This study was undertaken to identify the cytosolic 40-kDa zinc-containing alcohol dehydrogenases that oxidize all-trans-retinol and steroid alcohols in fetal tissues. Degenerate oligonucleotide primers were used to amplify by polymerase chain reaction 500-base pair fragments of alcohol dehydrogenase cDNAs from chick embryo limb buds and heart. cDNA fragments that encode an unknown putative alcohol dehydrogenase as well as the class III alcohol dehydrogenase were identified. The new cDNA hybridized with two messages of ~2 and 3 kilobase pairs in the adult chicken liver but not in the adult heart, muscle, testis, or brain. The corresponding complete cDNA clones with a total length of 1390 base pairs were isolated from a chicken liver λgt11 cDNA library. The open reading frame encoded a 375-amino acid polypeptide that exhibited 67 and 68% sequence identity with chicken class I and III alcohol dehydrogenases, respectively, and had lower identity with mammalian class II (55–58%) and IV (62%) isoforms. Expression of the new cDNA in Escherichia coli yielded an active alcohol dehydrogenase (ADH-F) with subunit molecular mass of ~40 kDa. The specific activity of the recombinant enzyme, calculated from active site titration of NADH binding, was 3.4 min$^{-1}$ for ethanol at pH 7.4 and 25 °C. ADH-F was stereospecific for the $\alpha$-hydroxysteroids. The $K_m$ value for ethanol at pH 7.4 was 17 mM compared with 56 mM for all-trans-retinol and 31 μM for epiandrosterone. Antiserum against ADH-F recognized corresponding protein in the chicken liver homogenate. We suggest that ADH-F represents a new class of alcohol dehydrogenase, class VII, based on its primary structure and catalytic properties.

Cytoplasmic zinc-containing alcohol dehydrogenases (ADH)$^1$ with 40-kDa subunits are capable of oxidizing a variety of primary, secondary, and aliphatic alcohols and a limited number of cyclic alcohols (1). Six classes of dimeric ADH isoforms have been identified in mammals (1). Except for class I, all other classes of ADH have high $K_m$ values for ethanol and oxidize medium-chain and long-chain alcohols most effectively.

Potential physiological substrates for ADH isoforms include retinoid and steroid alcohols. Human class IV is the most efficient retinol dehydrogenase, followed by class II and the class I $\alpha$ ADH (2). Class IV and II ADH are not active with steroid alcohols, whereas class I isoforms oxidize both retinoid and steroid substrates with relatively low catalytic efficiency. Class I isoforms$^2$ exhibit stereospecificity toward alcohol substrates. For example, horse SS and human γγ ADH oxidize 3β-hydroxyprogesterone but not 3α-hydroxyprogesterone (3). ADH isoforms vary in their tissue distribution; class IV ADH is expressed in the epithelial tissues of mammals and is present in the human stomach mucosa and esophagus (1), whereas class II $\pi\pi$, $\alpha\alpha$, and their heterodimers, as well as class II $\pi\tau$, are the predominant forms responsible for ethanol oxidation in the human adult liver. Class I isoforms are also expressed to a lesser extent in certain adult and fetal tissues, such as kidney, skin, gastrointestinal tract, and lung.

The physiological significance of the cytosolic ADHs for steroid and retinoid metabolism is not clear. The retinoid and steroid hormones play a major role in fetal development and are detected in the embryonal tissues during the early developmental stages. Since ADH isoforms appear at different stages of embryogenesis, it is important to determine which isoforms are present in the embryo during the stages when retinoid and steroid hormones are synthesized. The chick embryo is used as a model to study the effects of various hormones on gene expression during development. In this study, we analyzed the mRNA isolated from the chick fetal heart and limb buds at stage 21 for the presence of messages encoding cytosolic ADH.

EXPERIMENTAL PROCEDURES

PCR Amplification of ADH Isozymes—Degenerate oligonucleotide primers were synthesized based on the peptide sequences EID/EXUI/V/EVAP and FGLG/GV, which are conserved in all animal alcohol dehydrogenases (4). The first region corresponds to amino acids 24–30 (sense primer) and the second region to amino acids 198–204 (antisense primer) of the human class I $\beta_1$ ADH. Four oligonucleotides were synthesized for the sense orientation: 1), GA/GA/AG/TG/TGAG/G/A/GTGCC; 2), GA/GA/GA/CAT/CA/TG/GA/G/GTGCC; 3), GA/GA/GA/G/A/GTG/GCGC; and 4), GA/GA/GA/G/A/GAT/CA/TG/GA/G/GTGCC. Two oligonucleotides were synthesized for the antisense orientation: 1), TT/GA/GCG/G/G/TG; and 2), TT/GA/GCG/G/G/G/TG. Inosines were incorporated in

$^1$ The abbreviations used are: ADH, alcohol dehydrogenase; ADH-F, the new ADH in fetal chick described in this study; bp, base pairs; GST, glutathione S-transferase; PCR, polymerase chain reaction.

$^2$ Human class I alcohol dehydrogenases include the $\alpha$, $\beta_2$, and $\gamma$ isoforms; the human class II isoform is $\pi\pi$, the human class III isoform is $\chi\chi$, and the human gastric class IV isozyme is $\sigma\sigma$. Polymorphic variants of class I isoforms are designated by subscripts (e.g., $\beta_2$).
all positions that required a degeneracy of 4. Limb buds and hearts were dissected from 30 chick stage-21 embryos (3 days old). Total RNA was isolated from each tissue with RNAzol according to the manufacturer's protocol. Twenty micrograms of each RNA preparation were loaded onto a formaldehyde-agarose gel and RNA from each pool was reverse transcribed and used for PCR amplification. The amplified coding region of the new ADH mRNA were found to have an novel sequence with a high sequence identity with human class III ADH sequences, and 6 of the clones from limb bud individual clones were sequenced. Two of the clones from heart were built into the human stomach ADH-F. The expression of the fusion protein in the M13mp19RF (Life Technologies, Inc.) vector for sequencing (U.S. Biochemical Corp.) or with 0.2 mM NADH. The kinetic constants for alcohols other than retinol were obtained by monitoring the production of NADH at 340 nm (ε = 6.22 mε⁻¹ cm⁻¹). Reaction mixtures with steroid substrates contained 0.02% Tween 80. Steroid stock solutions were prepared in methanol, and concentration of methanol in the assay mixtures was kept constant at 0.3 M. Chick ADH-F was neither active toward nor inhibited by methanol up to 3 M at pH 7.5. The Vₘₐₓ and Kₘ values for alcohol substrates (at 2.4 mM NAD⁺) were calculated from a fit of the kinetic data to the Michaelis-Menten equation, V = Vₘₐₓ[A/Kₘ + A], where A is the concentration of the varied substrate. The kₗₒₜ (min⁻¹) was obtained by dividing Vₘₐₓ by the concentration of active sites assuming a subunit Mₘₐₓ of 40,000. The apparent Kₘ values for NAD⁺ and NADH were determined with 1 mM cinnamyl alcohol and 100 μM cinnamaldehyde, respectively. The inhibition constant for 4-methylpyrazole was determined with butanol as a substrate by varying both butanol (68–200 μM) and 4-methylpyrazole (75–350 μM) concentrations. The Kᵢ of 4-methylpyrazole was calculated from a fit of the kinetic data to the equation for competitive inhibition, V = VₘₐₓB/Kᵢ (1 + KᵢB⁻¹ + Kᵢ⁻¹). The Ki value for NADH was determined by varying NAD⁺ (15–60 μM) at 1 mM cinnamyl alcohol, using 0–10 μM NADH as the inhibitor.

The rabbit antiserum was raised against recombinant ADH-F. A 1:5,000 dilution of this antiserum detected 10 ng of purified ADH-F. Frozen chicken liver was homogenized in 10 mM Tris–HCl, pH 7.4, plus 5 mM benzamide and 1 mM diithiothreitol. The homogenate was centrifuged at 10,000 × g for 20 minutes, and the supernatant was concentrated twice. Glycerol was added to 50% concentration, and the liver extract was stored at −20°C. The proteins in the chicken liver homogenate were separated by isoelectric focusing using 3–10 pH gradient isoelectric focusing agarose plates (FMC Bioproducts, Inc.). After focusing, the separated proteins were transferred to nitrocellulose membrane, blocked with 3% bovine serum albumin in PBS-T, and incubated with the rabbit antiserum. The binding of anti-ADH-F antibody was visualized with 125I-protein A.

The amino acid substitutions occurring in chick ADH-F were modeled built into the human βₕ-structure using the molecular graphics program QUANTA (Molecular Simulations, Inc.). Following substitution of all amino acid side chains in the dimer, the model structure was subjected to 100 cycles of energy minimization using X-FLOR 3.1 with the x-ray energy term omitted (9). The position for the epoxide at the active site in the human βₕ-structure was found by manually adjusting its position to minimize close contacts between the enzyme active site and the substrate molecule.

Sequences of human class I βₕ, α and γ ADH; class I ADHs from the alligator, cod, frog, horse E and S, mouse, ostrich, quail, rabbit, and rat; class II ADHs from human and rat; class III ADHs from human, horse, mouse, and rat; class IV ADHs from human and mouse; human ADH6; and class VI from deer mouse were aligned with ADH-F by a progressive alignment method according to Feng and Doolittle (10). Sequences of ADHs were obtained from the GenBank™.

RESULTS

The pool of ~500-bp PCR products obtained with ADH-specific primers from the limb buds and heart mRNA of chick embryos at stage 21 was subcloned in M13 vector, and 48 individual clones were sequenced. Two of the clones from heart mRNA were found to have a novel sequence with a high resemblance to ADH sequences, while 4 of the clones from limb bud mRNA encoded a fragment that exhibited 87% peptide sequence identity with human γ-ADH (11, 12). The rest of the clones contained cDNA sequences that were not related to ADH. Since human class III ADH is not active with retinol, we did not pursue the cloning and characterization of this γ-ADH-like chick isozyme further.
The deduced protein sequence of the two novel identical PCR clones had a high resemblance to ADH sequences but was different from that of the \( \chi \)-ADH-like chick ADH and the chick class I ADH (13). A Northern blot analysis of adult chicken tissues demonstrated that this partial PCR product from embryonal heart hybridized with two messages of approximately 2 and 3 kilobase pairs in adult chicken liver (Fig. 1). Other tissues (brain, testis, skeletal muscle, and heart) did not show a detectable hybridization signal after 24 h of exposure. A chicken liver Agt11 cDNA library was used to isolate a full-length cDNA. Three independent clones hybridizing with the partial cDNA were isolated. Two clones encoded a complete cDNA, and one lacked the N terminus. The total composite cDNA sequence was 1408 bp long with the ATG starting codon at nucleotide 74 and the TGA stop codon at nucleotide 1202 (Fig. 2). The open reading frame encoded a 375-amino acid mature polypeptide with predicted Mr of 40,016.

The relationships of this presumed new ADH (ADH-F) with other ADH isozymes were analyzed by progressive alignment (10). Table I shows percentage identity of the new chick enzyme (ADH-F) with other ADH classes (11) as well as the range of percentage identity of the isozymes from different species within the same ADH class. The identity of the new ADH was highest with class I isozymes (69%). Class II and VI ADH were the least similar (about 60% identity) (Table I).

To characterize the catalytic properties of the new isozyme, the ADH-F cDNA was expressed in \( E. \text{coli} \) as fusion protein with GST (14). The recombinant enzyme separated from GST by thrombin cleavage had an apparent subunit Mr of 40,000 on SDS-polyacrylamide gel electrophoresis. 1 to 2 units of activity were obtained from a 1-liter culture, which corresponded to 12–24 mg of active enzyme. The specific activity of the ADH after thrombin cleavage was the same as that for the ADH-GST fusion protein.
the retinoid and steroid alcohols. The only ADH-related PCR product amplified from limb bud mRNA was identified as chick cinnamyl alcohol, and for NADH with saturating 100 μM cinnamyl alcohol, and for NADH with saturating 100 μM cinnamyl aldehyde.

### Table I

| ADH class | I  | II  | III | IV | V  | VI |
|-----------|----|-----|-----|----|----|----|
| ADH-F     | 61–69 | 54–62 | 62–64 | 62–63 | 63 | 57 |
| I         | 69–83 | 53–61 | 62–66 | 66–71 | 59–67 | 55–60 |
| II        | 66–72 | 55–63 | 51–58 | 54–60 | 47–51 |
| III       | 93–95 | 60–61 | 58 | 55 |
| IV        | 87–89 | 60° | 56–57 |
| V         | 100° | 67° |
| VI        | 100° |

a Includes isozymes from human (α, β, and γ1), horse (S and E), rat, mouse, rabbit, chicken, ostrich, alligator, and frog.

b Includes isozymes from human, rat, and ostrich.

c Includes isozymes from human, horse, rat, and mouse.

d Includes isozymes from human, rat, and mouse.

e A single representative of the class, human ADH6, is known.

f Class VI is represented by the deer mouse isozyme.

### Table II

| Substrate/cofactor | K<sub>m</sub> (mM) | ADH-F | Class I | Class II | Class IV |
|--------------------|------------------|-------|---------|----------|----------|
| Ethanol            | 0.17 ± 1.1       | 0.33<sup>a</sup> | 34<sup>b</sup> | 28       |
| Butanol            | 0.087 ± 0.008    | 0.040<sup>c</sup> | 0.15 ± 0.02<sup>c</sup> | 0.79     |
| (S)+(-)-2-Butanol  | 0.63 ± 0.10      | 1.9<sup>c</sup> | 24 ± 3<sup>c</sup> | 120      |
| (R)-(-)-2-Butanol  | 0.14 ± 0.02      | 1.2<sup>c</sup> | 49 ± 10<sup>c</sup> |           |
| Cyclohexanol       | 0.017 ± 0.002    | 0.03<sup>d</sup> NS | 0.014 | 0.009 |
| all-trans-Retinol  | 0.056 ± 0.005    | 0.29 | 0.014 | 0.009 |
| trans-2-Hexen-1-ol | 0.0081 ± 0.0004  | –    | 0.025 ± 0.003<sup>e</sup> | 0.019 ± 0.002<sup>e</sup> |
| NAD<sup>+</sup>     | 0.0054 ± 0.0005  | 0.0079<sup>f</sup> | 0.014<sup>f</sup> | 0.21     |
| NADH               | 0.0053 ± 0.0007  | 0.007<sup>f</sup> | 0.016<sup>f</sup> | 0.22     |

<sup>a</sup> Stone et al. (24).
<sup>b</sup> Bosron et al. (23).
<sup>c</sup> Data obtained in this study.
<sup>d</sup> NS, not saturable at the maximum solubility; NA, not active with 240 mM cyclohexanol at pH 7.5.

### Discussion

This study was undertaken to identify the isoforms of cytosolic 40-kDa (subunit) ADH in chick fetal tissues that oxidize the retinoid and steroid alcohols. The only ADH-related PCR product amplified from limb bud mRNA was identified as chick class III ADH based on the 87% protein sequence identity with human class III γ-ADH (12). Because human class III ADH is not active with retinol, we were not interested in characterizing
the corresponding chick enzyme. However, PCR amplification of embryonal heart mRNA yielded a novel ADH-related cDNA. This new cDNA encoded an active enzyme named ADH-F.

Northern blot analysis of tissues from a 7-week-old chicken showed that among seven tissues analyzed, only the liver contained an mRNA hybridizing with the partial PCR product from the fetal heart. The adult heart mRNA did not contain a sequence that hybridized with this PCR product. A change in the tissue-specific expression of ADH gene between fetal and adult organism was also observed for the class I ADH mRNA in rat (17) and may reflect different metabolic needs of the tissues at different stages of development. The complete cDNA isolated from the chicken liver cDNA library encoded an ADH (Fig. 2). ADH-F contained 13 residues (Fig. 2) that are conserved in the 47 members of the zinc-containing ADH family excluding a-crystallin (4). Sequence alignment with other animal ADHs indicated a single amino acid insertion after position 55. Therefore, the numbers of the conserved amino acid residues after Gly-55 were shifted by one position in chick ADH-F (Fig. 2) when compared with class I ADH (18). The conserved glycines and the value of the substrate binding domain were present at positions 67, 72, 78, 87, and 81 (Fig. 2). The four glycines of the coenzyme binding domain were in positions 193, 202, 205, and 237. Conserved ligands to the catalytic zinc, Cys-46 and His-68, were also present. The new ADH-F sequence had Asp-224, which has been suggested to determine the specificity for NAD$^+$ versus NADP$^+$ as a coenzyme, and Thr-48, which is thought to form a hydrogen bond with the alcohol hydroxyl group bound to the catalytic zinc (19). Cysteines 98, 101, 104, and 112, which are responsible for binding the noncatalytic zinc (20), were conserved in the new ADH. However, the sequence of this ADH had less than 68% identity with any of the known ADH isoforms (Table I); hence, we conclude that ADH-F belongs to a separate class in the family of ADHs, class VII.

The new ADH gene encoded an active enzyme when produced as a recombinant protein in E. coli. Antiserum against this recombinant ADH-F recognized protein bands in the chicken liver homogenate with the same range of isoelectric points as the multiple ADH-F forms (Fig. 3). The slightly more basic pI of the recombinant protein is consistent with the lack of N-acetylation in E. coli-expressed proteins (21).

The functional and kinetic properties of the new recombinant ADH-F were compared with those of other ADHs. The yield of ADH-F protein was high (up to 14 mg/L of E. coli culture), but the specific activity with saturating ethanol at pH 7.4 was relatively low (0.08 unit/mg), a value that is similar to that of the human class I (38). ADH-F activity (0.1 unit/mg) (22). The $K_a$ value for ethanol (17 mM) was close to that of human stomach class IV ADH (29 mM) (5) and human liver class II ADH (34 mM) (23) (Table II). NADH inhibited NAD$^+$ reduction competitively, and 4-methylpyrazole was a competitive inhibitor of butanol. These inhibition results are consistent with the Ordered Bi Bi mechanism suggested for other ADHs. ADH-F sensitivity to 4-methylpyrazole inhibition was similar to that of the human class IV ADH ($K_a = 350 \mu M$) (5). The $K_a$ value (300 \mu M) was greater than that of class I and less than that of class II ADH.

The catalytic efficiency of ADH-F toward the secondary al-

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**Table III**

| Substrate | $k_{cat}/K_m$ values of chick ADH-F for alcohol substrates compared with other ADH isoforms |
|-----------|--------------------------------------------------------------------------------|
| Ethanol   | 0.20 ± 0.01 | 150$^a$ | 150$^a$ | 0.6$^a$ | 65 |
| Butanol   | 50 ± 7      | 1,800$^b$ | 140 ± 13$^c$ | 2,600 |
| (S1(+)-2-Butanol | 3 ± 0.4 | 8.0$^c$ | 0.086 ± 0.006$^d$ | 1.2 |
| (R1(+)-2-Butanol | 24 ± 2 | 16$^c$ | 0.064 ± 0.008$^d$ | – |
| Cyclohexanol | 209 ± 15 | 600$^c$ | NS$^c$ | NA$^c$ |
| all-trans-Retinol | 26 ± 1 | 19 | 650 | 2,600 |
| trans-2-Hexen-1-ol | 1,009 ± 25 | – | 5,400 ± 470$^c$ | 64,000 ± 3,000$^c$ |

$^a$ Stone et al. (24).
$^b$ Bosron et al. (23).
$^c$ Data obtained in this study.
$^d$ NS, not saturable at the maximum solubility; NA, not active with 240 mM cyclohexanol at pH 7.4.

**Table IV**

| Substrate | $K_a$ | $K_m$ |
|-----------|-------|-------|
| 5x-Androstan-3β-ol-17-one | 0.031 ± 0.004 | 17 ± 2 |
| (epiandrosterone) | | |
| 5β-Androstan-3β-ol-17-one | – | Not active |
| (dihydrotestosterone) | | |
| 5β-Androstan-17β-ol-3-one | 0.079 ± 0.009 | 15.0 ± 0.3 |
| (dehydroepiandrosterone) | | |
| 5α-Androstan-3β-ol-17-one | 0.022 ± 0.003 | 23.0 ± 2.3 |
| (epiandrosterone) | | |
| 5β-Pregnan-3β-ol-20-one | – | Not active |
| (epipregnanolone) | | |

$^a$ 2.4 mM NAD$^+$.
$^b$ 0.2 mM NADH.

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**Fig. 3. Western blot analysis of ADH-F in chicken liver homogenate. Lane 1, 100 ng of recombinant ADH-F. Lanes 2 and 3, 1 and 10 μg of liver homogenate, respectively. Numbers on the right indicate isoelectric points of the isoelectric focusing standard proteins.**
Alcohol (R)-(2)-2-butanol was seven times that of (S)-(+)2-butanol. This specificity appeared to be similar to that of the human αα isozyme, where its catalytic efficiency was about four times higher with (R)-(−)-2-butanol (136 min⁻¹ mM⁻¹) than with (S)-(+)2-butanol (37.6 min⁻¹ mM⁻¹) (24). It has been suggested that the specificity of ADHs toward secondary alcohols is affected by amino acids at positions 48 and 93. Chick ADH-F has Thr-48 as in αα ADH and a unique Pro at the position homologous to residue 93 (94 in ADH-F). Modeling of the amino acid substitutions present in chick ADH shows that Pro can easily be accommodated at position 93 (Fig. 4) and that the region of the active site occupied by the secondary alcohol more closely resembles αα, with Ala at position 93, than ββ, with Phe at position 93. Thus, it is not surprising that this new ADH isozyme possesses a stereospecificity for small secondary alcohols that is more similar to αα than ββ (or horse ADH). The catalytic efficiency (kcat/Km) of ADH-F was higher for oxidation of large alcohols. For example, ADH-F was 5 × 10⁴ times more efficient with trans-2-hexen-1-ol than with ethanol.

Chick ADH-F was similar to human class I γγ isozyme in that it oxidized both retinoid and steroid alcohols (Tables III and IV). It was different from the other two retinol-oxidizing ADH isozymes, class IV σσ and class II ππ, which were not active with steroids (5, 25). ADH-F oxidized epiandrosterone about 88 times more slowly than horse SS ADH (0.51 min⁻¹ versus 44 min⁻¹) (26). The catalytic properties of ADH-F suggest that it may function as a steroid/retinoid dehydrogenase in chick. However, the physiological significance of chick ADH-F for steroid and retinoid metabolism will be clarified once the tissue-specific expression pattern during development and the amount of the active enzyme in tissues are determined.

In general, no single amino acid difference appears to be responsible for the unique kinetic properties of the new chick ADH. The ability to oxidize large hydrophobic alcohols, such as retinol and 3β,5α-hydroxysteroids, appears to be the result of several amino acid substitutions near the active site zinc atom and at the entrance to the alcohol binding pocket. With current knowledge, the ability of ADH isozymes to oxidize 3β-hydroxysteroids has been limited to those that possess a Ser at position 48 (human γγ and horse SS ADH). ADH-F has a Thr at position 48. Its ability to bind steroids productively may be due to substitutions in the vicinity of position 93. In steroid-oxidizing
horse SS and human γ, which have a Ser at position 48, there
is also a Phe at position 93, which is usually preceded by a
Pro-Leu sequence. The sequence Leu-Phe-Pro in chick ADH-F,
instead of Pro-Leu-Phe as in most class I isozymes, may ac-
count for the difference in steroid alcohol specificity. In addi-
tion, there are unusual substitutions at positions 318 and 319,
where Leu and Ala substitute for Ile and Phe, respectively.
Thus, the “floor” of the alcohol binding pocket appears to be
more open in ADH-F compared with class I ββα, and this could
make the site more accessible to large substrates (Fig. 4).
The enzyme appears to be sensitive to the configuration at the
5-position of steroid alcohol, since the 5α-hydroxysteroid alco-
hol is inactive (Table IV). Molecular modeling suggests that
the stereospecificity at the 5α-position of the steroid may be due
to the presence of the extra methyl group of Thr-48 and the
rearranged floor of the substrate binding pocket in the ADH-F
compared with ββα (Fig. 4).
The productive association of large hydrophobic substrates
leading to efficient oxidation is usually associated with rear-
rangements near the entrance to the alcohol binding pocket. It
was shown that both the horse SS and the human class IV σσσσ iso-
zyme efficiently oxidize sterols and retinol, respectively, due
to alterations in the loop at the entrance to the alcohol bind-
ing site comprising residues 112–119 (5, 27). Both of these
isozymes possess single amino acid deletions that appear to
widen the mouth of the substrate binding site, permitting easi-
er access for these large substrates. Although chick ADH-F
isozyme does not possess such a deletion, the presence of His
and Trp in positions 115 and 142, respectively, may affect
the conformation of this loop. A neutral or acidic residue at position
115 helps to correctly position this loop by hydrogen bonding
with the peptide nitrogen of residue 118 in most class I ADH
crystal structures. The His at position 115 will not perform the
same function to anchor this loop structure in place, and a
conformational change in the structure of this loop could cre-
ate a more open substrate binding site. The substitution of Asp-115
by Trp in the cod ADH crystal structure appears to be the
primary reason for the conformation of this loop to adopt an
α-helical structure (28). It is not clear whether the insertion of
one amino acid in the region between positions 55 and 60 will
also affect the structure at the entrance to the substrate bind-
ing site. Mutagenesis in class III χ-ADH, which also has an
insertion in this region, strongly implicates a role for Asp-57 in
binding of the substrate hydroxymethyl glutathione (29). Our
modeling of chick ADH would suggest that Phe-57 could form
favorable van der Waals contacts with the hydrophobic face of
hydroxysteroids.

Another interesting substitution occurs at position 173. In
most ADH isozymes the catalytic zinc ligand Cys-174 is sur-
rounded by two glycines. These glycines may provide the nec-
essary flexible linkage between the catalytic and coenzyme
binding domains to allow the large conformational change ob-
served upon coenzyme binding. The presence of Ala at this
position may impair the ability of this isozyme to undergo rapid
conformational shifts in its structure and may explain, at least
in part, the relatively low turnover rate of this isozyme.

Thus, ADH-F appears to be unique in terms of its structure-
function relationships. This enzyme has low specific activity; it
is active with 3β,5α-hydroxysteroids but not with 3β,5β-hy-
droxysteroids; it is active toward steroid substrates in the
absence of Ser-48; and it is active toward retinol in the absence
deletion in the loop between amino acids 115 and 120. Sev-
eral amino acid substitutions discussed above suggest an ex-
planation for some of its properties. X-ray structure determi-
nation of the enzyme will provide more complete insight into
the structural basis of its substrate specificity.

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