The three-dimensional structure of the human $\beta_3\beta_3$ dimeric alcohol dehydrogenase ($\beta_3$) was determined to 2.4 Å resolution. $\beta_3$ was crystallized as a ternary complex with the coenzyme NAD$^+$ and the competitive inhibitor 4-iodopyrazole. $\beta_3$ is a polymorphic variant at ADH2 that differs from $\beta_1$ by a single amino acid substitution of Arg-369 $\rightarrow$ Cys. The available x-ray structures of mammalian alcohol dehydrogenases show that the side chain of Arg-369 forms an ion pair with the NAD(H) pyrophosphate to stabilize the E-NAD(H) complex. The Cys-369 side chain of $\beta_3$ cannot form this interaction. The three-dimensional structures of $\beta_3$ and $\beta_1$ are virtually identical, with the exception that Cys-369 and two water molecules in $\beta_3$ occupy the position of Arg-369 in $\beta_1$. The two waters occupy the same positions as two guanidino nitrogens of Arg-369. Hence, the number of hydrogen bonding interactions between the enzyme and NAD(H) are the same for both isoenzymes. However, hydrogen bonding between the enzyme and guanidino nitrogens of Arg-369. Hence, the number of side chain. The equilibrium dissociation constants of $\beta_3$ differ from $\beta_1$ by the loss of the electrostatic interaction between the NAD(H) pyrophosphate and the Arg-369 side chain. The equilibrium dissociation constants of $\beta_3$ for NAD$^+$ and NADH are 350-fold and 4000-fold higher, respectively, than those for $\beta_1$. These changes correspond to binding free energy differences of 3.5 kcal/mol for NAD$^+$ and 4.9 kcal/mol for NADH. Thus, the Arg-369 $\rightarrow$ Cys substitution of $\beta_3$ isoenzyme destabilizes the interaction between coenzyme and $\beta_3$ alcohol dehydrogenase.

Alcohol dehydrogenase (ADH, EC 1.1.1.1) catalyzes the rate-limiting step in the oxidation of ethanol (1, 2). Human ADH isoenzymes are dimeric enzymes that contain one structural and one catalytic zinc per subunit. Human ADH isoenzyme subunits have between 373 and 379 amino acids and are encoded by the ADH1 (a), ADH2 (b), ADH3 (c), ADH4 (d), ADH5 (e), ADH6, and ADH7 (f) genes (3, 4). The $\alpha$, $\beta$, and $\gamma$ ADH subunits share greater than 93% sequence identity and form a complex group of homodimeric and heterodimeric isoenzymes. These isoenzymes have relatively high catalytic efficiency for ethanol oxidation and account for the majority of ethanol oxidation by the liver (3, 5–6).

Polymorphism at the ADH2 gene gives rise to the $\beta_1$, $\beta_2$, and $\beta_3$ subunits (3, 4). These polymorphic variants are the result of single amino acid substitutions. Arg-47 is substituted by His in $\beta_2$, and Arg-369 is substituted by Cys in $\beta_3$ (7, 8). The $\beta_2$ (ADH2*2) and $\beta_3$ (ADH2*3) alleles are observed in about 65% of Japanese and Chinese, and 25% of African-Americans, respectively (5, 9). The three human $\beta_3$ isofoms have considerably different steady-state kinetic properties (10). The $\beta_3$ isoenzyme exhibits a 50-fold greater $K_m$ for NAD$^+$, an 100-fold greater V$\max$ for ethanol oxidation, and a decreased pH optimum for ethanol oxidation, relative to $\beta_1$ (Table I) (11).

Three-dimensional structures have been determined for horse liver EE ADH as an apoenzyme and complexed with a variety of substrates or inhibitors (12, 13). Structures of $\beta_1$, complexed with NAD(H) and cyclohexanol, $\beta_3$, complexed with NAD$^+$ and 4-iodopyrazole, and 47Gly complexed with NAD$^+$ have been solved to 2.5 Å resolution (14). These structures show that residues 47 and 369 are located in the coenzyme binding cleft and provide electrostatic stabilization for coenzyme binding to both horse liver and human $\beta_3$ ADH (13, 14).

In this study, we report the structure of $\beta_3$ to 2.4 Å and the structure of $\beta_3$ to 2.2 Å in complexes with NAD$^+$ and 4-iodopyrazole. Structures of the $\beta_1$ and $\beta_3$ ternary complexes are compared in an effort to understand the molecular basis for differences in coenzyme binding affinity.

**MATERIALS AND METHODS**

Situated Mutagenesis—Single-stranded $\beta_1$ cDNA in the M13 HindIII vector (15) was used as a template for site-directed mutagenesis (Amersham) as described previously for $\beta_1$ mutants (16). The codon for Arg-369 (CGT) of the $\beta_1$ cDNA was mutated to TGT (Cys) using the oligonucleotide (5'-GGGAAAGATCTGTACCGTCCT-3'). After subcloning the $\beta_3$ cDNA into the plKZ223-3 expression plasmid (pKZ223-3), the coding sequence of the $\beta_3$ cDNA insert was completely sequenced with a Sequenase kit, version 2.0 (U.S. Biochemical Corp.) to verify that only the desired change was made in the $\beta_3$ cDNA sequence.

Enzyme Expression, Purification, and Crystallization—The $\beta_3$ plKZ223-3 expression plasmid was transformed into competent Escherichia coli TG-1 cells, and $\beta_3$ was expressed as described previously for $\beta_1$ ADH (16). $\beta_3$ was purified from an E. coli lysate in three chromatographic steps, DEAE-cellulose, S-Sepharose, and Affi-Gel Blue, as described for other $\beta_1$ ADH variants (17), except that zinc sulfate concentrations were increased to 0.1 molar in lysis and column buffers. Addition of zinc sulfate increased the enzyme's stability during purification (18).

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2. Recombinant homodimeric $\beta_1$, $\beta_2$, and $\beta_3$-ADHs are designated as $\beta_1$, $\beta_2$, and $\beta_3$ respectively. $\beta_1$ ADH with a Gly for Arg-47 mutation is designated as $\beta_1$.
Purified $\beta_b$ was dialyzed into 10 mM HEPES, pH 7.5, buffer containing 2 mM dithiothreitol. Enzyme purity was evaluated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (19), using a Hoefer Mighty Small apparatus (Hoefer Pharmacia Biotech, San Francisco, CA). Gels were stained using the alkaline silver stain method (20). Purified $\beta_b$ was concentrated with a Microcon 30 concentrator (Amicon, Beverly, MA) and $\beta_b$ protein was filtered through a sterile 0.2 μm cellulose-acetate membrane. Protein concentrations were determined using the Bio-Rad dye reagent protein assay (Bio-Rad) and bovine serum albumin as a standard (21).

Recombinant human $\beta_b$. 10 mg/ml, was crystallized as a ternary complex. Crystals were grown in 100 mM sodium phosphate, pH 7.5, 7.5 mM 4-iodopyrazole, 1 mM NAD$^+$, and 15.5% (w/v) polyethylene glycol 8000. The concentrations of NAD$^+$ and 4-iodopyrazole exceeded the $K_{\text{M, NAD}^+}$, by 9-fold and the $K_{\text{M, 4-iodopyrazole}}$, by 900-fold, respectively (Table I). $\beta_b$ was crystallized using CRYSTSMITH sitting drop plates (Charles Supper Co., Inc., Natik, MA) and equilibrium drop volumes of 3 μl. Crystals grew to maximal size in 4 to 5 days and were stable for up to 1 month. Recombinant human $\beta_b$ was expressed and purified as described previously (14). Crystals of the $\beta_b$-NAD$^+$-4-iodopyrazole complex with NAD$^+$ and 4-iodopyrazole were grown at a protein concentration of 15 mg/ml using equilibrium drop volumes of 4 μl. The crystalization buffer contained 50 mM sodium phosphate, pH 7.5, 2 mM NAD$^+$, 1 mM 4-iodopyrazole, and 13.5% (w/v) polyethylene glycol 8000.

X-ray Diffraction Data Collection and Processing—X-ray diffraction data were collected at 25°C on a Rigaku RAXIS image plate area detector equipped with a RAXIS IIC image plate area detector at a crystal-to-detector distance of 12.5 cm. The $\beta_b$ data collection was resolved to a resolution of 2.19 Å from two orientations of a single crystal. The $\beta_b$ data collection was resolved to a resolution of 2.19 Å from two orientations of a single crystal at a crystal-to-detector distance of 11 cm. RAXIS IIC data processing software (T. Hagashiki Corp., Japan) was used to index, merge, and scale the data sets.

Molecular Replacement Calculations and Structure Refinement—The enzyme and NAD$^+$ coordinates from the human $\beta_b$-NAD$^+$-cytochrome c oxidase ternary complex (14) were used directly to solve the structure of both the $\beta_b$ and $\beta_b$ ternary complexes. Refinements were performed using the CRYSTALS and X-PLOR (22). The $\beta_b$ and $\beta_b$ models were transferred from the original $\beta_b$ data (14). Each complex was oriented by rigid body refinement using the data between 8 and 2.8 Å. The resulting $R$-factors for the correctly oriented molecules were 21.5 and 28.7, for $\beta_b$ and $\beta_b$, respectively. Cys was substituted for Arg-369 in the $\beta_b$ model using CHAIN (23). The atomic positions were refined to 2.8 Å using the positional refinement protocol. $2F_o - F_c$ and $F_c - 2F_o$ maps were generated to 3.0 Å, and the maps were inspected with the program CHAIN. The position for 4-iodopyrazole was identified in the active sites and included in subsequent refinement steps. Independent refinement of each model was performed in stages with data of increasing resolution until all data between 8 Å and the limit of resolution (2.4 Å for $\beta_b$ and 2.2 Å for $\beta_b$ with Ficoll, greater than 1 were included. Water molecules were added independently to both models and inspected with the program CHAIN. The position for 4-iodopyrazole was identified in the active sites and included in subsequent refinement steps. Non-crystallographic symmetry restraints were removed during the last two rounds of refinement. Alignments of the backbone $\alpha$ carbons of $\beta_b$ and $\beta_b$ isoenzymes were performed with the programs O (Molecular Simulations Inc., San Diego, CA) and K-PLOR (22).

Steady-state and Stopped-flow Kinetics—Steady-state kinetic constants were evaluated in 0.1 M sodium phosphate buffer at pH 7.5 and 25°C, utilizing a Perkin Elmer Lambda 6 double beam spectrophotometer. Ethanol oxidation was followed by monitoring the production of NADH at 340 nm (molar extinction coefficient: 6.22 mM$^{-1}$ cm$^{-1}$). The inhibition by 4-methylpyrazole (K$^p_{\text{M,4-methylpyrazole}}$) exhibited by recombinant $\beta_b$ was calculated with Cleland’s programs (24). The inhibition kinetics were analyzed using both competitive and noncompetitive models. Since $K_p$ was 10 times $K_i$, in the noncompetitive fit, the competitive fit is reported in Table I.

Stopped-flow kinetics were evaluated in 15 mM PES, 15 mM Bisamide, pH 7.5, at 25°C. Data were collected on a HITech SF-51 instrument (17). Exponential traces were analyzed by HITech software. Linear regressions were analyzed with SAS (Cary, NC) and evaluations for goodness-of-fit were performed by comparing F-statistics and correlation coefficients.

**Table I**

| Constant          | $\beta_b$ | $\beta_b$ |
|-------------------|-----------|-----------|
| $K_{\text{M, NAD}^+}$ (μM) | 15        | 710       |
| $K_{\text{i,4-iodopyrazole}}$ (μM) | 0.60      | 1.1       |
| $V_{\text{max}}$ (s$^{-1}$) | 0.066     | 5.0       |
| $V_{\text{max}}/K_{\text{M, NAD}^+}$ (s$^{-1}$ μM$^{-1}$) | 1.3       | 0.14      |
| pH optimum$^a$    | 10.5      | 7.0       |

$^a$ $K_{\text{M, NAD}^+}$ and $V_{\text{max}}$ were determined as described (31).

**Table II**

| Data collection statistics |
|-----------------------------|
| $\beta_b$ | $\beta_b$ |
| NAD$^+$ | NAD$^+$ |
| 4-iodopyrazole | 4-iodopyrazole |
| Space group | P1       |
| Cell lengths a, b, c (Å) | 54.0, 44.4, 92.7 |
| Cell angles α, β, γ (degrees) | 92.7, 103.2, 69.2 |
| Number of observations | 53,103 |
| Number of unique reflections | 26,989 |
| Completeness | 86%  |
| Overall | 83%  |
| Highest resolution shell | 70% (2.51–2.4 Å) |
| Rmerge | 10%  |
| 10%  |
and the nicotinamide nitrogen are 3.9 Å, 6.7 Å, and 7.3 Å, respectively.

Solvent Accessibility Calculations—Solvent accessibility calculations for amino acid side chains were performed with QUANTA (Molecular Simulations Inc., Burlington, MA) using a spherical probe 2.8 Å in diameter (25).

| Table III | Refinement statistics |
|-----------|-----------------------|
|           | $\beta_3$ | $\beta_1$ |
| Data included (Å) | 8–2.4 | 8–2.2 |
| Number of reflections ($F/F_o > 1.0$) | 25,646 | 32,940 |
| R-factor (%) | 17.5 | 18.0 |
| Free R-factor (%) | 24.3 | 26.0 |
| Number of water molecules | 142 | 314 |
| R.m.s. deviation from ideal bond lengths | 0.008 Å | 0.009 Å |
| R.m.s. deviation from ideal bond angles | 1.65° | 1.68° |
| Luzatti estimate of coordinate error | 0.23 Å | 0.21 Å |

RESULTS

Purified recombinant $\beta_3$ exhibited a single band on a sodium dodecyl sulfate-polyacrylamide gel at the expected subunit molecular mass of 40 kDa (data not shown). Eighty milligrams of purified $\beta_3$ were routinely obtained from 9.8-liter cultures. These yields are similar to those obtained for $\beta_1$ (14). The specific activity of purified recombinant $\beta_3$ at 2.5 mM NAD$^+$ and 66 mM ethanol, 3.8 units/mg, was higher than that of purified $\beta_3$ from human liver obtained at autopsy, 2.8 units/mg (11). The $K_M$ for NAD$^+$, $K_i$ for 4MP, $V_{max}/K_M$ for ethanol oxidation, and pH optimum of recombinant $\beta_3$ are similar to $\beta_1$.

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Fig. 1. Electron density in the vicinity of residue 369. Omit 2$F_o - F$ electron density maps for (A) $\beta_3$ ADH and (B) $\beta_1$ ADH. Waters which occur between the amino acid at position 369 and coenzyme molecule are also displayed. The electron density maps were calculated after first removing those atoms displayed in the figure from the structure factor calculation. The maps are contoured at 1 S.D. of their respective electron density.
those reported for liver β3 (Table I).

The crystallization conditions for β3 were similar to those for the β1, β47Gly, and β2 isoenzymes (14, 26). β1 and β3 were crystallized in the presence of NAD⁺ and 4-iodopyrazole. Large β3 crystals, which diffracted to 2.4 Å, could be obtained from high specific activity enzyme within 4 days of enzyme preparation. X-ray diffraction data were collected to 2.37 Å for all Cα atoms within the coenzyme domain, and 0.20 Å for the catalytic domain of the A subunits; the corresponding values for alignment of the B subunits are 0.25, 0.19, and 0.24 Å.

The equilibrium binding differences were used to calculate the k_{off,NAD⁺} and k_{off,NADH} rate constants with β3 are 19- and 330-fold faster, respectively, than with β1. These differences result in 350- and 4000-fold increases in the K_{D,NAD⁺} and K_{D,NADH} values for β3 relative to β1. The equilibrium binding differences were used to calculate the free energy differences (ΔΔG) in coenzyme binding. These calculations show that NAD⁺ and NADH bind to β3 3.5 and 4.9 kcal/mol, respectively, less favorably than to β1.

**DISCUSSION**

The structure of β3 was determined to 2.4 Å resolution and that of β1 was extended to 2.2 Å. The final β3 model, containing 142 ordered water molecules and individual restrained isotropic temperature factors for all atoms, possesses a R-factor of 17.5%, a free R-factor of 24.3%, and good stereochemistry (Table III). The corresponding structure of β1 contains 314 water molecules, individual restrained isotropic temperature factors for all atoms, and a R-factor of 18.0% with a free R-factor of 26.0%. The structure of the β3 ternary complex with NAD⁺ and 4-iodopyrazole was determined in order to ensure that any differences between the two enzyme structures were not due to differences in the type of ligands bound in the substrate binding site.

An alignment of the β3 structure to the β1 structure indicates that they are virtually identical. The r.m.s. deviation of the alignments were similar to the Luzatti estimate of the coordinate error (Fig. 2 and Table III). The only substantial difference between the structures is found in the pyrophosphate anion binding site where the Arg-369 → Cys substitution occurs (Fig. 2). In β3, the Cys-369 side chain and two ordered water molecules occupy the space vacated by the side chain of Arg-369. These water molecules occupy similar positions and form the same hydrogen bonds as two of the guanidino nitrogens of Arg-369 (Fig. 2). The data suggest that the large changes in NAD⁺ and NADH binding affinity for β3 are not due to extensive structural rearrangements of local side chain conformations or movements of whole domains (Table III and Fig. 2), as was observed for β47Gly (14). β47Gly has no side chain at position 47 as a result of the Arg-47 → Gly substitution. Domain closure may be favorable in β47Gly since Gly-47 cannot form electrostatic or hydrogen bonding interactions. The Arg-47 → His substitution in β2 and the Arg-369 → Cys sub-

*Fig. 2. Aligned active sites of human β1 and β3 ADH. A stereo diagram of human β3 (thick lines) and β1 (thin lines) structures in the vicinity of amino acid 369. The structures were aligned by superposition of all their respective Cα atoms. R.m.s differences for alignment of β3 to β1 are 0.25 Å for all Cα atoms. Alignments of individual subunits or domains yielded r.m.s values of 0.19 Å for the Cα atoms within subunit A, 0.17 Å for the Cα atoms within the coenzyme domain, and 0.20 Å for the catalytic domain of the A subunits; the corresponding values for alignment of the B subunits are 0.25, 0.19, and 0.24 Å.*


in the Arg-47 Gly substitution because they do not cause major differences in the overall structure (Table III). Yet, from a functional viewpoint, the Arg-47 Gly substitution appears to be a more conservative substitution because it has only a 2-fold effect on coenzyme binding, relative to β3 (29).

In the β3 structure, the positively charged side chain of Arg-369 interacts with the negatively charged coenzyme pyrophosphate. The neutral thiol of Cys-369 of β3 cannot form a similar ion pair with a pyrophosphate oxygen. Thus, the difference in the coenzyme binding free energies between β3 and β1 isoenzymes may be due to the loss of the electrostatic interaction between Arg-369 and the coenzyme molecule. The difference in the free energy for NAD+ binding to β3 versus β1 (3.5 kcal/mol) is within the range of values calculated from the electrostatic differences in the enzyme/NAD(H) complexes alone (2.2–8.9 kcal/mol), using Coulomb's Law and a dielectric constant of either 40 or 10 (28). Solvent accessibility calculations show that Arg-369 of β3 cannot form a similar ion pair with a pyrophosphate oxygen. Thus, the differences in the Arg-369 interactions with the coenzyme molecule are due to the electrostatic repulsion between the positively charged side chain of Arg-369 and the negatively charged pyrophosphate oxygen. Thus, the differences in the Arg-369 interaction with the coenzyme molecule may be an important factor controlling the initial contact between Arg-369 and the coenzyme molecule. The dissociation constant of Arg-369 for NAD+ is 4000-fold higher than that for NADH (28). Solvent accessibility calculations show that Arg-369 of β3 contributes to the rate of coenzyme dissociation, most likely after the conformational change which occurs with coenzyme binding. The good correlation of Kd(NAD) with Vmax for ethanol oxidation in β1 and β3 (17), and not in Arg-47 Gly substitution at position 47 does not alter the rate-limiting step from that of coenzyme dissociation, but the Cys substitution at position 369 does alter the rate-limiting step. For β3, the rate of coenzyme dissociation is approximately 3-fold faster than the Vmax value. Detailed studies of the individual rate constants along the β3 reaction pathway are necessary to further define which step(s) control the overall rate of alcohol oxidation.

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TABLE IV

| Constant | β3 | β1 |
|----------|----|----|
| koff,NAD (μM s⁻¹) | 0.048 (0.006) | 0.90 |
| koff,NADH (μM s⁻¹) | 0.48 (0.03) | 5.8 |
| koff (s⁻¹) | 32 (5) | 1.7 |
| koff (s⁻¹) | 23 (1) | 0.070 |
| koff,NADH (μM) | 670 | 1.9 |
| koff,NADH (μM) | 48 | 0.012 |

369 is associated with large increases in the dissociation rate constant for NAD(H). The replacement of Arg-47 with Gin results in a 150-fold increase in the apparent dissociation rate constant for NADH (17), and in this study we show the replacement of Arg-369 with Cys results in a 330-fold increase in apparent dissociation rate constant for NADH. This data would suggest that once coenzyme is bound to the enzyme, Arg-47 contributes to the rate of coenzyme dissociation, most likely after the conformational change which occurs with coenzyme binding. The good correlation of Kd(NAD) with Vmax for ethanol oxidation in β1 and β3 (17), and not in Arg-47 Gly substitution at position 47 does not alter the rate-limiting step from that of coenzyme dissociation, but the Cys substitution at position 369 does alter the rate-limiting step. For β3, the rate of coenzyme dissociation is approximately 3-fold faster than the Vmax value. Detailed studies of the individual rate constants along the β3 reaction pathway are necessary to further define which step(s) control the overall rate of alcohol oxidation.

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