Human liver alcohol dehydrogenase: Amino acid substitution in the \( \beta_2\beta_2 \) Oriental isozyme explains functional properties, establishes an active site structure, and parallels mutational exchanges in the yeast enzyme

(bytypical enzyme/allelic variant/amino acid sequence/structure-function relationship/coenzyme binding)

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ABSTRACT The homodimeric Oriental \( \beta_2\beta_2 \) isozyme of human liver alcohol dehydrogenase, corresponding to an allelic variant at the \( ADH_2 \) gene locus, was studied in order to define the amino acid exchange in relation to the \( \beta_1\beta_1 \) isozyme, the predominant allelic form among Caucasians. Sequence analysis reveals that the amino acid substitution occurs at position 7 of the largest CNBr fragment, corresponding to position 47 of the whole protein chain. Here, the \( \beta_2 \) form has a histidine residue, whereas, in common with 
other characterized mammalian liver alcohol dehydrogenases, the \( \beta_1 \) form has an arginine residue. This exchange does not affect the adjacent cysteine-46 residue, which is a protein ligand to the active-site zinc atom, thus clarifying previously inconsistent results. The histidine/arginine-47 mutational replacement corresponds to a position that binds the pyrophosphate group of the coenzyme NAD(H); this explains the functional differences between the \( \beta_1\beta_1 \) and \( \beta_2\beta_2 \) isozymes, including both a lower pH optimum and higher turnover number of \( \beta_2\beta_2 \), which is likely to be the mutant form. The exchange demonstrates the existence of parallel but separate mutations in the evolution of alcohol dehydrogenases because these mammalian enzymes differ at exactly the same position by the same type of substitution as is found between a mutant and the wild-type constitutive forms of the corresponding yeast enzyme.

Alcohol dehydrogenase is a well-characterized enzyme. Both the primary and tertiary structures of the horse liver protein are known, as are catalytic mechanisms, isozyme differences, evolutionary divergence, and many enzyme properties (cf. 1–4). Similarly, for yeast alcohol dehydrogenases, primary structures, likely subunit conformation, mutant enzyme forms, and structures of genes have been characterized (5–11). Drosophila alcohol dehydrogenase also has been characterized both at the protein and gene level (12, 13). Finally, polyol dehydrogenases have been shown to be related to alcohol dehydrogenases, parallel evolutionary changes have been demonstrated, and both differences and similarities in a super-family of these and other (14) dehydrogenases have been discerned (15, 16).

In contrast, relatively little is known about human alcohol dehydrogenase, although there is extensive structural homology between the horse and human enzymes (17, 18). Many different isozymes of human liver alcohol dehydrogenase exist, but purification of all forms was difficult before effective affinity chromatography utilizing a pyrazole derivative became available (19, 20). Genes at three loci (\( ADH_1 \), \( ADH_2 \), and \( ADH_3 \)) code for three protein chains (\( \alpha, \beta, \) and \( \gamma \)), with allelic variants of \( \beta \) (and possibly several \( \beta_2 \)) and \( \gamma \) (\( \gamma_1 \) and \( \gamma_2 \)) (21, 22). The detection of yet other isozymes has resulted in the distinction of three classes of human alcohol dehydrogenases (23, 24). Dimers (homodimers and heterodimers) of the \( \alpha, \beta, \) and \( \gamma \) protein chains (21) constitute the isozymes of class I (23); \( \alpha \) isozymes are class II (25), and \( \chi \) isozymes (26) constitute class III. The three classes differ not only in net charge and sensitivity to inhibition by pyrazole but also in kinetic and immunological properties (24). One amino acid difference between class I subunits of nonallelic origin has been characterized, a valine/alanine exchange at position 43 (17), close to the active-site zinc ligand cysteine-46. This difference was detected before the complexity of isozyme patterns was known, but from the properties of the preparation investigated, the difference is likely to correspond to a \( \beta_1/\gamma \) difference, although such an assignment has been questioned (27).

Patterns of class I isozymes also vary because of allelic polymorphism within and among different racial groups. In particular, the \( ADH_2 \) locus has been studied because of readily distinguishable catalytic properties of the different phenotypes. An “atypical”, minor ADH phenotype in a Swiss population was described in 1965 (28); it exhibits an optimum for ethanol oxidation at about pH 8.5 instead of 10.0–10.5 for the “typical” enzyme. These findings are similar to those in other populations of Caucasian origin (21, 29) and to an atypical ADH main phenotype with similar catalytic properties in Oriental population groups (29, 30). The atypical phenotypes have been provisionally classified as \( ADH_2 \) in (31) and as \( ADH_\text{Oriental} \) (27) or \( ADH_\text{Honolulu} \) (31), respectively. The corresponding \( \beta_2 \) chains are called \( \beta_2 \)-Bern and \( \beta_2 \)-Oriental in the present report. Another and different variant involving the \( \beta \) subunit has been discovered in an American population of African origin. This allelic form exhibits a pH optimum for ethanol oxidation at pH 7.0 and has been designated \( \beta_\text{Indianapolis} \) (22, 31, 32).

Two reports have appeared on the structures of \( \beta_2 \) chains. One suggests that \( \beta_2 \)-Bern chains are due to a substitution at position 230 (18); the other unexpectedly suggests a change by one position in the \( \beta_1 \) chain of the zinc-liganding cysteine-46 in other mammalian alcohol dehydrogenases (1) and a replacement of this residue by histidine in the \( \beta_2 \)-Oriental subunit (27). Therefore, although alcohol dehydrogenase is generally well studied with known relationships to other enzymes (15, 16), the structure of the human enzyme is not established, not even the exact position of one of the ligands to its active-site zinc atom, and reports on the structure of atypical forms have differed.

Using a preparation of well-characterized homodimeric

Abbreviation: \( ADH \), alcohol dehydrogenase locus.
Oriental $\beta_2\beta_3$, we have now identified the amino acid substitution in the $\beta_2$-Oriental protein chain of human liver alcohol dehydrogenase. The data explain the functional differences between the isozymes containing the "atypical" $\beta_2$-Oriental and the "typical" $\beta_1$ subunits. They also confirm the structure at the active site region of the $\beta_1$ chain (17), which has been questioned, and relate the $\beta_2$-Oriental amino acid substitution to that of $\beta_2$-Bern chains (33). Hence, the genetic origin of $\beta_2$-Oriental and $\beta_2$-Bern can be identical. Finally, the type of $\beta_1/\beta_2$ substitution now established has been reported also in different yeast alcohol dehydrogenases, showing that parallel mutations have occurred in widely divergent evolutionary lines of this enzyme.

**MATERIALS AND METHODS**

The homodimeric form of human alcohol dehydrogenase with $\beta_2$-Oriental protein chains was prepared as described (34) from autopsy livers of Japanese donors with the homozygous ADH$^{32}$ phenotype (34). The enzyme (3.8 mg) was carboxymethylated with iodine$^{[14]}$ in 6 M guanidine-HCl/2 mM EDTA/0.1 M Tris, pH 8.15 after reduction with dithiothreitol (5). Cleavage with CNBr, prefractionation by chromatography on Sephadex G-50 in 30% acetic acid, final purification by reverse-phase high-performance liquid chromatography, and subsequent recleavage were carried out as for the related enzyme sorbitol dehydrogenase (35). Liquid-phase sequencer degradations, in a Beckman 890D instrument, used a 0.1 M Quadrol peptide program and application into glycine-pretreated Polybrene (36). Thiodyantoin derivatives were analyzed by reverse-phase high-performance liquid chromatography (37), supplemented at the position corresponding to the exchanged residue by back hydrolysis with HCl/SnCl$_2$ for independent residue confirmation.

**RESULTS**

The homodimeric variant of human liver alcohol dehydrogenase with $\beta_2$-Oriental protein chains, prepared from autopsy livers (34), was homogeneous on agarose isoelectric focusing, starch gel electrophoresis, and NaDodSO$_4$/polyacrylamide gel electrophoresis. Proof of its homodimeric structure was obtained by dissociation-recombination experiments. The reduced enzyme was $^{[14]}$C-carboxymethylated with labeled iodoacetate in guanidine-HCl, dialyzed to remove reagents, lyophilized, and cleaved with CNBr. Resulting peptides were purified by a two-step procedure utilizing prefractionation on Sephadex G-50 in 30% acetic acid, followed by final purification on reverse-phase high-performance liquid chromatography (35). Patterns of elution during prefractionation were indistinguishable from those obtained in parallel with the "typical" $\beta_1/\beta_1$ form in a separate study (unpublished results), and the peptide containing the amino acid substitution corresponding to the mutation in the $\beta_2$-Oriental chains could not be identified from the behavior during purification.

The CNBr fragments were submitted to structure analysis by determination of total compositions and by liquid-phase sequencer analysis as far inside each CNBr fragment as possible. In this way, a single amino acid substitution in relation to the $\beta_1$ enzyme was detected in the largest fragment. The purification of this fragment is shown in Fig. 1A; the composition, in Table 1; and the amino acid sequence data, in Table 2. The fragment corresponds to positions 41-208 of the $\beta_1$ subunit and is identical to $\beta_1$ at all positions checked except for the residue in cycle number 7, which corresponds to position 47 of the intact protein. Here, $\beta_2$-Oriental has a histidine residue (Table 2), while the $\beta_1$ chain has an arginine residue (17), exactly as do all other liver alcohol dehydrogenases (horse, rat, and chicken) that have been investigated (1, 38). This exchange is adjacent to the zinc-liganding cysteine-46 (39), which is unaltered. The conservation of cysteine-46 and the replacement of arginine by histidine at the adjacent position 47 were also identified by analysis of smaller enzymic peptides (especially peptide SP, Table 1) purified (Fig. 1B) from a digest with a glutamic acid-specific staphylococcal protease. Moreover, peptide SP completely lacks arginine (Table 1), excluding the presence of such a residue in this segment of the $\beta_2$ chain.

**DISCUSSION**

Nature of the Amino Acid Substitution in the $\beta_2$-Oriental Allelic Chain. The sequence analysis (Table 2) clearly identifies histidine in position 7 of the largest CNBr fragment, corresponding to position 47 of the enzyme. The residue was identified both by high-performance liquid chromatography and amino acid analysis after back hydrolysis (Table 2). Similarly, all residues in the surrounding sequence were fully identified, including the cysteine derivative corresponding to position 46 (Table 2). Both the histidine and cysteine residues were identified also in a smaller peptide after digestion.
Table 1. Amino acid compositions*  

<table>
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<th>Amino acid composition</th>
<th>CNBr fragment acid hydrolysis</th>
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* Amino acid compositions of the CNBr fragment containing the amino acid exchange in carboxymethylated β1-Oriental alcohol dehydrogenase chains and of the constituent peptide obtained by digestion with staphylococcal protease (SP peptide). For comparison, the corresponding values for the α1 CNBr fragment are also shown as the integer numbers obtained from sequence analysis of the β2 and β1 SP peptides.

with staphylococcal protease (SP peptide, Table 1). Accordingly, the primary structure of the β2-Oriental protein chain around the zinc-eldiganding cysteine residue, which corresponds to position 46 in other liver alcohol dehydrogenases, is as shown in Table 2.

This structure differs from that of the β1 chain in having a histidine residue instead of arginine at position 47. The arginine at this position in “typical” human liver alcohol dehydrogenase was reported previously by using an isozyme mixture (17) and is now further known to be present also in the human γ1 subunit (unpublished results), derived from the ADH2 locus (21), as well as in all alcohol dehydrogenase forms investigated from horse, rat, and chicken (1, 38).

The arginine/histidine exchange between human β2 and β1 chains is completely compatible with the absence of any differences in elution patterns of CNBr fragments from these two isozyme forms during purification (Fig. 1). The exchange is also visible directly in the total composition of the β2 CNBr fragment (Table 1), which suggests the presence of one more histidine and one less arginine residue than in the β1 form. Thus, the conclusion about the histidine/arginine exchange at position 47 is incontrovertably established with respect to the type of residues involved, their positions, and the surrounding amino acid sequence.

Structure of the β1 Subunit of Human Liver Alcohol Dehydrogenase and Correlation with Previous Data. The results also allow a judgment and interpretation of two previously incompatible reports on the structure surrounding cysteine-46 in the β1 forms of human liver alcohol dehydrogenase. The present analysis shows position 46 to be occupied by a cysteine residue, exactly as in the first of the previous reports (17) and as confirmed recently by an independent analysis of the β1 chain in another study. In contrast, it has been suggested that cysteine occupies position 47 in both the β1 and the β2-Oriental forms (27). That suggestion is incompatible with the present analysis of pure homodimeric Oriental ββ2, with separate analysis of homodimeric ββ1, with analyses of isozyme mixtures (17), and with those of the pure γγ1 form.

Consequently, it can now be considered established that the β1 chain has the Cys-Arg structure reported originally (17), which differs from that of the β2-Oriental chains by the Arg/His substitution at position 47. These results further suggest that the mutant allele is that for β2 (ADH2'), not that for β1 (ADH1'), because the β2 protein chain is the one that deviates at this position from all other liver alcohol dehydrogenase chains characterized (human β1, γ1; horse E and S; rat, chicken), while β1 at this position is identical with all those forms. Therefore, the report suggesting an inversion of residues 46 and 47 due to separate but adjacent mutations (27) is without support.†

The data establish that cysteine-46, the protein ligand to the catalytic zinc atom in other liver alcohol dehydrogenases (33), is conserved also in human liver alcohol dehydrogenases, including the Oriental ββ2 allele variant. As shown in Table 2, the second ligand to the active-site atom, histidine-

†Those assignments were based on tryptic analyses of the aminomethylated enzymes (27). However, trypsin can cleave both at arginine and aminooxyethylcysteine, thus complicating distinctions between these two residues and likely accounting for previous interpretations.
67, also remains unchanged. Finally, the sequence around cysteine-46 shows that the isozyme subunits investigated previously (17) were $\beta_1$ and $\gamma$ and that $\beta_2$-Oriental and $\beta_2$-Bern chains have the same amino acid exchange (33). Thus, $\beta_2$-Oriental and $\beta_2$-Bern can have the same mutational origin.

Correlation with Functional Properties (Coenzyme Binding). The pH optimum of alcohol oxidation for the $\beta_2\beta_2$ Oriental isozyme is known to be 8.5, which is lower than that for $\beta_B\beta_2$, pH 10.0. Similarly, the $K_m$ values for NAD$^+$ and NADH are approximately 20 times higher with the $\beta_2$-Oriental chains than with the $\beta_2$ chains. These properties are consistent with the observed substitution of histidine for arginine at position 47. Thus, labeling experiments (40, 41) suggest that arginine-47 in horse liver alcohol dehydrogenase forms charge interactions with the pyrophosphate moiety of the coenzyme; the lower $K_m$ value of the imidazole group of histidine, as compared with that of the guanido group of arginine, is compatible with a lower pH for optimal interaction in coenzyme binding. Notably, the pH optimum for ethanol oxidation of the wild-type constitutive yeast enzyme is also at pH 8.6 (42), and it, too, has a histidine residue in this position (7), whereas a mutant form with arginine has lower $K_m$ values for NAD$^+$ and NADH (11). Further, since the replacement of arginine by histidine is compatible with a weaker interaction and since coenzyme dissociation is the rate-limiting step (1), a histidine enzyme form would be expected to have a higher turnover number, exactly as has been observed for the yeast enzyme form with histidine at that position (11). In the latter case, identical measurements on activities showed the histidine form to reduce 4- to 5-fold more NAD$^+$ per mg of protein (activities of 352 versus 79 units/mg) (43). Similarly, direct measurements on Oriental $\beta_2\beta_2$ human alcohol dehydrogenase reveal this form to have a higher specific activity than that of $\beta_2\beta_1$. Therefore, the histidine/arginine exchange now characterized explains all aspects of the known functional differences between the $\beta_2\beta_2$ and $\beta_2\beta_1$ isozymes.

Parallel Mutations in Separate Forms of an Enzyme Family. Apart from establishing the structural basis for the variant $\beta_2$-Oriental chain, confirming the $\beta_2$ structure, and explaining the functional properties of the variant "atypical" chain, the present mutation also illustrates parallel mutational events in an enzyme family. Thus, a histidine/arginine exchange at the same position (corresponding to position 47 in the human enzyme) has now been found in alcohol dehydrogenases as results of three completely separate mutational events:

First, wild-type main forms of characterized yeast alcohol dehydrogenases have histidine, and known mammalian alcohol dehydrogenases (except human $\beta_2$ subunits) have arginine, at this position (1, 7-10, 17). The ancestral, mutational event that gave rise to this difference must be very old because it is reflected in most known forms in the respective divergent lines.

Second, the human $\beta_2$ protein chain differs from the $\beta_1$ chain by having histidine instead of arginine at this position. This difference corresponds to a single comparatively recent mutation and explains an allelic variant.

Third, in relation to constitutive yeast alcohol dehydrogenase from Saccharomyces cerevisiae, a mutant form (S-AA-5) demonstrates a substitution at the same position, involving the same residues but in a direction opposite to that of the human $\beta_2$ variant (11). Again, it is a single mutational event. It is recent and artificially produced in yeast.

Fig. 2 shows these relationships, delineating the three separate mutations. Not only is the coincidence in position of interest but also the common nature of only two residues affected, histidine and arginine. The restricted deviations suggest that these residues may be among the only ones capable of binding the pyrophosphate of the coenzyme at this position. Therefore, the mutational data emphasize the general role of this position in coenzyme binding, in agreement with crystallographic observations and labeling experiments (39-41). The present characterization greatly reinforces the correlation between functional properties and structural changes, while remaining compatible with previous deductions on the mode of coenzyme binding. In fact, the naturally occurring isozyme variants allow direct and consistent functional conclusions in the same way as if they were derived from artificial, selected site-directed mutagenesis. They also constitute an exceptionally good example of separate, parallel evolutionary changes of a protein.

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**Fig. 2.** Demonstration of three separate mutational exchanges at the same position of alcohol dehydrogenase (position 47 of the liver enzyme); 1, 2, and 3 indicate three separate mutations, all affecting the same position and the same residues. YADH in the figure represents all characterized major wild-type forms of yeast alcohol dehydrogenase—i.e., the constitutive and inducible Saccharomyces cerevisiae enzymes (7-9) and the enzyme from Schizosaccharomyces pombe (10). The mutant YADH represents the S-AA-5 form (11). LADH represents all characterized parent forms of liver alcohol dehydrogenase—i.e., the E- and S-isozyme chains of the horse liver enzyme, the $\beta$ and $\gamma$ isozyme chains of the human liver enzyme, and the subunits of the rat and chicken liver enzymes (1, 17, 38). A direct correlation between the histidine/arginine exchange and the binding of NAD$^+$ is obvious from kinetic properties of the mutant forms and the parent LADH and YADH forms, respectively (cf. text).
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