Structure of human $\beta_1\beta_1$ alcohol dehydrogenase: Catalytic effects of non-active-site substitutions

(x-ray diffraction/enzyme-cofactor complex/NAD⁺)

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ABSTRACT The three-dimensional structure of human $\beta_1\beta_1$ alcohol dehydrogenase (ADH; EC 1.1.1.1) complexed with NAD⁺ has been determined by x-ray crystallography to 3.0-Å resolution. The amino acids directly involved in coenzyme binding are conserved between horse EE and human $\beta_1\beta_1$ alcohol dehydrogenase in all but one case [serine (horse) vs. threonine (human) at position 48]. As a result, the coenzyme molecule is bound in a similar manner in the two enzymes. However, the strength of the interactions in the vicinity of the pyrophosphate bridge of NAD⁺ appears to be enhanced in the human enzyme. Side-chain movements of Arg-47 and Asp-50 and a shift in the position of the helix comprising residues 202–212 may explain both the decreased $V_{\max}$ and the decreased rate of NADH dissociation observed in the human enzyme vs. the horse enzyme. It appears that these catalytic differences are not due to substitutions of any amino acids directly involved in coenzyme binding but are the result of structural rearrangements resulting from multiple sequence differences between the two enzymes.

There are multiple molecular forms of human alcohol dehydrogenase (ADH; alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1), all of them dimeric molecules containing 374 amino acids and two zinc atoms per subunit (1). Together, they catalyze the rate-limiting step for ethanol metabolism: the NAD⁺-dependent oxidation of alcohol to acetaldehyde. The individual human ADH subunits are the products of five separate gene loci, *ADH1*–*ADH5*, producing the α , β , γ , π , and χ subunits, respectively; heterodimers can be formed among the α , β , and γ subunits. This multiplicity of forms is further increased by polymorphism at *ADH2* and *ADH3* (2).

The human ADH isoenzymes exhibit distinct enzymatic properties in spite of extensive sequence similarity. For example, $\alpha\alpha$, $\beta\beta$, and $\gamma\gamma$ enzymes, and their polymorphic variants, share >93% sequence identity yet oxidize ethanol with $V_{\max}$ values at pH 7.5 that vary over a range of >40-fold (3). The structure of the horse liver ADH enzyme—determined to high resolution for the native enzyme and a variety of substrate and inhibitor complexes—has proved to be a useful starting point for the interpretation of the kinetic properties of the human ADH isoenzymes (4–6). The human and horse enzymes share >86% sequence identity, and certain kinetic properties of the human isoenzymes, such as the substrate specificity of $\alpha\alpha$ and the coenzyme-binding properties of the $\beta_2\beta_2$ and $\beta_3\beta_3$ isoenzymes (3, 7), can be easily explained based on the horse structure. However, other steady-state kinetic and affinity-labeling properties of the human enzymes have been difficult to explain using models based on the horse structure (3, 8, 9).

One of the most intriguing differences in the kinetic properties of the human $\beta_1\beta_1$ and horse enzymes is the $\approx$ 50-fold

lower $V_{\max}$ of the human isoenzyme for ethanol oxidation (refs. 7 and 10; C. L. Stone, W.F.B., and M. Dunn, unpublished observations). Although the dissociation of NADH is partially rate-limiting for both enzymes (3, 7, 10), there are no obvious amino acid substitutions in the coenzyme binding site of the human enzyme that could account for a slower release of NADH; in fact, the only substitution among the residues that directly contact the coenzyme is a substitution of threonine (human) for serine (horse) at position 48 that would not seem to greatly alter the coenzyme–protein interactions (6). To gain further insight into the mechanism of alcohol oxidation and the specific properties of the human isoenzymes, we determined the three-dimensional structure[§] of the binary complex of the $\beta_1\beta_1$ isoenzyme of human ADH with NAD⁺.

METHODS

Preparation and Crystallization of Human $\beta_1\beta_1$ ADH. The recombinant human β_1 enzyme used in these studies was purified as described (9). Crystals of the binary ADH–NAD⁺ complex were grown using the hanging-drop method with 2- μ l drops. The crystallization medium contained 50 mM sodium phosphate (pH 7.5), 1 mM NAD⁺, and 12.5% (wt/vol) PEG 8000, with a protein concentration of 10–15 mg/ml. The crystals formed as thin, flat parallelepipeds after 3–4 days and grew to maximal size in an additional 2–3 days.

Data Collection. Two crystals (approximate dimensions, 0.5 $\times$ 0.2 $\times$ 0.03 mm) were used to collect the native data set at 23°C with a Nicolet multiwire area detector equipped with a Rigaku Rotaflex RU-200B rotating-anode generator. The data sets were scaled and processed using the software package XENGEN (11). The final merged data set contained 11,114 reflections to 2.88 Å with intensities >0.2 σ . The R_{sym} values for the individual crystals were 4% and 5% and the R_{merge} between crystals was 6%.

Molecular Replacement and Crystallographic Refinement. The structure of the human enzyme was solved by molecular replacement using as the search model the horse ternary complex dimer (12) with NADH and dimethyl sulfoxide. The Crowther rotation function (13), as implemented in the package MERLOT (14), with the data between 10 and 4 Å, was used to calculate the function. The direct R -factor search of X-PLOR (15) with data between 8 and 3.3 Å was used to refine further the solution from MERLOT.

After the amino acid sequence of the human enzyme was introduced, crystallographic refinement was carried out using both X-PLOR and PROLSQ (16) on a Silicon Graphics 4D/80GT, a VaxStation 3100, and a Vax 8530. The refinement by X-PLOR was accomplished by using the heating and fast-cool protocols with all the data between 8 and 3.3 Å.

Abbreviation: ADH, alcohol dehydrogenase.

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§The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973.

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Noncrystallographic symmetry constraints were used throughout the refinement procedure and were applied with a weight of 100 kcal/(mol·Å²) during simulated annealing and 30 kcal/(mol·Å²) during positional refinement. The NAD⁺ molecule and the zinc atoms were refined with the structure throughout this process. For refinement by PROLSQ, the data between 5.5 and 3.0 Å with F/σ_F values >2.0 were used (7775 reflections). During this refinement, noncrystallographic symmetry constraints were applied with weights based on σ values of 0.15, 0.5, and 2.0 Å for main-chain, NAD⁺, and side-chain atoms, respectively. In addition, dihedral constraints on the α -helices were applied. At various stages of refinement, $2F_o - F_c$ maps were inspected using TOM (FRODO for the Iris computer), and various portions of the structure were rebuilt as necessary.

RESULTS

The conditions for the crystallization of human ADH (50 mM sodium phosphate, pH 7.5/1 mM NAD⁺/12.5% PEG 8000) are very different from those used for the horse enzyme. Crystals usually grow to an average size of $0.5 \times 0.1 \times 0.02$ mm, although drops containing fewer crystals can produce crystals that are substantially larger in all dimensions. Two large crystals were used to collect a total of 10,213 independent reflections to 3.3 Å. Due to the data collection strategy used, an additional 901 reflections between 3.3- and 2.9-Å resolution were also collected in this data set. With the exception of the angle α , which changed by about 10%, the cell parameters of the human crystal are within 3% of those of the horse ternary complex crystals with two monomers in the asymmetric unit (Table 1).

The large difference in the cell angle α made it necessary to use a rotation function search (as opposed to using the human amplitudes with the horse phases). A self-rotation function gave a strong dimer peak, and the cross-rotation function (13, 14) using the horse ternary complex dimer and data between 10 and 4 Å gave two solutions related to each other by the dimer local twofold axis. Since a solution was found for the orientation of the dimer, no translation function was needed in the space group *P1*. Further refinement using the direct *R*-factor search in X-PLOR gave an overall *R* factor of 0.537 for all reflections with $F \geq 0.2\sigma$ between 8.0 and 3.3 Å (84% complete; 9280 reflections). After X-PLOR refinement, the *R* factor for all reflections between 8 and 3.3 Å was 0.29. For further refinement and stereochemical optimization, this model was subjected to nine iterations of manual rebuilding using $2F_o - F_c$ maps and $2F_o - F_c$ omit maps followed by PROLSQ (16) refinement until convergence after each rebuilding. The corrected model was then resubmitted for refinement by PROLSQ. The final model has excellent geometry and an *R* factor of 0.266 for the reflections between 5.5 and 3.0 Å with $F \geq 2\sigma$ (7775 reflections; 63% complete; 78% complete to 3.2 Å) for a single isotropic temperature factor and of 0.259 for correlated individual isotropic temperature factors. The average bond length deviation from ideality is 0.019 Å and the average bond angle deviation is 2.3°. Even at this resolution the maps exhibited sufficient detail to locate 44 solvent molecules. Many of the solvent molecules adjacent to lysines

and arginines were refined to extremely low-temperature factors and high occupancies. Those that exhibited good ion-pair geometry with the positive side chains were then replaced by phosphate ions that showed normal refinement behavior (a total of 15 phosphates were introduced).

In the final structure, alignment of the two monomers showed a root-mean-square (rms) deviation of 0.32 Å for the main-chain atoms not involved in lattice contacts, while comparison of all the α -carbons yielded a rms deviation of 0.93 Å. Most of the large deviations occur in the external loops of the coenzyme-binding domain, where most of the lattice contacts are found: alignment of the individual domains yields 1.29-Å rms deviation for the coenzyme-binding domain (residues 180–335) and 0.45-Å rms deviation for the catalytic domain (residues 1–175 and 340–374).

All of the elements of secondary structure present in the horse enzyme are present in the human enzyme with very similar lengths, and the connecting loops have, in most cases, similar paths (Fig. 1A). There are sufficient differences between the two structures to assure that the extensive rebuilding and refinement have yielded a structure of the human enzyme that is independent of the search model.

DISCUSSION

The structure of the human enzyme was solved by molecular replacement using the horse triclinic ternary-complex dimer as the search model. A self-rotation function on the human data gave a strong peak, and the two best solutions from the cross-rotation function were related to each other by this local twofold axis. These solutions were essentially the only strong features of the rotation function; the next highest peak was <50% of the height of the strongest peak.

The refinement of the structure was accomplished using both X-PLOR and PROLSQ with data to 3.0-Å resolution. PROLSQ was used during the final stages of model building and stereochemical refinement because the stereochemistry of the X-PLOR model structure was not well optimized with the weights used (0.034-Å rms deviation in bond lengths and 5.9° rms deviation in bond angles). The final model has both a good *R* factor for this resolution and excellent geometry.

The 1.57-Å rms deviation of the α -carbon positions in the human and horse enzyme is much greater than expected for enzymes that share 86% sequence identity. These differences seem to be randomly distributed throughout the two structures (Fig. 1A), since an alignment of the individual catalytic and coenzyme-binding domains produces similar results (1.59 Å for the coenzyme-binding domain and 1.53 Å for the catalytic domain). In addition, the structure of the substrate-binding pocket in this complex appears to require no additional changes to accommodate an alcohol substrate, only the displacement of the water molecules in this region. Thus, it appears that the large rms deviation in the structures is not simply the result of a different conformation of the catalytic domain.

The overall conformation of the bound NAD⁺ molecule is very similar to that observed in the horse enzyme, with the exception of the pucker of the adenosine ribose, which is C3'-endo in the human structure (Fig. 1B) but C2'-endo in the horse structure. When the rotation matrix obtained from the alignment of the α -carbon atoms between the horse and human enzymes is used to transform the coordinates of the coenzymes (Fig. 1B), the overlap is essentially identical to that obtained when the NAD⁺ molecules are aligned directly with each other. This suggests that the NAD⁺ molecules are bound in almost identical positions and conformations in the two proteins. In the discussion that follows, we will emphasize the similarities and differences between the horse and human structures in these protein-coenzyme interactions.

Table 1. Space group and cell dimensions of human and horse ADH

Species	Space group	Dimensions, Å			Angles, degrees		
		<i>a</i>	<i>b</i>	<i>c</i>	α	β	γ
Human	<i>P1</i>	53.6	45.7	91.5	95.7	101.6	68.4
Horse	<i>P1</i>	52.0	44.6	94.4	104.4	101.9	70.7

There is one ADH dimer with 374 amino acids per monomer in the cell. Data for horse ADH are from ref. 5.

FIG. 1. Stereo pairs of the aligned human and horse subunits and coenzyme molecules. (A) The α -carbon tracing of subunit A of the human enzyme (thick lines) is compared with that of the horse subunit A (thin lines). The positions of the catalytic and structural zinc atoms are displayed as the large (human) and small (horse) rings. The positions of the bound coenzyme molecules are also shown. (B) Isolated coenzyme molecules in the same orientation as in A (thick lines, human; thin lines, horse).

As in the horse enzyme, the adenine ring of the coenzyme in human $\beta_1\beta_1$ is bound between the side chains of Ile-224 and Ile-269 (Fig. 2), but the hydrogen bonds to the adenine nitrogens are different in the two enzymes. In human $\beta_1\beta_1$, the N7 atom of the adenine interacts through a water molecule with the NH1 atom of Arg-271, and the N6' atom of the adenine ring forms a hydrogen bond with the OG1 atom of Thr-274. This is in contrast with the horse enzyme, where Thr-274 has a solvent-mediated interaction with the N6' atom of the adenine and Arg-271 is in van der Waals contact with the edge of the adenine ring but forms no apparent hydrogen bonds to the coenzyme (12).

Other differences are seen in the interactions of the adenine ribose hydroxyls with the enzymes. In the human enzyme Asp-223 and Lys-228 interact only with the 2'-hydroxyl of the ribose molecule (2.6 Å from the Asp-223 carboxylate and 2.9 Å from the Lys-228 amine; Lys-228 and Asp-223 are 3.1 Å from each other), and the 3'-hydroxyl forms a hydrogen bond to the peptide carbonyl of residue 364 (Fig. 2). In the horse enzyme residues 223 and 228 interact with both the 2'- and 3'-hydroxyl oxygens (12). These alterations in the ribose hydroxyl interactions are primarily the result of a difference in the ribose puckering, C3'-endo in the human enzyme but C2'-endo in the horse enzyme (12). In both enzymes the

FIG. 2. A summary of the important interactions between NAD^+ and the amino acids of human $\beta_1\beta_1$ alcohol dehydrogenase. (A) A stereo pair showing the important contacts between the coenzyme and protein atoms. Amino acids are indicated by single-letter symbols and position numbers. A bound water molecule (WAT) is shown. (B) A two-dimensional representation of the interactions shown in A. The donor/acceptor assignments were made using the local chemistry as a guide.

ribose approaches main-chain atoms very closely in the region of residues 199–202 (not shown).

The nicotinamide ribose interacts with the side chains of Thr-48 and His-51 and the peptide nitrogen of residue 294. Residue 48 is the only substitution between horse (serine) and human ADH (threonine) that is directly involved in coenzyme binding. The hydroxyl of Thr-48 forms a 2.8-Å hydrogen bond with the 2'-hydroxyl of the ribose (Fig. 3A) that is very similar to that formed by Ser-48 in the horse enzyme (Fig. 3B), suggesting that the substitution has little or no effect on ligand binding. The interaction between His-51 and the nicotinamide ribose hydroxyls in the human enzyme is not well defined in the A subunit; there is poor density for the imidazole ring. In the B subunit the density indicates that an interaction occurs with the 3' (not the 2'-) hydroxyl group of the nicotinamide ribose. As a result of this arrangement, only half of the proton-relay system that was proposed for the horse enzyme (17) is correctly formed. It is possible that the contribution of His-51 is fully formed only when a substrate is bound to the catalytic zinc atom.

The nicotinamide ring appears to be well ordered even in the absence of a second substrate (the map has well-defined densities for the plane of the ring and the carboxamide group) and is held in position by van der Waals contacts with the side chains of Thr-178, Val-203, and Val-294 (Figs. 2 and 3A). In addition, the carboxamide nitrogen forms a hydrogen bond with the peptide carbonyl of residue 317 (not shown). The region that includes residues 290–295 is considered to be important in the mechanism of the horse enzyme, because it undergoes a large conformational change between the open and closed forms of the enzyme (5). This change is not induced by coenzyme analogs that lack the nicotinamide ring. No clear explanation of this observation was provided by the structures of the horse enzyme (apoenzyme and ternary complex; refs. 5 and 12). In the structure of the human $\beta_1\beta_1$ —an ADH-NAD⁺ binary complex—there is a close interaction between the side

chain of Val-294 and the nicotinamide ring (Figs. 2 and 4) that could provide an explanation for this phenomenon.

As mentioned above, one of the major kinetic differences between the human $\beta_1\beta_1$ and the horse EE ADH enzymes is the ≈ 50 -fold lower $V_{\max}$ and NADH dissociation rate of $\beta_1\beta_1$ at pH 8.0 (refs. 7 and 10; C. L. Stone, W.F.B., and M. Dunn, unpublished observations). This difference is very difficult to explain using only the model of the horse enzyme, because there are no major amino acid substitutions between the two enzymes in the residues that directly contact the cofactor. The most important structural rearrangements in the coenzyme-binding site involve the interactions with the pyrophosphate bridge, since the differences we observed in the interactions with the adenine and nicotinamide rings and the riboses do not appear substantial enough to account for the observed kinetic differences. In both enzymes the phosphates are at the amino-terminal end of the helix spanning residues 202–212. This arrangement is commonly observed in nucleotide-binding domains (18), where it is thought that the helix dipole helps to stabilize the negative charge(s) of the phosphate(s). As in other nucleotide-binding enzymes (18), the other side of the phosphate is stabilized by interactions with positively charged residues, in this case Arg-47 and Arg-369. In the horse EE enzyme, the adenosine phosphate oxygen interacts with the terminal side-chain nitrogen of Arg-47, while the ϵ -nitrogen forms a salt bridge to the carboxylate of Asp-50 (Fig. 3B). In the human $\beta_1\beta_1$ enzyme, the ϵ -nitrogen of Arg-47 interacts with the adenosine phosphate oxygen (Figs. 2 and 3A). Since Arg-47 is not involved in a salt bridge with Asp-50 (Asp-50 interacts with His-363, which is Arg-363 in the horse enzyme; Fig. 3A and B), the interaction between Arg-47 and this phosphate oxygen appears to be strengthened in the human enzyme. This change is in the right direction to explain the kinetic differences. However, additional changes between the human $\beta_1\beta_1$ and the horse enzyme can be found in the interactions of the nicotinamide phosphate with the helix comprising residues

FIG. 3. A structure comparison of the amino acids involved in adenosine phosphate and nicotinamide ribose binding in human $\beta_1\beta_1$ ADH (A) and horse EE ADH (B). The single-letter amino acid code is used, except for the NAD⁺ molecule (NAD). Dashed lines indicate potential hydrogen bonds. V203 and T178 are within van der Waals contact distance to the nicotinamide ring in both structures. A water molecule (WAT) forms a hydrogen bond with Thr-48 in the human structure, and the inhibitor dimethyl sulfoxide (DMSO) is bound to the zinc atom (ZN) in the horse structure.

FIG. 4. The $\beta_1\beta_1$ structure showing the interactions involved in the binding of the nicotinamide phosphate of NAD⁺ (NAD) and the nicotinamide ring. The coloring scheme of this stereo pair is as follows: white for carbon, blue for nitrogen, red for oxygen, and pale yellow for phosphorus. The ribbon represents the path of the main-chain atoms of the helix comprising residues 201–212. The ribbon was created using the C α , C, and N atoms as guides. Potential hydrogen bonds between the main-chain nitrogen atoms of residues 203 and 204 and the nicotinamide phosphate oxygens are represented by the white dashed lines. The main-chain nitrogen atom of residue 202 is also within hydrogen-bonding distance of both the nicotinamide phosphate oxygens. The first and last amino acids in the first turn of the helix (A201 and A204, respectively) and the amino acids involved in van der Waals contacts with the nicotinamide ring, Thr-178 (A178), Val-203 (not labeled), and Val-294 (A294), are represented. Additionally, the side chains of Arg-369 (A369) and Glu-68 (A68) are displayed, because they are involved in an internal ion pair near the nicotinamide phosphate oxygens.

202–212. In horse ADH, in addition to the interactions with the helix dipole, the OP5 atom of the nicotinamide phosphate makes a hydrogen bond with the peptide NH of residue 203—the second of the three non-hydrogen-bonded NH groups at the beginning of the helix. In the human $\beta_1\beta_1$ structure, the amino terminus of the helix is shifted by ≈ 0.9 Å, so that the NH groups of residues 203 and 204—the second and third non-hydrogen-bonded NH groups of the helix—are now able to form hydrogen bonds with the OP5 and OP4 atoms, respectively (Fig. 4). This arrangement, involving hydrogen bonds from two consecutive NH groups at the beginning of an α -helix with the two oxygens of the same phosphate group, has not been observed in other nucleotide-binding proteins even though it appears to be an excellent way of stabilizing a phosphate group partially buried in a protein. The NH–NH lateral distance (3.5 Å) is similar to the O–O distance (2.6 Å), allowing for good geometry in both hydrogen bonds. In this arrangement, the free-energy difference between the phosphate-free and the phosphate-bound forms is further enhanced by the intrinsic instability of the free amide NH groups at the beginning of the helix (19, 20) in the coenzyme-free form.

Careful comparison of the structures of the human $\beta_1\beta_1$ and the horse EE enzyme did not identify specific amino acid substitutions directly involved in coenzyme binding responsible for the observed kinetic differences. On the contrary, the difference in NAD(H)–phosphate binding is due to small structural rearrangements (the change in the position of several side chains and the movement of the N-terminus of the 202–212 helix by 0.9 Å) caused by multiple sequence differences between the two enzymes. These results have important implications with respect to the interpretation of kinetic studies using site-directed mutagenesis. In the absence of structural information, an amino acid substitution that affects the kinetics of the enzyme is usually identified as a binding- or catalytic-site residue. The kinetic differences in ADH are the result of structural rearrangements caused by substitutions in residues not directly involved in catalysis or binding. This kind of phenomenon is probably more common than previously believed.

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