Amino acids important in enzyme activity and dimer stability for *Drosophila* alcohol dehydrogenase

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We have determined the nucleotide sequences of eight ethyl methanesulphonate-induced mutants in *Drosophila* alcohol dehydrogenase (ADH), of which six were previously characterized by Hollocher and Place [(1988) Genetics **116**, 253–263 and 265–274]. Four of these ADH mutants contain a single amino acid change: glycine-17 to arginine, glycine-93 to glutamic acid, alanine-159 to threonine, and glycine-184 to aspartic acid. Although these mutants are inactive, three mutants (Gly17Arg, Gly93Glu and Gly184Asp) form stable homodimers, as well as heterodimers with wild-type ADH, in which the wild-type ADH subunit retains full enzyme activity [Hollocher and Place (1988) Genetics **116**, 265–274]. Interestingly, the Ala159Thr mutant does not form either stable homodimers or heterodimers with wild-type ADH, suggesting that alanine-159 is important in stabilizing ADH dimers. The mutations were analysed in terms of a three-dimensional model of ADH using bacterial 20β-hydroxysteroid dehydrogenase and rat dihydropteridine reductase as templates. The model indicates that mutations in glycine-17 and glycine-93 affect the binding of NAD^+. It also shows that alanine-159 is part of a hydrophobic anchor on the dimer interface of ADH. Replacement of alanine-159 with threonine, which has a larger side chain and can hydrogen bond with water, is likely to reduce the strength of the hydrophobic interaction. The three-dimensional model shows that glycine-184 is close to the substrate binding site. Replacement of glycine-184 with aspartic acid is likely to alter the position of threonine-186, which we propose hydrogen bonds to the carboxamide moiety of NAD^+. Also, the negative charge on the aspartic acid side chain may interact with the substrate and/or residues in the substrate binding site. These mutations provide information about ADH catalysis and the stability of dimers, which may also be useful in understanding homologous dehydrogenases, which include the human 17β-hydroxysteroid, 11β-hydroxysteroid and 15-hydroxyprostaglandin dehydrogenases.

INTRODUCTION

The alcohol dehydrogenase (ADH) locus (*Adh*) in *Drosophila melanogaster* has long been used as a model system for studying gene regulation, evolution and mechanisms of gene mutation [1,2] because of several advantageous properties: ADH activity is easily measured; the enzyme is so abundant that activity in a single fly can be determined; and naturally occurring mutations and those induced by chemicals and X-rays can be conveniently studied because the gene is small and readily sequenced [3–7]. ADH is active as a dimer, and there are electrophoretic variants available for separating homodimers from heterodimers [8–10]; therefore flies with an interesting *Adh* mutation can be crossed to flies with either wild-type or mutant *Adh* alleles to investigate both *in vivo* and *in vitro* properties of the hybrid molecules [8–10].

Examination of wild-type *Drosophila* for mutations in the coding and non-coding parts of *Adh* and its neighbouring 5′ and 3′ segments reveals that the ADH protein is more conserved than would be expected within *D. melanogaster* and between sibling species [3,11,12] suggesting the importance of this locus in natural selection.

Using a group of ethyl methanesulphonate (EMS)-induced *Adh* null-mutations in *D. melanogaster*, Hollocher and Place [8,9] investigated the enzyme activity and the stability of interallelic heterodimers among null mutations and with wild-type *D. melanogaster Adh*. Interestingly, Adh^{n7} had partial restoration of enzyme activity when heterozygous with Adh^S and Adh^F. Unfortunately, the DNA sequence of Adh^{n7} and the other mutants was not determined. DNA sequence analysis of these mutants would provide important information about determinants of enzyme activity and protein–protein interactions in ADH. Moreover, information about residues that are important in ADH activity and dimer stability would also be useful in understanding catalysis by ADH homologues [13–17], such as 11β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase and 15-hydroxyprostaglandin dehydrogenase, enzymes that regulate the concentration of glucocorticoids, oestrogens and prostaglandins respectively in humans.

Here we report the DNA sequences of six EMS mutants studied by Hollocher and Place and of two EMS mutants (nBr4 and nBr18) induced in our Baton Rouge laboratory. To elucidate the structural basis of these mutations, we constructed a three-dimensional (3D) model of ADH using the tertiary structures of two homologues, *Streptomyces hydrogenans* 20β-hydroxysteroid dehydrogenase [18,19] and rat dihydropteridine reductase [20,21], as templates. The 3D model, together with Hollocher and Place's data [8,9], provide additional information about ADH catalysis and the stability of ADH dimers.

EXPERIMENTAL

Source of mutations

A number of *D. melanogaster Adh* null-mutations have been induced with EMS, but DNA from these mutations has not been previously sequenced. Eight of these EMS-induced *Adh* null-mutations have been sequenced and are described in this paper.

Abbreviations used: ADH, alcohol dehydrogenase; EMS, ethyl methanesulphonate; 3D, three-dimensional.

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Alleles (n1, n2, n7, n10 and n11) are described in Lindsley and Zimm [22] and were obtained from the Mid-America Drosophila Stock Center, Bowling Green State University, Bowling Green, OH 43403, U.S.A. The amino acid sequence of $Adh^{n\,11}$ was initially determined by amino acid sequence analysis [23] $Adh^{n\,14}$ was obtained from Dr. W. Sofer before 1980 and maintained in the Baton Rouge Drosophila Laboratory. Three additional Adh^{n} mutations were induced with EMS in our Baton Rouge laboratory, one of which was a deletion and will be reported elsewhere. Of the intragenic EMS-induced mutations, two, Adh^{nBR4} and Adh^{nBR14}, have not been previously described in the literature. They were induced in spermatozoa with 10 mM and 5 mM EMS respectively, using methods of treatment and mutation recovery described in Fossett et al. [6].

Enzymes

Thermus aquaticus (*Taq*) polymerase enzyme and reagent kit and Circumvent cycle sequencing kit were purchased from Cetus and New England Biolabs respectively.

DNA isolation and PCR gene amplification

DNA was isolated from 50 flies, homozygous or hemizygous for the Adh^{n} mutation, using the method of Chia et al. [4]. A 1146 bp *Adh* fragment was amplified using *Adh*-specific primers, one that binds a specific sequence that is 121 bp 5′ to the initiation codon and a second primer that binds a sequence 82 bp 3′ from the termination codon. *D. melanogaster* genomic DNA (3 μg) and 25 pmol of each primer were added to nucleotide triphosphates and reaction buffer according to the Perkin–Elmer Cetus protocol. The reaction mixture was heated to 94 °C for 10 min to denature the genomic DNA. Subsequently, 2.5 units of *Taq* polymerase was added to the reaction mixture and *Adh* fragment amplification was achieved using a 1 min, 95 °C denaturing step, a 1 min, 55 °C annealing step and a 1.5 min, 72 °C primer extension step for 25 cycles. Excess primers and nucleotide triphosphates were removed from the amplified fragment by diluting the 100 μl reaction mixture to a total volume of 2 ml with distilled water and concentrating the mixture using Centricon 30 (Amicon) microconcentrators [24]. The fragment concentration was then determined spectrophotometrically at 260 nm.

Gene amplification using *Taq* polymerase has two potential problems: lack of specificity for the amplified gene and infidelity of the *Taq* polymerase [24]. To control both of these sources of error, the same mutant sequence was required from two separate amplifications and sequencing analyses. To minimize discordance between the two amplifications from genomic DNA, stringent primer annealing and extension conditions, coupled with the unique primer sequences for the *Adh* gene, greatly reduced the problem of non-specificity for the amplified gene. Infidelity of *Taq* polymerase was minimized by starting the gene amplification with at least 10^6 copies of the *Adh* gene.

Oligonucleotides that bind specifically to the *Adh* gene were used for DNA sequence analysis. The primers bind to either the coding or non-coding strand at intervals of approx. 200 bp. DNA sequence analysis was performed according to the methods described in the New England Biolabs Circumvent kit. A rapid screen for single nucleotide changes and small intragenic insertions or deletions was achieved by loading the sequencing gel as follows: three samples of mutant template DNA and one wild-type template DNA were used to produce four primer extensions with the same primer; the four samples were loaded so that all strands that terminated with dideoxyadenosine were loaded adjacently; this loading scheme was repeated with ddC, ddG and ddT samples. The single-base changes, deletions and insertions can be quickly visualized from the resulting autoradiograph [25].

Criteria for a mutant sequence were: (1) the entire gene must be amplified using PCR amplification followed by cycle sequencing of the entire gene using the vent DNA polymerase, and (2) the mutant sequence is confirmed from an independent amplification from genomic DNA.

3D modelling of ADH

We used the Homology program (Biosym, 1994) to model the ADH structure using as a template the reported tertiary structures of *S. hydrogenans* 20β-hydroxysteroid dehydrogenase [18] and rat dihydropteridine reductase [20]. The NAD^+ structure was obtained from the Brookhaven Protein Database. The position of NAD^+ was extracted from rat dihydropteridine reductase. Then the ADH structure with NAD^+ was subjected to extensive energy minimization. The backbone of ADH was constrained during minimization of 2000 iterations. Molecular graphics were created using the MolScript program [26].

RESULTS AND DISCUSSION

Sequence of *Adh* mutations

All of the eight EMS-induced intragenic mutations are single-base substitutions, with seven GC to AT transitions and one AT to TA transversion (Table 1). Four of the GC to AT transitions are at a glycine codon. One multilocus deletion, Df(2L)BR3, was recovered following EMS treatment and will be reported with other chemically induced multilocus deletions. The explanation for null activity is evident in three of the mutations. $Adh^{n\,14}$ is an AT to TA transversion of the first base pair of the start codon; Adh^{nBR4} is a GC to AT transition in the third base pair of the start codon. Neither of these mutations in the start codon results in a transcript. $Adh^{n\,10}$ is a GC to AT transition in a tryptophan codon that results in a premature stop codon and hence does not produce a polypeptide capable of cross-reacting with goat anti-ADH antibody [8]. Of the other five mutations shown in Table 1, four are at sites that have not previously been investigated.

Structural analysis of the mutants

We use the 3D model of ADH to understand the structural effects of the different mutations and how they may affect catalysis and dimer stability. Figures 1 and 2 show the four mutations in ADH that are discussed below.

Glycine-17 to arginine mutation

EMS mutant Adh^{nBrR8} has glycine-17 mutated to arginine. This mutation occurs in the AMP-binding domain, which consists of a β-strand, α-helix, β-strand structure, in which three highly conserved glycine residues form a tight loop between the first β-strand and the α-helix [27–30]. In ADH, the glycine motif is Gly^{15}-Xaa-Xaa-Gly^{18}-Xaa-Gly^{20}. This differs from that in most homologues, which have a Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly motif [27]. As a result, the turn is sharper in ADH. In ADH and many of its homologues, extra glycine residues, such as glycine-17, are found between the canonical glycine residues. The glycines provide flexibility for this part of ADH, facilitating close contact between AMP and the enzyme and hydrogen bonding between the 2′ and 3′ hydroxyls on the adenosine ribose and the C-terminal part of the second β-strand.

Table 1 DNA sequence analysis of EMS-induced intragenic *Adh* null-mutations

Mutation	Parental allele	Type	Sequence	Position	Codon change
Adh^{n14}	Adh^{F}	Transversion	ACC ATG TCG TTG	778	Start codon
Adh^{nBR4}	Adh^{F}	Transition	ACC ATG TCG ATA	780	Start codon
Adh^{n11}	Adh^{F}	Transition	GCC GGT CTG GAT	821	Gly-15 to Asp
Adh^{nBR18}	Adh^{F}	Transition	CTG GGA GGC AGA	826	Gly-17 to Arg
Adh^{n1}	Adh^{S}	Transition	AAC GGA GCT GAA	1120	Gly-93 to Glu
Adh^{n10}	Adh^{F}	Transition	TTC TGG GAC TGA	1213	Trp to Stop
Adh^{n2}	Adh^{S}	Transition	GCG GCC GTG ACC	1317	Ala-159 to Thr
Adh^{n7}	Adh^{F}	Transition	CCC GGC ATC GAC	1463	Gly-184 to Asp

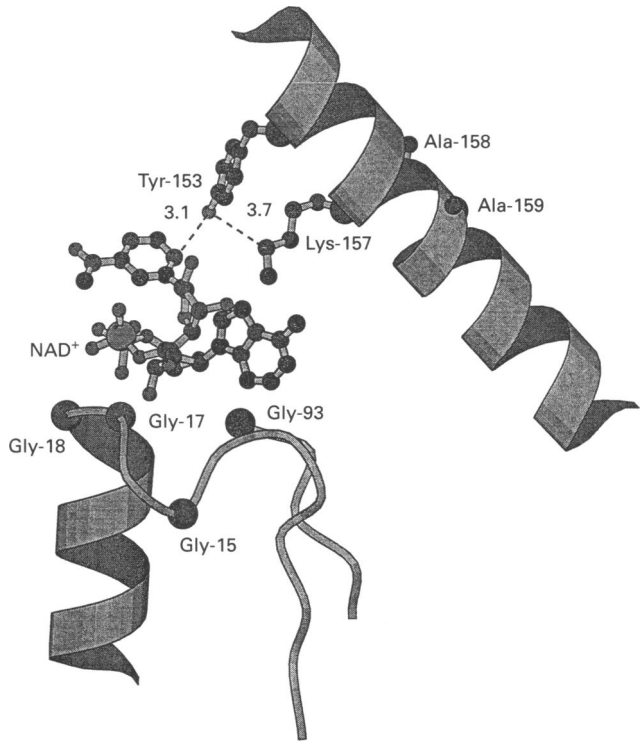

Figure 1 3D model of the N-terminal part of *Drosophila* ADH

Distances (Å) are indicated. Glycine-17 is in a glycine-rich hydrophobic segment that forms a binding pocket for the AMP part of NAD^+. Glycine-93 is in a hydrophobic β-strand that is close to AMP.

Figure 1 shows the region around glycine-17. This residue is part of a tight turn in a hydrophobic binding pocket that is close to the adenine in AMP. Replacement of glycine-17 with arginine is likely to interfere with binding of NAD^+, because arginine adds a bulky positively charged side chain and removes a residue that both lacks a side chain and contributes to flexibility between the first two canonical glycines. These changes are likely to interfere with close contact between ADH and the AMP part of the cofactor, leading to loss of activity.

The effect of the mutation at glycine-17 is consistent with the effects of mutations at glycine-15, the first glycine in the turn. Thatcher [23] found that substitution of aspartic acid for glycine-15 eliminated enzyme activity. Chen et al. [31] found that substitution of alanine lowered enzyme activity by about 30%, due to a decreased affinity of NAD^+ for ADH. A valine substitution at this position eliminates enzyme activity, probably due to the bulky isopropyl side chain obstructing binding of NAD^+. This also is consistent with the effect of the replacement of glycine-20 with alanine, which reduces the affinity of ADH for NAD^+ to about 55% of that of wild-type ADH [32].

Glycine-93 to glutamic acid mutation

EMS mutant Adh^{n1} has glycine-93 mutated to glutamic acid. This mutation occurs in a highly conserved hydrophobic β-strand that is close to NAD^+. Replacement of glycine-93 with glutamic acid adds a negatively charged side chain to a closely packed hydrophobic region. The 3D model indicates that the glutamic acid side chain will be in the pocket that binds NAD^+, where the negative side chain is likely to complex with water. This means that when NAD^+ binds to this region it must displace the bound water and interact with a less hydrophobic region, which should reduce the affinity for NAD^+. In addition, the negative charge of glutamic acid is likely to disrupt the hydrophobic β-strand that binds NAD^+. Hollocher and Place [9] found that although the peptide produced by Adh^{n1} is inactive, it can form stable homodimers. Interallelic complementation of Adh^{n1} with Adh^{S} leads to a heterodimer that has half the activity of the wild-type homodimer. Interestingly, the peptide produced by Adh^{n1} can form a functional heterodimer with the inactive EMS mutant Adh^{n7} [9].

Alanine-159 to threonine mutation

EMS mutant Adh^{n2} has alanine-159 mutated to threonine. This occurs in a highly conserved segment containing tyrosine-153 and lysine-157, which are essential for catalysis [33–35]. Tyrosine-

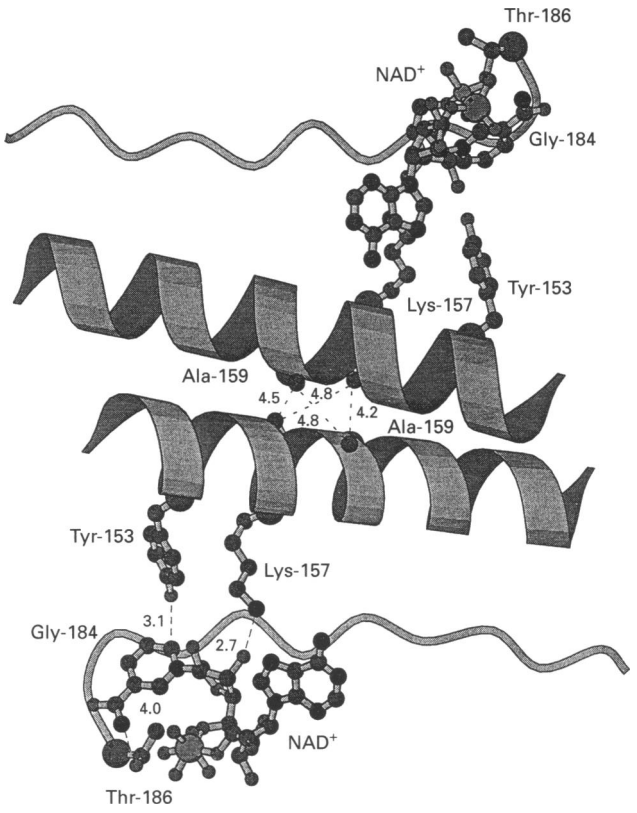

Figure 2 3D model of the dimer interface in *Drosophila* ADH

Distances (Å) are indicated. The α-helix F interface is shown with Ala-159 in a hydrophobic anchor in the dimer interface. This allows the close approach of Ala-158 and Ala-159 on the two ADH monomers. Replacement with threonine would increase the distance between the dimers. Water also could enter this region and hydrogen bond to the secondary hydroxyl on threonine. Also shown are the proposed [33–35] catalytic Tyr-153 and Lys-157 on the inside surface of α-helix F and NAD^+. Gly-184 is shown at the end of a β-strand, where we propose that it provides flexibility for the close approach of Thr-186 to the carboxamide group on nicotinamide. The distance of 4.0 Å is that for the final energy-minimized model. However, computer modelling shows that there is space for the alcohol side chain to move to as close as 2.8 Å to the carboxamide moiety, supporting the hypothesis [36] for a strong hydrogen bond between Thr-186 and NAD^+. The 3D model shows that replacement of Gly-184 with aspartic acid would alter the position of Thr-186. Moreover, the negative charge on aspartic acid is close to the substrate binding site and may interact with the substrate and nearby amino acids that participate in hydride transfer.

153 is proposed to abstract a proton from the alcohol side chain; lysine-157 is proposed to lower the pK_a of tyrosine from ~ 10 to ~ 7.6 [34]. Figure 2 shows the model of α-helix F, which contains tyrosine-153, lysine-157 and NAD^+ in close spatial orientation. Lysine-157 in ADH is close to the ribose 2′-hydroxyl on NAD^+ [26], in agreement with Varughese et al.'s analysis of dihydropteridine reductase [21]. The 3D model shows that replacement of alanine-159 with a threonine residue places an extra methyl side chain and a secondary alcohol in α-helix F, which could alter its conformation and displace either the essential lysine-157 and/or tyrosine-153 at the catalytic site, leading to loss of enzyme activity.

However, another interpretation is possible because Hollocher and Place found that Adh^{n2} does not form dimers; nor does interallelic complementation of Adh^{n2} with either Adh^S or Adh^F lead to heterodimers. This indicates that the alanine-159 to threonine mutation in Adh^{n2} affects the stability of functional dimers, leading to loss of enzyme activity. Figure 2 shows α-helix F with alanine-159 and alanine-158 from each subunit forming a hydrophobic anchor in the dimer interface. The four alanine residues from the dimer fit nicely into a hydrophobic tetrahedral configuration, which we propose is important in stabilizing the ADH dimer. Replacement of alanine-159 with threonine would disrupt this tetrahedral configuration and its hydrophobicity by increasing the distance between the two subunits and adding an alcohol side chain that can hydrogen bond to water in solvent. These changes would be expected to reduce the strength of the hydrophobic attraction between this part of the subunits. This would, of course, distort α-helix F and displace tyrosine-153 and/or lysine-157 in the catalytic site.

Glycine-184 to aspartic acid mutation

EMS mutant Adh^{n7} has glycine-184 mutated to aspartic acid. This mutation occurs in a glycine that is conserved among many members of this protein superfamily. The glycine is at the end of a β-strand that appears to be important in the close approach of threonine-186 to the nicotinamide ring. Although we show this distance as 4 Å in Figure 2, computer modelling indicates that the alcohol side chain can move to as close as 2.8 Å to the carboxamide moiety in nicotinamide. This indicates that hydrogen bonding between threonine-186 and NAD^+ is likely to be important in stabilizing NAD^+ in ADH. The loss of flexibility at residue 184, as a result of replacement of glycine with aspartic acid, will interfere with close association of threonine-186 with NAD^+. This could explain the finding of Cols et al. [35] that mutation of glycine-184 to leucine results in an inactive protein. The other effect of Asp-184 is to add a negative charge that could interact with the substrate and/or residues at the catalytic site, which would be expected to affect catalysis. Although Adh^{n7} forms homodimers that are inactive, interallelic complementation with Adh^{n1} leads to expression of an active heterodimer. Moreover, a cross with Adh^S leads to a heterodimer with greater specific activity than expected with Adh^S [9].

Another EMS mutant, Adh^{n11}, was shown by Thatcher [23] to have glycine-15 replaced by aspartic acid. Indeed, it was this mutation that first provided experimental evidence for the location of the canonical glycine motif that binds AMP. Interestingly, Hollocher and Place [8,9] found that Adh^{n11} is the most successful complementing subunit. It forms active heterodimers with two inactive mutants, Adh^{n1} and Adh^{n7}, as well as with Adh^F and Adh^S.

Dimer stability

Wild-type *Drosophila* ADH is active as a dimer. Activity for monomers has not been reported. Human ADH monomers have reduced activity under normal assay conditions [37,38]. It is interesting that all but one of the mutations we have identified do not eliminate formation of homodimers, suggesting that there are localized regions that are important in dimer stability. Varughese et al. [21] found that a four-helix bundle, consisting of α-helices E and F, forms the dimer interface in rat dihydropteridine reductase. Our 3D model of ADH predicts a similar role for the homologous helices in ADH. In particular, the outer surface of α-helix F contains alanine-158 and alanine-159, which form a tetrahedral hydrophobic anchor between the two subunits.

It is interesting that not all mutations on α-helix F lead to loss of dimer stability. We previously reported that mutation of tyrosine-153 to either phenylalanine or glutamic acid, or mutation

of lysine-157 to isoleucine, yielded mutants that could form dimers, even if they were enzymically inactive [34]. The lysine-157 to isoleucine mutation is especially interesting because it involves the loss of a positive charge and is close to alanine-158 and alanine-159, that we propose form an anchor for the dimer interface. It appears that the hydrophobic interaction between the outer surfaces on α-helix F in each monomer is strong enough to withstand significant changes in nearby residues in the interior of ADH.

The presence of the catalytically important tyrosine and lysine residues on the α-helix that is part of the dimer interface may explain why monomers are not active. Hydrophobic residues on the outside of the α-helix would be exposed to solvent, distorting α-helix F and the conformation of tyrosine-153 and lysine-157 in the catalytic site.

Interallelic complementation

The mechanism for interallelic complementation, in which inactive and partially active subunits in complexes with each other or with wild-type subunits can restore some enzyme activity in the mutants, is not known [39,40]. Crick and Orgel [39] suggested that a defect in the folding of a mutant subunit could be corrected by association with a complementing subunit. In this model, the effect of a mutation must be localized so that a subunit that is correctly folded can restore the active configuration to the mutant subunit.

Hollocher and Place [8,9] examined the different mutants for interallelic complementation. They found that in heterodimers of wild-type ADH and inactive ADH with mutations in glycine-15, glycine-17, glycine-93 or glycine-184, the wild-type subunit retains its activity. The first three mutations are in the AMP binding domain; the last one affects threonine-186, which is important in orienting NAD^+ in the catalytic site [36]. Evidently, the changes due to these mutations are sufficiently localized so as not to affect catalytically important residues on the wild-type subunit.

Hollocher and Place [8,9] found that the glycine-184 mutant has some activity in a heterodimer with wild-type ADH, suggesting some complementation of this mutation. Even more surprisingly, various heterodimers of the inactive homodimer ADH enzymes have about 5% of wild-type ADH activity. Thus heterodimers between ADHs with mutations in glycine-15 and glycine-93, glycine-15 and glycine-184, and glycine-93 and glycine-184 lead to an active enzyme, while all of these have no activity as homozygotes. These results indicate that X-ray analyses of these heterodimers crystallized with cofactor and substrate are likely to be useful in elucidating the mechanism of interallelic complementation.

Steroid and prostaglandin dehydrogenases

As with ADH, the residues that are important for catalysis by the homologues of ADH, which include steroid and prostaglandin dehydrogenases, are still being elucidated. ADH has been useful for this purpose because the effects of mutations in ADH [33–35] correlate with the effects of mutations at homologous sites in 11β-hydroxysteroid dehydrogenase [41] and 15-hydroxyprostaglandin dehydrogenase [42]. The four sites that we have identified in this paper have not been investigated in these or other ADH homologues. Mutations at the four homologous positions in these enzymes could be useful in helping to gain an understanding of the metabolism of steroids and prostaglandins.

This research was supported by NIH grant ES03347. The support of the Supercomputer Center at the University of California, San Diego, is gratefully acknowledged.

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Received 31 August 1994/10 January 1995; accepted 13 January 1995