of NADH association for 847H and 847K are consistent with a two-step mechanism in which the bimolecular binding step is coupled to a unimolecular process. These findings indicate that the greater role of position 47 in coenzyme dissociation may occur after a coenzyme-induced isomerization. Substitutions at position 47 also strongly influence apparent DACA hydride transfer rate constants; hydride transfer is faster with mutant enzymes containing weak bases like histidine at this position. Steady-state kinetics, however, reveal that the rate-limiting step of both 847R and 847H for acetaldehyde reduction and for ethanol oxidation is coenzyme product dissociation. Thus, the different activities of \( \beta_1 \beta_1 \) and \( \beta_2 \beta_2 \) for ethanol oxidation and acetaldehyde reduction are caused primarily by different coenzyme dissociation rates.

**Amino Acid Substitutions at Position 47 of Human \( \beta_1 \beta_1 \) and \( \beta_2 \beta_2 \) Alcohol Dehydrogenases Affect Hydride Transfer and Coenzyme Dissociation Rate Constants**

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Human liver NAD\(^+\)-dependent alcohol dehydrogenase (EC 1.1.1.1) catalyzes the reversible oxidation of alcohols. The majority of ingested ethanol is metabolized by this dimeric enzyme. Multiple isoenzyme forms exist in human tissues that arise from subunits encoded at ADH2, \( \beta_1 \beta_1 \), \( \beta_2 \beta_2 \), and \( \beta_1 \beta_2 \), exhibit strikingly different steady-state kinetic properties for ethanol oxidation with NAD\(^+\) and for acetaldehyde reduction with NADH (3-6). For example, at pH 7.5, the \( V_{\text{max}} \) for acetaldehyde reduction and \( K_m \) for NADH of \( \beta_2 \beta_2 \) is 10-fold greater than those of \( \beta_1 \beta_1 \) and \( \beta_1 \beta_2 \) has a 10-fold greater \( V_{\text{max}} \) for acetaldehyde reduction and a 40-fold greater \( K_m \) for NADH than \( \beta_1 \beta_1 \) (4, 5, 7). These large kinetic differences are the result of single amino acid substitutions at position 47 (histidine in \( \beta_2 \) and arginine in \( \beta_1 \)).

The recent x-ray crystallographic structure of \( \beta_1 \beta_1 \) complexed with NAD\(^+\) indicates that, like the horse EE enzyme (10), the guanidino group of Arg-47 forms a salt bridge with the coenzyme pyrophosphoryl oxygens and stabilizes the enzyme(coenzyme) complex (11). Site-directed mutagenesis studies of Arg-47 revealed that coenzyme steady-state kinetic parameters (\( K_m \) and \( V_{\text{max}} \)) increase with substitution of a weaker base like histidine or a neutral residue like glutamine at position 47 (6). The component rate constants of these kinetic parameters are composed (coenzyme dissociation and association rate constants), however, have not been determined with \( \beta_1 \beta_1 \) or \( \beta_2 \beta_2 \) or with mutants at position 47. Moreover, it was not known which step in the catalytic mechanism is rate-limiting for these enzymes.

In this study, we used stopped-flow kinetics to evaluate apparent coenzyme dissociation and association rate constants of \( \beta_1 \beta_1 \) as a function of pH and of site-specific mutants containing histidine (847H), lysine, or glutamine at position 47. The chromophoric aldehyde, 4-trans-(N,N-dimethylamino)cinnamaldehyde (DACA)\(^3\) was used both as a chromophoric indicator of coenzyme binding and as a substrate. The effect of these substitutions on the apparent DACA hydride transfer rate was also obtained. The individual rate constants were compared to steady-state kinetic parameters.

The abbreviations used are: DACA, 4-trans-(N,N-dimethylamino)cinnamaldehyde; \( k_{\text{DAC}} \), \( k_{\text{AD}} \), \( k_{\text{ADH}} \), NAD\(^+\) and NADH apparent association rate constants, respectively, \( \mu M^{-1} s^{-1} \); \( k_{\text{DAC}}^{\text{NAD}} \), \( k_{\text{AD}}^{\text{NAD}} \), \( k_{\text{ADH}}^{\text{NAD}} \); NAD\(^+\) and NADH apparent dissociation rate constants, respectively, \( s^{-1} \); \( k_{\text{DAC}}^{\text{HAD}} \), apparent limiting hydride transfer rate of DACA, \( s^{-1} \); \( k_{\text{DAC}}^{\text{HAD}} \), apparent limiting rate of butanol oxidation, \( s^{-1} \); \( k_{\text{DAC}}^{\text{HAD}} \), apparent limiting rate of butanol oxidation, \( s^{-1} \); (this value may include butanol association, butanol hydride transfer, or butanol dissociation); \( k_{\text{DAC}}^{\text{HAD}} \), association of 4-methylpyrazole to the enzyme(NAD\(^+\)) complex, \( s^{-1} \); \( \bar{r} \), \( m \), pH-corrected limiting rates at high and low pH, respectively; mutant designations are designated by the amino acid at position 47 (e.g. 847H is the \( \beta_1 \beta_1 \) isoenzyme with a histidine at position 47); PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); Bicine, N,N-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylammonio)-1-propanesulfonic acid; ACES, N-(2-acetamido)-2-aminoethanesulfonic acid.
Human Alcohol Dehydrogenase Kinetics

**Experimental Procedures**

**Reagents**—The chromophore 4-trans-(N,N-dimethylaminocinnamaldehyde (DACA) was obtained from Aldrich and purified by sublimation. Coenzyme NADH, 4-methylpyrazole, 1-butanol, glycylglycine, sodium carbonate buffers were obtained from Sigma; Bicine was recrystallized once in water. Horse liver alcohol dehydrogenase and NAD⁺ were obtained from Boehringer Mannheim. Horse alcohol dehydrogenase was dialyzed overnight into 0.1 M Tris·Cl, pH 9.5, heated to 50 °C for 10 min, and centrifuged at 5100 g for 20 min to remove particulate matter. This procedure increased the activity to about 6 units/mg.

**Enzyme Purification and Preparation**

Native human liver ββ₂ (β47R) and ββ₂β (β47H) enzymes were prepared from human autopsy livers as described (4, 12). Recombinant β47R was purified from Escherichia coli transformed with the human β cDNA as described (6), with the following modifications. 5 μM zinc sulfate was added to the growing cultures at the time of induction; this increased the final yield of active protein 2-fold. Sometimes, E. coli cell concentrates were frozen at −70 °C in 0.1 M Tris, pH 8.5, 5% (w/v) sucrose, 2.0 mM benzamidine, and 2.0 mM dithiothreitol for future use. For purification, the frozen cells were thawed quickly in a microwave on the "defrost" setting for intervals of 2 to 3 min to avoid local heating and then centrifuged at 8670 × g for 20 min.

Cell lysates, prepared with a bead beater, were centrifuged and initially purified by DEAE-cellulose chromatography (6). This DEAE-52 activity was further concentrated and equilibrated overnight in the presence of 0.1 M Tris·Cl, 20 mM dithiothreitol, 1.0 mM benzamidine, and 5.0 μM zinc sulfate, pH 7.5 (conductivity approximately 0.9 mS−1), with a Millipore Minimac concentrator (30,000-dalton membrane). The enzyme sample was then loaded at 5 ml/min onto a 5.9-cm S-Sepharose Fast Flow column (Bio-Rad), equilibrated with the same buffer. After washing the column to remove unbound protein, the enzyme was eluted with a linear gradient of 7 to 100 mM sodium phosphate (400 ml in each reservoir). Fractions containing activity were pooled and dialyzed against 10 mM sodium phosphate, 0.5 mM dithiothreitol, 5 μM zinc sulfate, pH 7.4, and then further purified with 75 ml of Affi-Gel Blue (13). This resin was added to the activity pool and stirred at room temperature for 1 h. Excess buffer was filtered through a glass-sintered funnel, and the resin was packed in a 1.9-cm column. The column was washed until the absorbance at 280 nm was less than 0.01, and then enzyme was eluted with 0.8 M NaCl in buffer. As determined by absorbance at 280 nm, the resulting β47R and β47K activity pools contained less than 15% coenzyme-bound enzyme active sites. The β47H and β47Q enzymes contained no detectable amount of bound coenzyme. Enzyme was homogeneous by SDS-polyacrylamide gel electrophoresis (14). Purified enzyme was dialyzed first into 10 mM sodium phosphate, pH 7.5, and then dialyzed into 0.5 mM NADH, 2.5 mM NAD⁺, and 25% ethanol with 50% glycerol and stored at −20 °C after filtering through a 0.2-μm membrane.

Activity assays of the horse liver alcohol dehydrogenase, β47K, and β47R were performed at 25 °C, with 2.4 mM NAD⁺ and 33 mM ethanol in 0.1 M glycine buffer, pH 10. Assays of β47H and β47Q were performed in 0.1 M sodium pyrophosphate, pH 8.5. Production of NADH was monitored at 340 nm, assuming an extinction coefficient of 6.22 mM−1 cm−1. Activity site concentrations were obtained by direct site titrations (15), or by activity assays assuming 6 units/mg for horse alcohol dehydrogenase, 19 units/mg for β47H and β47Q, 0.24 unit/mg for β47R, and 0.45 unit/mg for β47K (6).

**Buffers, Activity Assays, and Enzyme Stability**

A three-component buffer was used for pH-dependent studies to minimize ionic strength differences across a wide pH range while maintaining buffering capacity (16). A buffer containing 15 mM ACES, 15 mM glycylglycine, and 60 mM CAPS was used for steady-state kinetics from pH 6 to 10. Since DACA forms Schiff's base complexes with the enzymes, another buffer was developed for studies with this substrate. The buffer consisted of 15 mM PIPES and 15 mM Bicine, with the addition of 50 mM sodium carbonate for experiments at pH 9 and 10 or 50 mM sodium acetate at pH 5 and 6. Buffers were titrated to the desired pH with sodium hydroxide or acetic acid. Long-term storage (from pH 7 to 8.5) with the ACES-glycylglycine-CAPS buffer, and from 1.3 to 11.3 mM with the PIPES-Bicine (carbonate) buffer. No significant differences in activity at pH 8 were seen with β47R or β47H using either buffer or when compared to 0.1 M sodium pyrophosphate. At pH 6, all mutant enzymes were fully active for at least 85 min at 25 °C; at pH 5, β47R was fully active while β47H and β47Q exhibited inactivation rates of 0.05 unit/ml·s and 0.01 unit/ml·s, respectively. At pH 10, β47R remained fully active, while β47H exhibited a rate of inactivation of only 0.15 unit/ml·s. The pH 8.5 of each experiment varied from the initial pH by no more than 0.2 pH unit.

**Stopped-flow Kinetics**

All absorbance experiments were performed on a Durrum stopped-flow instrument, and traces were analyzed with KINFIT (OLIS; Jefferson, GA). Fluorescence experiments were performed on a HI-TECH SF5i instrument, and traces were analyzed with HI-TECH software. Goodness-of-fit was determined by F-statistic or correlation coefficient. Standard errors were within 10% of the estimates. Apparent rate constants obtained with horse EE alcohol dehydrogenase did not differ from previously reported values (18–20).

**Apparent DACA Association Rate Constants and Equilibrium Dissociation Constants**—Apparent DACA association rate constants (kₐDACA μM⁻¹ s⁻¹) and DACA equilibrium dissociation constants (KᵦDACA) were determined by mixing enzyme and saturating concentrations of NADH with DACA. Relaxations (1/t) were determined by exponential regression of absorbance at 454 nm versus time. 9 to 11 data points from 0.2 μM to 7.7 μM were used for linear regression of 1/t versus DADA concentration as described (17). Final concentrations of DACA were varied from 0.2 μM to 32 μM in the presence of 3 to 4 mM enzyme active sites, and 0.5 mM NADH or 2.5 mM NAD⁺ at low or high pH, respectively. Values of KᵦDADA were determined by the change in final versus initial absorbance as described (17). 6 to 15 data points at 0.2 to 13 μM DADA were used for this analysis. The apparent DADA association rate constants with the mutants were approximately 11 μM⁻¹ s⁻¹, and equilibrium dissociation constants were 3 to 4 μM. These values did not change with pH. Concentrations of 75 μM DADA ensured pseudo-first order kinetics for the other studies. The concentration of DADA was confirmed by absorbance at 398 nm, assuming an extinction coefficient of 3.1 × 10⁴ M⁻¹ cm⁻¹ (17). Tertiary (E-NADH-DADA) complex formation for this and all other experiments with DADA were monitored at 464 nm, a wavelength that easily distinguished this complex from free DADA (Fig. 1). Apparent DADA limiting hydride transfer rates—Apparent DADA limiting hydride transfer rates (kₚDADA μM⁻¹ s⁻¹) were determined by mixing enzyme, preincubated with excess NADH, with excess 4-methylpyrazole and varying DADA concentrations (Equation 1) (18).

\[
(E \cdot \text{NADH}) + \text{DADA} \rightleftharpoons (E \cdot \text{NADH} \cdot \text{DADA}) \quad \text{ADACA} \quad \text{ADACA} \quad \text{ADACA}
\]

(E-NAD⁰ + DADA) + 4-methylpyrazole → (E-NAD° + DADA + 4-methylpyrazole) → (E-NAD° - 4-methylpyrazole)

In this reaction, hydride transfer between NADH and DADA produced NAD⁺ and the 4-methylpyrazole rendered the reaction quasi-irreversible by rapid formation of the (E-NAD° + 4-methylpyrazole) adduct (18). The depletion of DADA at 464 nm was monitored. Monitoring NADH depletion in the reaction mixture at 340 nm yielded the same rates, ensuring that hydride transfer had occurred; no increase in absorbance at 398 nm was seen, indicating that DADA did not simply diffuse from the tertiary (E-NAD⁺-DADA) complex. Also, acid-catalyzed decomposition of DADA and NAD⁺ is negligible (18).

Final concentrations of reaction mixtures were 3 to 4 μM enzyme active sites, 0.2 mM NADH with β47R and β47K, or 1.5 mM with β47H and β47Q, 15 mM 4-methylpyrazole, and 5 to 75 μM DADA. All traces fit a single exponential model across the pH range studied, indicating that any enzyme inactivation at low pH was minimal. Estimates of kₚDADA and KᵦDADA were determined by nonlinear regression of a first order hydride transfer versus time (10 to 15 data points at 5 to 7 DADA concentrations) (18). To minimize exposure time of the enzymes below pH 6, pH-jump experiments were performed as described (18). Final pH did not differ from the intended pH by more than 0.2 pH unit. The concentration of 4-methylpyrazole used in these experiments varied; pH did not change more than 0.5. To ensure pseudo-first order conditions, the apparent 4-methylpyrazole association rate to the (E-NAD⁺) complex, kᵦDADA, must not be rate-limiting. The value of this apparent rate was obtained by
mixing 30 mM 4-methylpyrazole with enzyme, pre-equilibrated with 2 mM NAD+. These rates with β47H and β47R were not limiting across the pH range studied (Table I). The β47Q mutant appeared to be limited by apparent 4-methylpyrazole association rates below pH 6; therefore, measurements were not recorded below this pH.

**Apparent NADH Dissociation Rate Constants**—Apparent NADH dissociation rate constants (kDADCA, s−1) were obtained by monitoring either the fluorescence decrease of the enzyme(NADH) complex (19), or the absorbance decrease of the enzyme(NADH-DACA) complex (Equation 2).

\[
(E\text{-NADH}) + NAD^+ \leftrightarrow (E\text{-NAD}^+)+ \text{4-methylpyrazole} \rightarrow (E\text{-NAD}^+\text{-4mpyr})
\]

Fluorescence measurements were made by monitoring emission of NADH through Schott glass No. 7 (Bb24) and No. 15 (Bb470) filters (excitation at 340 nm). Enzyme at a final concentration of 5 to 8 μM active sites was pre-equilibrated with enough NADH to bind 95% of the sites. A final NADH concentration of 6 μM with β47R, β47K, and the horse enzyme at all pH values or 0.5 mM (based on NADH affinity) with β47H and β47Q was used. The enzyme(NADH) mixture was then mixed with excess 4-methylpyrazole and NAD+ at final concentrations of 15 mM 4-methylpyrazole and 0.5 to 17 mM NAD+. When used as an indicator, a trace amount of NADH was included in the enzyme(NADH) mixture at a final concentration of 0.5 μM with β47K and β47R or 5.0 μM with β47H and β47Q. Concentrations of NADH were confirmed by absorbance measurements at 340 nm, assuming an extinction coefficient of 6.22 mM−1 cm−1. Eight to ten data points at 4 to 5 NAD+ concentrations were used to determine kDADH values by linear regression of 1/r versus pH values or 45 μM to 1.0 mM (Equation 3).

\[
(E\text{-NAD}) + NAD^+ \leftrightarrow (E\text{-NAD}^+) + \text{DACA} \leftrightarrow (E\text{-NAD}^+\text{-DACA})
\]

Here, NADH binding drove NAD+ dissociation, and excess DACA trapped the ternary (E-NAD-DACA) complex. Final concentrations were 3 to 4 μM enzyme active sites, 75 μM DACA, 10 to 250 μM NAD+, and 25 μM NAD+ for β47R and β47Q. Experiments with β47H and β47Q contained 250 μM NAD+ at low pH and 1.0 mM NAD+ at high pH. Estimates of kDADH were determined by linear regressions of 1/r versus 1/NAD+ concentration (20), using 8 to 14 data points at 5 to 7 NAD+ concentrations. At low pH with β47Q, a slow drop in absorbance at 464 nm was observed, sometimes with appreciable amplitude. The same phenomenon occurred to a lesser extent with β47H. This second rate was identified as reductive hydride transfer of DACA.

**Apparent NADH Association Rate Constants**—Apparent NADH association rate constants (kaDADH, μM−1 s−1) were determined by mixing enzyme with excess DACA and varying NADH concentrations (Equation 4).

\[
E + NAD^+ \leftrightarrow (E\text{-NAD}^+) + \text{DACA}
\]

After NAD+ binding, the excess 1-butanol was rapidly oxidized, forming butanol and NADH. Excess DACA was used to drive butanol dissociation and form the (E-NAD-DACA) complex. Final reaction concentrations were 3 to 4 μM enzyme active sites, 75 μM DACA, 5 mM 1-butanol, and 20 to 60 μM NAD+ at low pH or 30 to 250 μM NAD+ above pH 6. Relaxations (1/r) were determined by exponential regression of absorbance versus time. Estimates of kaDADH were determined by linear regressions of 1/r versus NAD+ concentration (19), using 14 to 15 data points of 7 to 8 NAD+ concentrations. At low pH, a slow decreasing drift in absorbance was observed and was most strongly observed with β47R. This drift was identified as reductive hydride transfer of DACA.

For these experiments to be pseudo-first order, kD must not be rate-limiting. The kD values, obtained by mixing enzyme, pre-equilibrated with 10 mM NAD+ for β47R or 60 mM for β47H, with 150 μM DACA and 10 mM 1-butanol, did not limit the reaction sequence at any pH with β47H, but did limit the reaction with β47R (Table I). The apparent kD values with β47R was therefore determined by monitoring intrinsic fluorescence (excitation = 280 nm, 5 nm slit; emission filter > 320 nm), mixing enzyme with varying NAD+ concentrations in the absence of butanol and DACA.

**Steady-state Kinetics**

Steady-state kinetics were performed on a Gilford Response spectrophotometer. The Vmax for acetaldelyde reduction was obtained by varying NADH in the presence of excess acetaldelyde. Enzyme (0.2 to 5.2 μg/ml) was preincubated 3 min at 25°C with 3 μM NADH to 1.7 mM NADH (based on affinity) for β47H or with 0.2 to 60 μM NADH for β47R before initiating the reaction with 10 μM acetaldelyde for β47H or 1 mM acetaldelyde for β47R. 17 to 26 data points at 8 to 13 NADH concentrations were used for each experiment.

Estimates of Vmax for ethanol oxidation were obtained by varying NADH and ethanol simultaneously. With β47R, NAD+ and ethanol concentrations were varied from 10 to 300 μM, and 80 μM to 2 mM, respectively, using 39 to 58 data points. With β47H below pH 9, NAD+ and ethanol concentrations were varied from 50 μM to 1.6 mM and 50 μM to 15 mM, respectively, using 34 to 64 data points; at
higher pH values, NAD\textsuperscript{+} concentrations ranged from 2 to 15 mM. Liver enzyme or enzyme expressed in \textit{E. coli} (0.5 to 13 \mu g/ml) was preincubated with ethanol for 2 to 3 min at 25 °C before initiating the reaction with NAD\textsuperscript{+}. Kinetics from either source were identical.

Estimates of initial rates were obtained from linear regression fits over a reaction period of 1 to 4 min, monitoring NADH production at 340 nm. Less than 10% of the substrate was used over the reaction time course of 1 to 4 min. Initial rates were analyzed by nonlinear regression, assuming a standard Michaelis-Menten or Sequential mechanism (21, 22). Regressions were analyzed for goodness-of-fit by residual analysis. All \( V_{\text{max}} \) values were corrected for any decrease in activity and converted to units of time with the specific activities listed above and assuming 40 mg/m\text{mol}/min. Ethanol oxidation of \( \beta 47R \) was not examined below pH 7.5 because the enzyme activity was too low to obtain reproducible kinetic constants.

\textbf{Apparent pK Values}

Apparent pK values were estimated with the NLIN regression program of SAS (Cary, NC). This program requires input of a nonlinear model, initial estimates of each parameter, and partial first derivatives of the model with respect to each parameter (23). Apparent pK values for \( k^\text{NADH} \), \( k^\text{NAD\textsuperscript{+}} \), and \( k^\text{NADH} \) with \( \beta 47Q \) were determined by nonlinear regression of the equation \( r = \frac{r}{(1 + K/H^+)} \), where \( r \) = limiting rate at low pH. Apparent pK values for \( k^\text{NAD\textsuperscript{+}} \) were determined by regression against the equation \( r = \frac{r}{(1 + H^+/K)} \), where \( r \) = limiting rate at high pH. These models assume that rate changes correspond to a single ionization. Apparent pK values for \( k^\text{NAD\textsuperscript{+}} \) with \( \beta 47R \) were determined by nonlinear regression of the equation \( r = \frac{r}{(1 + K/K_H)} \), where \( r \) = limiting rate at low pH and \( m \) = limiting rate at high pH. This model assumes that rate changes correspond to a single ionization, with limiting rates at low and high pH. Apparent pK values for \( k^\text{NAD\textsuperscript{+}} \) with \( \beta 47H \) were determined by regression of a model assuming two ionizations, rate \( r = \frac{r}{(1 + H^+/K_1 + K_2/H^+)} \), where \( K_1 \) and \( K_2 \) occur at low and high pH, respectively. Apparent pK values for \( k^\text{NAD\textsuperscript{+}} \) with \( \beta 47H \) were determined with the model rate \( r = \frac{r}{(1 + H^+/K)} \). Each nonlinear regression was analyzed by standard error, F-statistic, and Durbin-Watson statistic.

\textbf{RESULTS}

\textbf{DACA Binding to the Enzyme-Coenzyme Binary Complex—} The UV-visible spectrum of the chromophoric substrate DACA when bound in a ternary complex with human \( \beta 47R \) and NADH is shown in Fig. 1. The absorbance maximum of DACA shifted from 400 nm when free in solution to 457 nm when bound in the ternary complex. These spectra were similar to those reported with the horse enzyme, in which the maximal absorbances of the free and ternary complex were 398 nm and 464 nm, respectively (17). Spectra of the DACA complexes with \( \beta 47H \) were identical with those with \( \beta 47R \), and no bound DACA was observed in the absence of NADH.

\textbf{Coenzyme Dissociation Rate Constants—} Apparent NAD\textsuperscript{+} dissociation rates, \( k^\text{NAD\textsuperscript{+}} \), with \( \beta 47R \) gave a pH-invariant value of approximately 0.07 s\textsuperscript{-1} from pH 6 to 9 (Fig. 2A); above pH 9, the rates increased. The pH-profile with \( \beta 47K \) was similar to that with \( \beta 47R \), but the rates with the lysine mutant were slightly faster at low pH and showed a greater increase above pH 9. From pH 6 to 10, horse alcohol dehydrogenase exhibited \( k^\text{NAD\textsuperscript{+}} \) values that were pH-independent, but that were more than 10 times faster than those values with \( \beta 47R \) and \( \beta 47K \).

The \( k^\text{NAD\textsuperscript{+}} \) values determined with \( \beta 47H \) and \( \beta 47Q \) were identical above pH 8 and were much faster than the values obtained with \( \beta 47R \) (Fig. 2B). At pH 10, the rate with \( \beta 47H \) and \( \beta 47Q \) was 15 s\textsuperscript{-1}, a value 100 times faster than that obtained with \( \beta 47R \). Below pH 8, the \( k^\text{NAD\textsuperscript{+}} \) values with \( \beta 47H \) decreased and fit the pH-profile expected for a single ionizable group with an apparent pK of 7.0 ± 0.2. The values determined with \( \beta 47Q \) below pH 8 remained at 15 s\textsuperscript{-1}.

Apparent NAD\textsuperscript{+} dissociation rate constants, \( k^\text{NAD\textsuperscript{+}} \), with \( \beta 47R \) decreased about 10-fold from pH 6 to 10 (Fig. 3A). The data fit a model for a single ionizable group involving a pH-dependent transition between limiting values of \( k^\text{NAD\textsuperscript{+}} \) at low and high pH (apparent pK value = 7.4 ± 0.1). Values of \( k^\text{NAD\textsuperscript{+}} \) determined with \( \beta 47K \) were slightly faster than those determined with \( \beta 47R \), but produced a pH-profile similar to that with \( \beta 47R \) (apparent pK value = 6.8 ± 0.1). The \( k^\text{NAD\textsuperscript{+}} \) values determined for \( \beta 47R \) and \( \beta 47K \) were nearly an order of magnitude slower than the values determined for horse alcohol dehydrogenase between pH 6 and 10. The rates with the horse enzyme showed no tendency to approach a minimum

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Absorbance spectra of human alcohol dehydrogenase with DACA. Absorbance scans of \( \beta 47R \) (72 \mu g active sites) with 7 \mu M DACA were determined in the absence (solid lines) or presence (dashed lines) of 0.13 mM NADH at pH 8. The absorbance at 405 nm (DACA\textsubscript{free}) is 0.36, at 457 nm (DACA\textsubscript{bound}) is 0.17, and at 330 nm (NADH\textsubscript{bound}) is 0.94.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Dependence of apparent NAD\textsuperscript{+} dissociation rate constants on pH. In A, apparent NAD\textsuperscript{+} dissociation rate constants with \( \beta 47R \) (\( \bullet \), \( \beta 47K \) (\( \circ \)), and horse liver alcohol dehydrogenase (\( \circ \)) were measured at different pH values. In B, apparent NAD\textsuperscript{+} dissociation rate constants with \( \beta 47H \) (\( \triangle \)) and \( \beta 47Q \) (\( \triangledown \)) are shown. A nonlinear regression fit of the \( \beta 47H \) data (solid line) produced pK = 7.0 (± 0.2) and \( r = 9.9 (± 1.9) \) s\textsuperscript{-1}. Data points of \( \beta 47Q \) are connected by dashed lines. The fitted pH-profile of \( \beta 47R \) (\( \times \)) is included for reference (dotted line). Standard errors do not reach beyond the symbols.}
\end{figure}
plateau at high pH. The apparent pK value determined from the pH dependence of $k^{D+}$ for horse alcohol dehydrogenase was 7.7 ± 0.1.

The $k^{D+}$ values determined with β47Q exhibited a pH-profile similar to that with horse alcohol dehydrogenase, producing an apparent pK value of 8.2 ± 0.2 (Fig. 3B). The values of $k^{D+}$ with β47Q were 100 times faster at low pH and 50 times faster at high pH than those with β47R. The rates with β47H above pH 8 were similar to those of β47Q. Below this pH, however, $k^{D+}$ values decreased as the pH approached the expected solution pK of the histidine imidazole. The data fit best a model involving two ionizations with a limiting value of 7.7 ± 0.2 for P47R and 8.5 ± 0.2 for P47K. The nonlinear regression analysis of the enzyme fit a single ionization with a single limiting rate at low pH and produced $k^{D+} = 7.7 \pm 0.1$ and $\tau = 162 \pm 20 \mu M^{-1} s^{-1}$. In B, the pH dependence of the apparent NAD$^+$ dissociation rate constants with β47H (Δ) and β47Q (△) are shown. Nonlinear regression fits of a single ionization with a limiting value at intermediate pH and produced $k^{D+} = 6.7 \pm 0.2$ and $\tau = 222 \pm 90 \mu M^{-1} s^{-1}$ for β47Q. The data of β47H fit two ionizations with a limiting value at intermediate pH and produced $k^{D+} = 7.6 \pm 0.3$ and $\tau = 136 \pm 41 \mu M^{-1} s^{-1}$. The fitted pH-profile of β47R is included as a function of pH. The pH-profiles fit nonlinear regression models of a single ionization and a single limiting rate at intermediate pH produced $k^{D+} = 6.7 \pm 0.2$ and $\tau = 222 \pm 90 \mu M^{-1} s^{-1}$ for β47Q. The data of β47H fit two ionizations with a limiting value at intermediate pH and produced $k^{D+} = 7.6 \pm 0.3$ and $\tau = 136 \pm 41 \mu M^{-1} s^{-1}$. The fitted pH-profile of β47R is included as a function of pH. The pH-profiles fit nonlinear regression models of a single ionization and a single limiting rate at intermediate pH produced $k^{D+} = 6.7 \pm 0.2$ and $\tau = 222 \pm 90 \mu M^{-1} s^{-1}$ for β47Q. The data of β47H fit two ionizations with a limiting value at intermediate pH and produced $k^{D+} = 7.6 \pm 0.3$ and $\tau = 136 \pm 41 \mu M^{-1} s^{-1}$. The fitted pH-profile of β47R is included as a function of pH. The pH-profiles fit nonlinear regression models of a single ionization and a single limiting rate at intermediate pH produced $k^{D+} = 6.7 \pm 0.2$ and $\tau = 222 \pm 90 \mu M^{-1} s^{-1}$ for β47Q. The data of β47H fit two ionizations with a limiting value at intermediate pH and produced $k^{D+} = 7.6 \pm 0.3$ and $\tau = 136 \pm 41 \mu M^{-1} s^{-1}$.
dependent on a single ionizable group with an apparent pK of 8.1 ± 0.2. The data with β47K did not differ from those with β47R (data not shown). The pH-profile of k$AD'H with β47H was similar to that with β47R (pK = 7.7 ± 0.2), and the rates were approximately 5 times slower at all pH values than the corresponding values with β47R. Rates with β47H were the same as those with β47Q (data for β47Q not shown).

Apparent association rate constants for NAD+, $k_{AD'}^A$, with β47R were approximately 1.4 ± 0.5 μM⁻¹s⁻¹ from pH 6 to pH 7.5 (Fig. 5B); the values dropped to 0.007 μM⁻¹s⁻¹ at pH 10. The dependence fit a single ionization with an apparent pK of 7.8 ± 0.2. The apparent rate constants determined with β47K were the same (within limits of error) as those with β47R (data not shown). Values of $k_{AD'}^A$ determined for β47H and β47Q did not differ from each other (data for β47Q not shown), and the rates showed pH-profiles similar to that seen with β47R (apparent pK value = 8.4 ± 0.1). At low pH, the $k_{AD'}^A$ values measured with β47H and β47Q were nearly 10 times slower than those measured with β47R. At high pH, these three mutants exhibited identical values.

The equilibrium dissociation constant for NADH binding with β47R, $K_{AD''}^N$, calculated from the ratio $K_{AD''}^N/K_{AD}'$ at pH 6, was 20 times less than the corresponding values determined with β47H and over 200 times less than that determined with β47Q (Table II). Above pH 8, the NADH affinity with β47H and β47Q were similar, but the $K_{AD'}^N$ values obtained with both enzymes were 200 to 600 times higher than those values with β47R. The $K_{AD'}^N$, calculated from the ratio $K_{AD''}^N/k_{AD'}^N$, with β47R was approximately 2 μM from pH 6 to pH 9 and increased to 19 μM at pH 10 (Table II). The $K_{AD'}^N$ with β47H was 48-fold greater than the value determined with β47R at pH 6, and the difference increased to over 400-fold at pH 9. At pH 6, the ratio measured with β47Q was 10-fold higher than that measured with β47H, but at higher pH the two mutants gave similar values.

**DACA Limiting Hydride Transfer Rates**—Hydride transfer rates, measured at saturating DACA concentrations, $k_{AD'ACCA}$, with all mutants were faster at low pH than at high pH (Table III). At pH 6.5, the rate with β47H was 10-fold faster than that with β47R. At this pH, the rate with β47Q was 175-fold faster than that with β47R and similar to that reported with horse alcohol dehydrogenase (18).

**Steady-state Kinetics**—With acetaldehyde as a substrate, the $V_{max}$ values with β47R decreased from 6 s⁻¹ at low pH to 0.1 s⁻¹ at high pH and coincided with the fitted curve of $k_{AD'}^A$ across the pH range studied (Fig. 6A). The $V_{max}$ values with β47H were nearly identical with the corresponding $k_{AD'}^A$ values except below pH 7, at which the observed values deviated from the fitted $k_{AD'}^A$ values. Over the pH range examined, the $V_{max}$ values for ethanol oxidation with β47R and β47H were identical with $k_{AD'}^A$ values (Fig. 6B).

**DISCUSSION**

Steady-state kinetics of human liver ββH, (β47R), ββH (β47H), and the β47K and β47Q mutants showed that when strong bases at position 47 (arginine or lysine) were substituted by a weak base (histidine) or a neutral side chain (glutamine), the $K_m$ and $K_v$ values for coenzymes were increased (6). It was not known, however, which individual kinetic rate constants were altered by this perturbation. Using stopped-flow kinetics, we find that both the apparent coenzyme dissociation rate constants and the apparent DACA hydride transfer rate constants are greatly altered by these mutations. We also observe that the pH-profiles of these apparent rate constants are altered.

The values of $k_{AD''}^A$ and $k_{AD''}^N$ determined with the human alcohol dehydrogenase mutants increase considerably when the arginine or lysine at position 47 (arginine or lysine) were substituted by a weak base (histidine) or a neutral side chain (glutamine), the $K_m$ and $K_v$ values for coenzymes were increased (6). It was not known, however, which individual kinetic rate constants were altered by this perturbation. Using stopped-flow kinetics, we find that both the apparent coenzyme dissociation rate constants and the apparent DACA hydride transfer rate constants are greatly altered by these mutations. We also observe that the pH-profiles of these apparent rate constants are altered.

**TABLE II**

Coenzyme equilibrium dissociation constants

<table>
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<th>pH</th>
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<th>β47H</th>
<th>β47Q</th>
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<tbody>
<tr>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>10</td>
<td>2.0</td>
<td>19</td>
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</table>

**TABLE III**

DACA hydride transfer rates, $k_{AD'ACCA}$

Values of $k_{AD'ACCA}$ were obtained as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>pH</th>
<th>β47R</th>
<th>β47H</th>
<th>β47Q</th>
</tr>
</thead>
<tbody>
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<td>s⁻¹</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>3.0</td>
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<td>8</td>
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</table>

*ND, not determined because $k_{AD''}^N$ rates were rate-limiting with this mutant at this pH.

**Fig. 6.** $V_{max}$ for ethanol oxidation and acetaldehyde reduction. The pH dependences of $V_{max}$ for ethanol oxidation with β47R (●) and β47H (△) are shown as A. Profiles of fitted $k_{AD'}^A$ values with the enzymes are also included (dashed lines). In B, the pH dependences of $V_{max}$ for ethanol oxidation with β47R (●) and β47H (△) are shown. Profiles of $k_{AD'}^N$ with β47R and β47H are also included (dotted and respectively dashed lines). Except where indicated, standard errors do not reach beyond the symbols.
charge at position 47 of the yeast alcohol dehydrogenase does not affect the pH-profile of $k_{\text{ADV}}^{\text{NADH}}$. The importance of position 47 in coenzyme dissociation is most obvious with the $\beta 47H$ mutant, in which an apparent pK value of 6.7 to 7.0 influences the value of $k_{\text{ADV}}^{\text{cozyme}}$, but not that of $k_{\text{ADV}}^{\text{enzym}}$. Since this apparent pK value is only observed in the histidine mutant, we assign this pH transition to the deprotonation of His-47 (Figs. 2 and 3).

The increase in $k_\text{on}$ values with $\beta 47Q$ versus $\beta 47R$ range from 300- to 500-fold at most pH values (Table II). At physiological pH, a $\Delta G$ value of about 3.4 to 3.7 kcal/mol can be calculated from this increase ($\Delta G = [1.9 \times 10^{-7} \text{ kcal-}\text{mol}^{-1}\cdot\text{K}^{-1}][298 \text{ K}][\ln(K_{\text{P}}/K_{\text{R}})]$). This value for $\Delta G$ is reasonably expected by the change in a single charge interaction, assuming that the charge is located in a dipolar environment (with effective dielectric constant = 20) and assuming that the distance between charges is 4.8 Å (force = $(4.8 \times 10^{-9} \text{ electronostatic units})(1.4 \times 10^{-6} \text{ kcal-}\text{mol}^{-1}\cdot\text{cm}^{-1})(4.8 \times 10^{-6} \text{ cm})(20)$). This calculation suggests that at neutral pH, ionic interactions can account for the differences in coenzyme affinity between $\beta 47Q$ and $\beta 47R$. The observed increase in $k_{\text{ADV}}^{\text{cozyme}}$ values with $\beta 47Q$ versus $\beta 47R$ accounts for nearly all the changes in $k_\text{on}$ values; $k_{\text{on}}^{\text{cozyme}}$ increases only 2-fold (Figs. 2 and 3).

The oxidized and reduced coenzymes interact with the human mutants differently. First, the charge on the nicotinamide ring of NAD$^+$ increases $k_{\text{ADV}}^{\text{NAD}}$ values and slows $k_{\text{ADV}}^{\text{cozyme}}$ values (Figs. 2 and 3). The enzyme affinity for NAD$^+$ is therefore less than that for NADH (Table II). Second, the pH dependence of $k_{\text{ADV}}^{\text{NAD}}$ values is very different from that of $k_{\text{ADV}}^{\text{NADH}}$ values (Figs. 2 and 3). The human enzymes with an arginine or lysine at position 47 have a lower apparent pK value for $k_{\text{ADV}}^{\text{NAD}}$ than that with a histidine or glutamine at this position (Fig. 3). This finding suggests that a positive charge at position 47 facilitates a deprotonation event that is important for strong coenzyme affinity.

The results presented here do not elucidate the constituent(s) causing the pH dependence for $k_{\text{ADV}}^{\text{NAD}}$ or for $k_{\text{ADV}}^{\text{NADH}}$ and $k_{\text{ADV}}^{\text{cozyme}}$. The hypothesis that zinc-bound water mediates coenzyme binding in the horse enzyme (20, 25, 26), however, is consistent with the results of these human mutants. The zinc-bound water molecule in the coenzyme-free enzyme may exhibit a pK value of 7.8 with $\beta 47R$ and $\beta 47K$ and 8.4 with $\beta 47H$ and $\beta 47Q$ (Fig. 5). When coenzyme binds, that pK value becomes sensitive both to the charge on the nicotinamide ring(s) causing the pH dependence for $k_{\text{ADV}}^{\text{cozyme}}$ values. Since the mutations at position 47 do not perturb the spectrum of inner sphere-coordinated DACa within the enzyme/coenzyme, Daca complex, we conclude that the strength of the bonding interaction between Daca and the active site zinc ion is not altered. Therefore, it would appear that the Lewis acid strength of the catalytic metal ion is unchanged (27). Because catalysis of hydride transfer requires activation of both the aldehyde carbonyl and the 1,4-dihy- drodinitocarnamide ring of NADH, it seems likely that the enhanced hydride transfer rates of $\beta 47Q$ and $\beta 47H$ result from perturbations that activate bound NADH. Considerable development of positive charge on the nicotinamide ring would occur in the transition state for hydride transfer. The low hydride transfer rate of the $\beta 47R$ species may result from a destabilizing interaction associated with the positively charged side chain and the developing charge on the nicotinamide ring in the activated complex. Mutation to histidine or glutamine would lower the activation energy by alleviating this unfavorable interaction.

The $k_{\text{ADV}}^{\text{cozyme}}$ values with the human $\beta 47R$ enzyme are at least 10-fold slower at all pH values than those with horse alcohol dehydrogenase, which also contains an Arg-47. The $k_{\text{ADV}}^{\text{cozyme}}$ values with $\beta 47R$ are also slower than those with the horse enzyme, and the apparent pK value for $k_{\text{ADV}}^{\text{cozyme}}$ is shifted to a lower value by nearly 2 pH units. Therefore, although the human and horse enzymes function similarly, Arg-47 influences kinetics of the enzymes differently. In fact, $k_{\text{ADV}}^{\text{cozyme}}$ and $k_{\text{ADV}}^{\text{DACa}}$ values with horse alcohol dehydrogenase are more similar to those with the $\beta 47H$ and $\beta 47Q$ mutants than with $\beta 47R$. The x-ray crystal structure of the human $\beta 47R$, (NAD$^+$) binary complex differs from the horse(NAD(H)-dimethyl sulfoxide) complex in two distinct ways (10,11). First, the residues at the end of an $\alpha$-helix (residues 202–204) are oriented such that the hydrogen bonding with the NAD$^+$ pyrophosphate group is more optimal in the $\beta 47L$ subunit than in the horse subunit. Second, whereas Arg-47 in the horse enzyme is hydrogen-bonded to both Asp-50 and the pyrophosphoryl moiety of NAD(H), the Arg-47 e-nitrogen of $\beta 47L$ interacts solely with the pyrophosphate group. These cumulative effects appear to account for the differences between human $\beta 47R$ and horse alcohol dehydrogenase with respect to $k_{\text{ADV}}^{\text{cozyme}}$ values. The differences between the two enzymes in $k_{\text{ADV}}^{\text{cozyme}}$ values are less dramatic.

Sekhar and Plapp observed a saturation of apparent first order association rates in the binding of NAD$^+$ to horse alcohol dehydrogenase (28), and, based on existing steady-state evidence (29), they hypothesized that this phenomenon represented a kinetically significant enzyme(NAD$^+$) isomerization. We have seen a similar saturation phenomenon for apparent first order association rates of NADH with $\beta 47H$ and $\beta 47Q$ at pH 10 (Fig. 4). This is the first stopped-flow kinetic evidence that an isomerization occurs with the human enzymes and that an isomerization of the enzyme(NADH) complex occurs with alcohol dehydrogenases. The data indicate that a kinetically significant step, $k_i$, exists between NADH association, $k_1$, and Daca association, $k_2$ (see Scheme I). This could represent the kinetic equivalent of an open to closed conformational change, which has been suggested by x-ray structures of the coenzyme-free and coenzyme-bound horse alcohol dehydrogenase complexes (10,30). At pH 10, $k_i$ becomes rate-limiting with $\beta 47H$ and $\beta 47Q$ at a high NADH concentration. If such an isomerization also occurs with $\beta 47R$ or $\beta 47K$, then the positive charge at position 47 must increase $k_i$ to rates that are faster than can be detected by the stopped-flow instrument.

From Scheme I, apparent $k_{\text{ADV}}^{\text{NADH}}$ values do not represent $k_i$,
but instead represent the product of the NAD equilibrium association constant ($k_{1}/k_{-1}$) and $K_i$. From these studies, we are unable to determine whether the pH dependence of apparent $k_{1}^{NADH}$ values (Fig. 5) is caused by a pH dependence of $K_i$ or of $k_{1}/k_{-1}$, or of both. This ambiguity is represented in Scheme I, by allowing enzyme deprotonation to occur with several enzyme species (movement from the upper to the lower pathway in Scheme I). Dependence of apparent $k_{1}^{NADH}$ values on pH is caused by deprotonation of either the free enzyme species ($H^{+}E_{0} \rightarrow E_{0}$) or the open enzyme (NADH) complex ($H^{+}E_{0}(NADH) \rightarrow E_{0}(NADH)$).

With horse alcohol dehydrogenase, $V_{max}$ values for acetaldehyde reduction and ethanol oxidation were shown with stopped-flow kinetics to be limited by coenzyme dissociation (19, 20). We found with the human enzymes that the $V_{max}$ for acetaldehyde reduction and ethanol oxidation with $\beta 47R$ and $\beta 47H$ also coincide with $K_{1}^{NADH}$ and $k_{1}^{NADH}$ values, respectively (Fig. 6). This result indicates that with $\beta 47R$ and $\beta 47H$, coenzyme dissociation is rate-limiting for acetaldehyde reduction and ethanol oxidation.

The pH-profile of $V_{max}$ values for ethanol with $\beta 2\beta 2$, or $\beta 47H$ (Fig. 6B), does not coincide with the pH-optimum of activity (pH 8.5) reported with the $\beta 2\beta 2$ isoenzyme (4). In these studies, the pH optimum was obtained with assays containing 2.4 mM NAD$. The $K_{i}$ value for NAD$, however, increases from 0.3 mM at pH 8 to over 12 mM at pH 10 (data not shown). Therefore, the decrease in activity above pH 8.5 was not due to a decrease in $V_{max}$, but was due to decreased coenzyme affinity. The pH-profile of $V_{max}$ is accurately described in Fig. 6B, in which the enzyme remains optimally active above pH 8.5.

Asian individuals who are homozygous for $\beta 2$ metabolize ethanol at significantly higher rates than those who are heterozygous for $\beta 2$ (31). In this in vitro kinetic study, we find that the differences in coenzyme affinity and $V_{max}$ values for ethanol and acetaldehyde with human $\beta 2\beta 2$ and $\beta 2\beta 2$ alcohol dehydrogenase are explained by changes in apparent coenzyme dissociation rate constants. Other steady-state parameters, however, such as $K_i$ values for coenzyme and ethanol, and $K_i$ values for products, may be affected by hydride transfer rates. Whereas the constituents mediating apparent coenzyme association rate constants are altered only slightly by mutations at position 47, the constituents mediating apparent coenzyme dissociation rate constants and apparent DACA hydride transfer rate constants are greatly affected by charge at this position.

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