Structure of the Class II Enzyme of Human Liver Alcohol Dehydrogenase: Combined cDNA and Protein Sequence Determination of the π Subunit†

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ABSTRACT: The class II enzyme of human liver alcohol dehydrogenase was isolated, carboxymethylated, and cleaved with CNBr and proteolytic enzymes. Sequence analysis of peptides established structures corresponding to the π subunit. Two segments from the C-terminal region unique to π were selected for synthesis of oligodeoxyribonucleotide probes to screen a human liver cDNA library constructed in plasmid pT4. Sequence analysis of two identical hybridization-positive clones with cDNA inserts of about 2000 nucleotides gave the entire coding region of the π subunit, a 61-nucleotide 5′ noncoding region and a 741-nucleotide 3′ noncoding region containing four possible polyadenylation sites. Translation of the coding region yields a 391-residue polypeptide, which in all regions except the C-terminal segment corresponds to the protein structure as determined directly by peptide analysis. With the class I numbering system, the exception concerns a residue exchange at position 368, the actual C-terminus which is Phe-374 by peptide data but a 12-residue extension by cDNA data, and possibly two further residue exchanges at positions 303 and 312. The size difference might indicate the existence of posttranslational modifications of the mature protein or, in combination with the residue exchanges, the existence of polymorphism at the locus for class II subunits. The π subunit analyzed directly results in a 379-residue polypeptide and is the only class II size thus far known to occur in the mature protein. Comparison of the π structure with those of the class I subunits (α, β, and γ) reveals a homology with extensive differences (positional identity: 60–65% in the different pairwise comparisons between π and the other three subunits). Large variations in segments affecting relationships at the active site and the area of subunit interactions account for the significant alterations of enzymatic specificities and other properties that differentiate class II from class I enzymes.

Detailed studies of mammalian alcohol dehydrogenases initially concerned the horse liver enzyme (Brändén et al., 1975). Subsequent work established structural, functional, and evolutionary relationships between that and other alcohol dehydrogenases, including the enzyme from highly divergent organisms, such as yeast and maize (Jörnvall et al., 1978; Dennis et al., 1984; Brändén et al., 1984; Young & Pilgrim, 1985; Duester et al., 1986a), as well as other enzymes, such as polyol and sugar dehydrogenases (Jörnvall et al., 1981, 1984b,c; Jeffery & Jörnvall, 1983). For a time, the knowledge regarding human alcohol dehydrogenase was less extensive but the enzyme was known to be composed of multiple isozymes (Blair & Vallee, 1966; Smith et al., 1971; Li & Magnes, 1975; Li et al., 1977). Introduction of an efficient affinity chromatography procedure (Lange & Vallee, 1976) has made possible purifications of all the different forms (Wagner et al., 1983). Three enzyme classes with different enzymatic, immunological, and electrophoretic properties have been identified (Strydom & Vallee, 1982; Vallee & Bazzone, 1983).

Class I isozymes migrate cathodically on electrophoresis and are sensitive to the competitive inhibitor pyrazole. The class I isozymes are homo- and heterodimers of the α, β, and γ subunits (Smith et al., 1971); their structures are known at both the protein and cDNA levels (Hempel et al., 1984, 1985; Bühler et al., 1984, Jörnvall et al., 1984a; Duester et al., 1984; Ikuta et al., 1985, 1986; Hedén et al., 1986; von Bahr-Lindström et al., 1986; Höög et al., 1986). Class II isozymes are dimers of the π subunits, which are less cathodic, and less sensitive to pyrazole, than the class I forms (Li & Magnes, 1975; Bosron et al., 1979). Dimers of the χ subunits constitute the anodic and pyrazole-insensitive class III isozymes (Parés & Vallee, 1981).

Thus far, the $\pi\pi$ isozyme has been detected in liver. However, the organ distribution of all the isozymes differs and is specific (Li et al., 1977; Parés & Vallee, 1981; Vallee & Bazzone, 1983; Beisswenger et al., 1985). Like other alcohol dehydrogenase isozymes, the $\pi\pi$ isozyme catalyzes the oxidation of a variety of alcohols, preferably hydrophobic alcohols, though its uniquely specific substrates have not been identified. The $\pi\pi$ isozyme fails to oxidize methanol, glycerol (Bosron et al., 1977), ethylene glycol, intermediates in the oxidation pathway of norepinephrine (Mårdh et al., 1985), or any of the metabolites of the cardiac sterols (Ditlow et al., 1984).

At least five gene loci, ADH1–ADH5, code for the different isozyme subunits. All loci for class I, and perhaps also the remaining loci, are located on the long arm of chromosome 4 (Duester et al., 1985). The exon–intron arrangement of the ADH2 gene that encodes the class I β subunit has been determined (Duester et al., 1986b).

In this study, we present the first structural analysis of the π subunit. The structure, established by peptide and cDNA analyses in parallel, shows that the π subunit is clearly but

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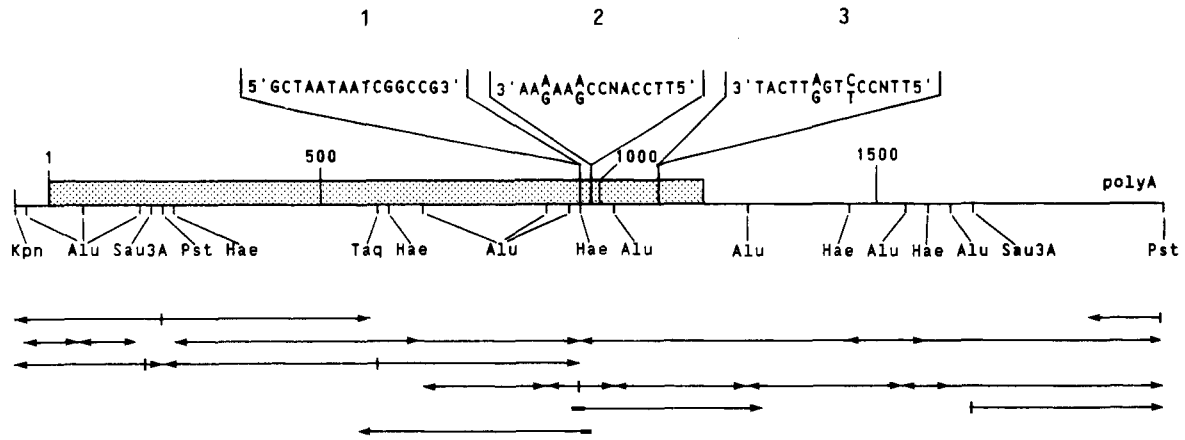

FIGURE 1: Restriction map of cDNA clones investigated corresponding to the π subunit of human liver alcohol dehydrogenase and synthetic oligodeoxyribonucleotides utilized as hybridization probes and sequencing primers. The cDNA insert is drawn to scale (continuous line). Probes 2 and 3 are enlarged (top line) at their positions in the continuous cDNA sequence and were used to screen the cDNA library. Oligodeoxyribonucleotides 1 and 2 (also enlarged at their correct positions) were used as sequencing primers. Only restriction sites used for the dideoxy sequence method are shown. Arrows indicate the direction of sequencing, and the scale at the top refers to base pairs. The stippled region corresponds to the coding sequence.

distantly related to those of the class I enzymes, with differences that can be interpreted to account for the altered enzymatic properties and subunit interactions.

Materials and Methods

Protein Analysis. The $\pi\pi$ enzyme of human liver alcohol dehydrogenase was isolated as described (Bosron et al., 1977), reduced, carboxymethylated, and fragmented with CNBr, Glu-specific protease from *Staphylococcus aureus* strain V8, or trypsin. Peptides were purified by exclusion chromatography on Sephadex G-50 and by reverse-phase high-performance liquid chromatography (Jeffery et al., 1984a). Amino acid compositions were determined, after hydrolysis for 24 h at 110 °C in 6 M HCl/0.5% phenol, on a Beckman 121M amino acid analyzer. Amino acid sequence analysis was performed by the manual (dimethylamino)azobenzene isothiocyanate method as described (von Bahr-Lindström et al., 1982) and by liquid-phase sequencer degradations with phenylthiohydantoin identification by high-performance liquid chromatography (Jeffery et al., 1984a).

Identification of cDNA Clones. A human liver cDNA library constructed in plasmid pT4 by the method of Okayama and Berg (1982) and earlier found to contain cDNA clones corresponding to class I ADH (Hedén et al., 1986; von Bahr-Lindström et al., 1986; Höög et al., 1986) was screened by hybridization with π-specific probes.

On the basis of peptide sequences, two mixed oligodeoxyribonucleotides (Figure 1) were synthesized by the solid-phase phosphoramidite method with an automatic synthesizer (Josephson et al., 1984). The oligodeoxyribonucleotides were labeled at the 5'-end with ^{32}P from [γ-^{32}P]ATP (Amersham; 3000 Ci/mmol), by use of T4 polynucleotide kinase (Amersham; Sgaramella & Khorana, 1972). Colonies were screened for hybridization with the oligodeoxyribonucleotides, first screened in an unordered fashion and then rescreened after restreaking of putative positive colonies. Hybridization conditions were as described (Wallace et al., 1979); after being washed, the filters were subjected to autoradiography with intensifying screens.

Isolated plasmid DNA from hybridization-positive clones was cleaved with restriction enzymes *Kpn*I and *Pst*I (Amersham), and the fragments were separated in low melting point agarose (Bio-Rad). Large fragments were recleaved in agarose slices, with *Alu*I, *Hae*III, *Sau*3AI, or *Taq*I (Boehringer and Pharmacia). All cleavages were performed under the conditions suggested by the manufacturers.

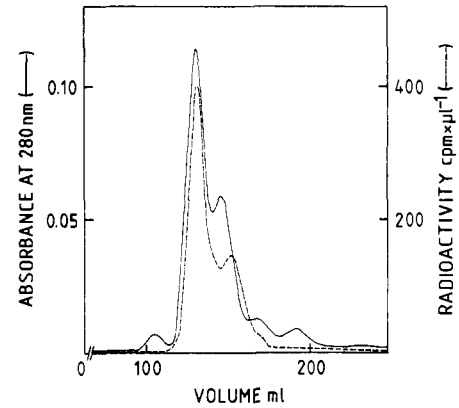

FIGURE 2: Exclusion chromatography on Sephadex G-50 in 30% acetic acid of CNBr fragments obtained from the carboxymethylated π subunit of class II human liver alcohol dehydrogenase.

DNA Sequence Analysis. Restriction enzyme fragments, in agarose slices, were ligated into M13 vectors (Messing & Vieira, 1982; Norrander et al., 1983). Sequence analysis was carried out by the dideoxy method (Sanger et al., 1977) with single-stranded M13 templates (Schreier & Cortese, 1979) and an M13-specific universal primer (17-mer; Amersham) or a π-specific primer (Figure 1) synthesized on a Pharmacia Gene Assembler. The labeled nucleotide was [α-^{35}S]dATP (Amersham; 600 Ci/mmol), and the sequence mixture was separated on ultrathin (0.2 mm) urea–polyacrylamide gels.

Results

Direct Amino Acid Sequence Analysis of the π Protein Subunit. Separate samples of the reduced and carboxymethylated π subunit were cleaved with CNBr, staphylococcal Glu-specific protease, and trypsin. Peptides obtained were purified by exclusion chromatography and reverse-phase high-performance liquid chromatography. The elution pattern of CNBr fragments from the initial Sephadex G-50 step (Figure 2) suggests the presence of few methionine residues, in agreement with the total composition (Table I). Results of structural analyses for all peptides analyzed are summarized in Figure 3 and given in detail in Table II for those peptides that were found not to be in complete agreement with the structure deduced from the cDNA. Peptides obtained were homologous to class I enzymes, but differences were extensive. Therefore, before knowledge of the entire structures, peptides

Table I: Amino Acid Composition of the π Subunit of Class II of Human Liver Alcohol Dehydrogenase[a]

residue	acid hydrolysis	final sequence (Figure 3)
Cys	13.4 (14)	16
Asx	34.6 (32)	33
Thr	27.4 (24)	26
Ser	23.1 (22)	24
Glx	25.3 (27)	23
Pro	18.4 (23)	17
Gly	41.2 (38)	39
Ala	35.7 (33)	35
Val	25.2 (29)	26
Met	3.9 (6)	4
Ile	25.5 (26)	32
Leu	31.3 (31)	30
Tyr	5.4 (8)	5
Phe	18.7 (17)	19
Trp	ND (3)	3
Lys	34.2 (29)	33
His	5.6 (7)	6
Arg	7.2 (10)	8

[a] The first column shows presently obtained analytical values from hydrolysis of the protein (Trp not determined, ND). For comparison, earlier published data from hydrolysis of the π subunit (Bosron et al., 1979), recalculated to the now-known size, are given in the second column. The last column shows the composition from the 379-residue subunit given in Figure 3.

from corresponding segments of the class II and class I subunits were often not identified as such from compositions or properties only but required long regions of sequence characterizations before final alignments could be ascertained. The C-terminal segment, including the C-terminal phenylalanine, was detected by direct peptide analyses and was recovered in both a CNBr fragment (Table II) and a tryptic peptide (Figure 3). Similarly, all the CNBr fragments expected from the final structure in Figure 3 were traced by direct degradations, except the N-terminal CNBr fragment, which is therefore concluded to be blocked by α-amino acetylation, as in other mammalian alcohol dehydrogenases (Persson et al., 1985).

Screening of a cDNA Library. A total of 10 000 colonies was screened with the two oligodeoxyribonucleotide probes synthesized to correspond to parts of the peptide structures determined. Four colonies gave positive hybridization signals with both probes, and two of these were selected for further analysis, on the basis of the size of the insert and of partial nucleotide and peptide sequence data. The total lengths of their cDNA inserts were estimated from electrophoresis in agarose gels to be about 2000 nucleotides.

Nucleotide Sequence Analysis. The DNA sequences of the cDNA inserts in the two plasmids were determined according to the strategy shown in Figure 1. The plasmids were cleaved with *Kpn*I and *Pst*I, and the resulting fragments were separated by electrophoresis in agarose. Large fragments were cleaved in agarose before ligation. All fragments were ligated in both directions into appropriate M13 vectors and were subjected to dideoxy sequence analysis on both strands. The 3′-end fragments, however, did not yield interpretable sequences from the poly(A)-tailed ends. The inserts of the two plasmids both comprise 1981 nucleotides plus a poly(A) tail of about 100 nucleotides, as shown in Figures 1 and 3. The 5′ noncoding region consists of 61 nucleotides and the 3′ noncoding region of 741 nucleotides, which contains four possible polyadenylation sites as shown in Figure 3.

Correlation of Peptide and DNA Results. The complete primary structure deduced for the π subunit is shown in Figure 3. For sake of clarity, residue numbers previously used for alcohol dehydrogenases have not been changed in this text. Therefore, Figure 3 employs the numbering system of the class I enzymes, which is equal to that of the horse enzyme subunit, although the π subunits contain extra residues that change the true residue numbers after position 60 (as explained in Figure 3).

Table II: Data from Direct Analysis of Peptides of Importance in Comparison of Protein and cDNA Data[a]

(A) Composition

residue	CNBr 276–362	CNBr 363–374
Cys	2.1 (2)	
Asx	9.1 (9)	1.0 (1)
Thr	7.5 (9)	1.0 (1)
Ser	6.3 (5)	1.0 (1)
Glx	4.1 (3)	1.0 (1)
Pro	3.7 (3)	
Gly	10.2 (9)	1.1 (1)
Ala	6.6 (7)	
Val	4.2 (5)	
Met	0.6 (1)	
Ile	3.6 (6)	2.7 (3)
Leu	7.3 (8)	1.0 (1)
Tyr	1.1 (1)	
Phe	6.1 (7)	1.0 (1)
Trp	ND (2)	
Lys	6.7 (8)	0.9 (1)
His	0.9 (1)	
Arg	1.2 (1)	0.8 (1)
sum	87	12

(B) Sequence

CNBr 276–362						CNBr 363–374		
cycle		nmol	cycle		nmol	cycle		nmol
1	Lys	14	23	Ser	1	1	Asn	7
2	Ala	13	24	Lys	4	2	Gln	5
3	Ala	14	25	Gly	1	3	Gly	7
4	Leu	11	26	Leu	3	4	Lys	8
5	Asp	10	27	Thr	1	5	Ser	2
6	Cys	4	28	Val	2	6	Ile	7
7	Thr	6	29	Phe	2	7	Arg	2
8	Thr	8	30	Pro	1	8	Thr	4
9	Ala	10	31	Glu	1	9	Ile	4
10	Gly	5	32	Glu	2	10	Leu	4
11	Trp	1	33	Leu	2	11	Ile	2
12	Gly	6	34	Ile	2	12	Phe	1
13	Ser	1	35	Ile	2			
14	Cys	3	36	Gly	+			
15	Thr	4	37	Lys	1			
16	Phe	6	38	Thr	+			
17	Ile	5	39	Ile	1			
18	Gly	4	40	Asn	+			
19	Val	4	41	Gly	+			
20	Ala	5	42	Thr	+			
21	Ala	6	43	Phe	1			
22	Gly	3						

[a] Two peptides gave results different from those of cDNA. Both are CNBr fragments, located (Figure 3) at the positions shown by their start and end numbers (CNBr 276–362 and CNBr 363–374). (A) Compositions after acid hydrolysis (ND for Trp, not determined); (B) results of liquid-phase sequencer degradation (values listed show nanomoles recovered in each cycle). Fragment CNBr 276–362 was large, incompletely pure, and with the suggested differences (at cycles 28 and 37) late in the degradations. Consequently, these differences, although apparently clear, need not be final and should not alone be taken as argument against the cDNA data. However, fragment CNBr 363–374 gave unambiguous data, proving the residue at cycle 6 to be Ile; Val as in Figure 3 is even absent from the composition (A), and the total size (from both A and B) ends with Phe (corresponding to Phe-374 and in Figure 3).

The amino acid residues identified in peptides by direct analysis are identical with the amino acid sequence deduced indirectly from the cDNA data in all regions except the C-terminal segment. Here, position 368 (class I isozyme numbering), recovered in both a tryptic peptide and a CNBr fragment, is clearly Ile in the protein analyzed (Table II) but Val in the structure deduced indirectly (Figure 3). Similarly,

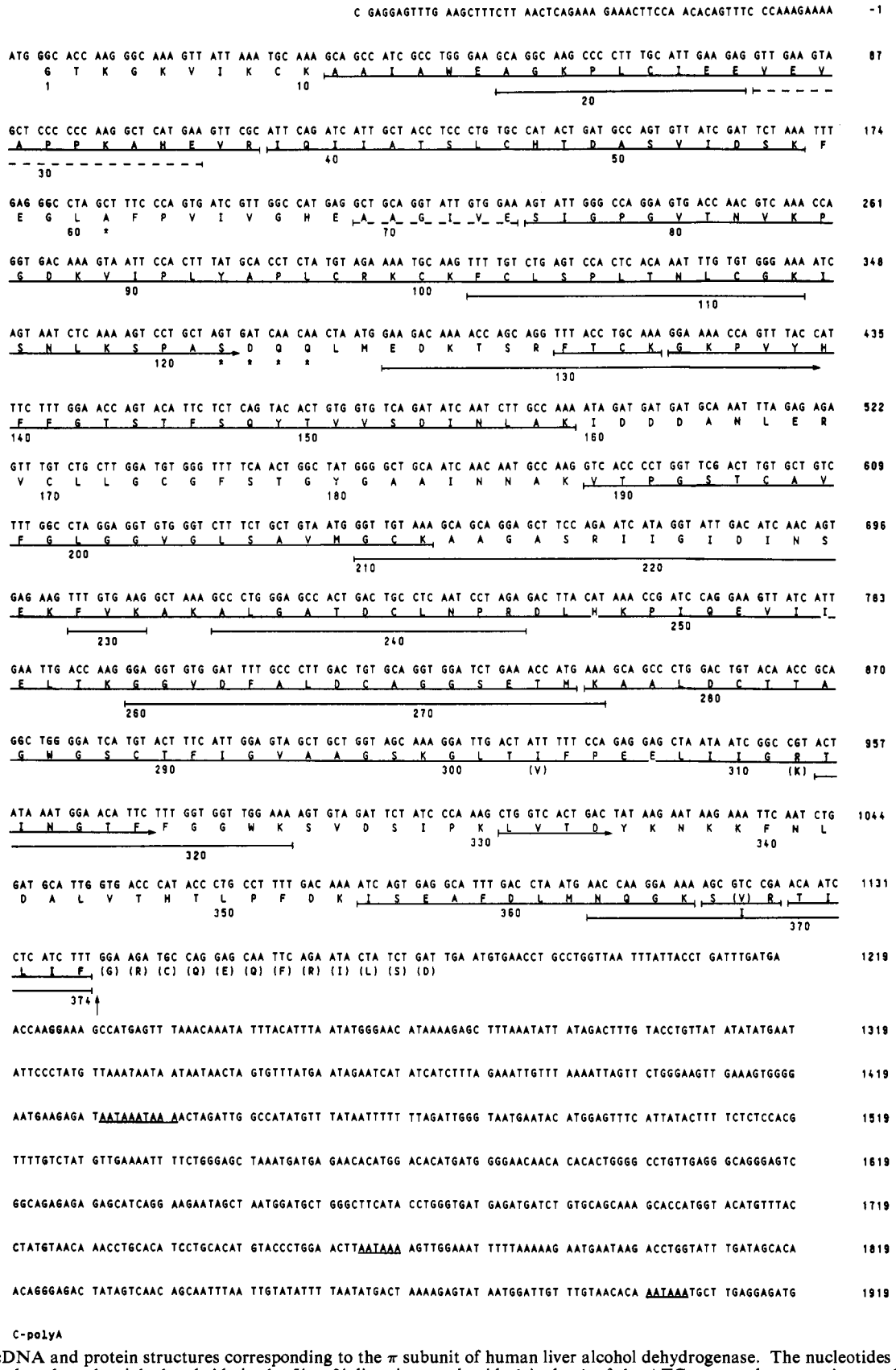

FIGURE 3: cDNA and protein structures corresponding to the π subunit of human liver alcohol dehydrogenase. The nucleotides (top in each line) are numbered on the right-hand side in the 5′ to 3′ direction; nucleotide 1 is the A of the ATG start codon; negative numbers refer to the 5′ untranslated region. The four putative polyadenylation signals in the 3′ untranslated region are underlined. The amino acid sequence (bottom in each line) is numbered underneath the residues according to the class I isozyme positional numbering system in order to keep residue numbers unchanged on comparisons with previously available data for the class I human enzyme subunits and the horse enzyme E-type subunit. Additional residues, relative to those of class I, after positions 60 and 121 in the π subunit are marked with asterisks. For the C-terminal segment where protein and nucleotide data deviate, the amino acid residues are given with the cDNA-deduced alternative at the top and the peptide analyzed residue below. For positions 303 and 312, the peptide-deduced structure is given within parentheses and the cDNA deduced structure without parentheses. For position 368 and positions 375 and onward, the opposite use of parentheses is utilized to show the interpretations of the analytical data if the results should represent only one structure. However, a π polymorphism may be a likely alternative as an explanation to the results, but the structure from the peptide data is the only one thus far detected from direct analysis of the mature protein, with a C-terminus at the arrow. Lines below the amino acid sequence show the peptides directly analyzed (continuous lines denote regions proved by sequence degradations; broken lines denote remaining regions analyzed by amino acid compositions only).

positions 303 and 312 also suggest single residue exchanges of a conservative nature (Figure 3), although for those positions peptide data need not be equally definitive (cf. Table II). Finally, the C-terminus is formed by Phe-374 (in the class I numbering system), unambigously identified in fragments purified from both the tryptic digest and the CNBr cleavage (Table II). Consequently, the mature protein analyzed ends at this position, and the protein data results in a 379-residue polypeptide (in absolute numbers), as shown in Figure 3. However, the cDNA clones investigated gave a nucleotide sequence that did not have a stop codon after this position but continued for 12 further amino acid positions, as shown in Figure 3. The polypeptide translated from cDNA data would thus consist of 391 amino acid residues with an elongated C-terminus.

Discussion

Structure of the π Subunit. The structure of the π subunit has been determined by combined protein and cDNA analyses. The structure obtained with the two methods differs at a few positions. Figure 3 shows both alternatives. However, thus far only the unambiguous protein data at two of the four positions with deviations are known to represent true π subunits, as they are derived from analysis of the mature, functional ππ enzyme. The differences between the two sets of data are concentrated to the C-terminal region and affect position 368 and positions 375 and onward. There are at least three possible reasons for the discrepancies at the C-terminus.

The cDNA data can be results of cloning artifacts. The cDNA library used in this study was also employed to determine the cDNA sequences corresponding to the α, β_1, γ_1, and γ_2 subunits. Overall, isolated differences that resulted in amino acid replacements were not found. However, for γ_1 at positions 275–276, a difference between protein and cDNA data has not been excluded (Höög et al., 1986), and in the case of the α subunit, one of two cDNA clones clearly differs by virtue of an internal deletion of a 139-bp segment (von Bahr-Lindström et al., 1986). The difference in length of the α cDNAs coincides with exon–intron boundaries, but this is not the case in the present π extension difference. In summary, previous data tend, if anything, to argue against cloning artifacts as a reason for the π differences.

Posttranslational processing of the polypeptide could be another reason for the difference in length. In several peptide hormones, proteolytic C-terminal cleavages and further modification result in an amidated C-terminus, but thus far this has never been demonstrated for a protein. This type of structure for cleavage recognition and amidation, with a mature C-terminus initially followed by Gly and a basic residue, is typical for the proforms of amidated hormones. For example, the sequence Phe-Gly-Arg occurs in preprogastrin (Yoo et al., 1982). Interestingly, this sequence is identical with the Phe-Gly-Arg deduced for the π subunit around the mature C-terminus at the arrow in Figure 3. However, typically in prepro hormones, though not necessarily (Mayo et al., 1985; Schwartz, 1986), the basic residue is in a sequence with two adjacent basic residues.

Polymorphism at the ADH4 locus is a third and perhaps most likely explanation that would account for both size and residue differences. However, a polymorphism of π subunits has not yet been reported positively at protein or DNA levels. The differences now found between protein and cDNA data would not result in a charge difference, and the two corresponding variants of π subunits that could result would therefore be indistinguishable upon electrophoresis, the conventional isozyme identification method. In summary, the existence of multiple forms of the π subunit appears likely and should be studied further, but the possible existence of a C-terminal posttranslational modification must also be considered.

cDNA Structure. A comparison of the cDNA structures encoding the class II subunits (π) and class I subunits (α, β_1, γ_1, and γ_2) shows that the largest differences are in the noncoding regions, as expected. The positional nucleotide identity is only 25% or very close to random when the sequences are aligned without gaps. In the coding region, however, the positional identity is as large as 67% at the nucleotide level, only marginally larger than that for the alignment at the protein level. In contrast to the class I/II case, the intra class I homologies are markedly similar in extent for the coding and noncoding regions (Höög et al., 1986). Also, a remarkably high number of silent mutations are found between class II and class I alcohol dehydrogenases. Of all codon differences in the classes compared, 228 result in unaltered amino acid residues, and of these 119 differ at the wobbling position. Both these observations stress that class I and class II alcohol dehydrogenases diverged long ago.

Protein Structure. Table III summarizes the differences between the π subunit and all those of the class I subunits. It should be noted that differences are extensive, over 4-fold those for the variability of the class I enzyme subunits only. There are two internal chain-length differences, totaling five residues. A one-residue insertion is found after position 60, and a four-residue insertion is present after position 121. Both these insertions in the π subunit occur in greatly altered regions, and their exact positions can therefore be nominally shifted slightly since the exact homology is weak. It should also be noted that the same two regions contain insertions/deletions also when sorbitol dehydrogenase from sheep and alcohol dehydrogenase from maize and yeast are aligned with horse liver alcohol dehydrogenase (Jörnvall et al., 1978; Brändén et al., 1984; Eklund et al., 1985). In space, the two inserted regions are close to each other and superficially positioned, allowing for the extra extensions/deletions. While possibly indicating some hypervariability as in a "junction region", these regions do not coincide with the exon/intron boundaries that have been established for the human ADH2 gene (Duester et al., 1986b).

In total, the π subunit and those of the class I enzymes differ by 146 residues. The sum of the charge differences (gained net negative charges are 3, 4, and 5 vs. α, γ_1, and β_1 subunits, respectively) explains the lesser cathodic mobility of the ππ enzyme.

The residue differences are distributed over the entire polypeptide chains but cluster in three regions, around positions 39–61, 93–128, and 265–328 (Table III). Functionally important residues are those at positions 47 and 48, which explain catalytic differences for the class I human isozymes (Jörnvall et al., 1984a; von Bahr-Lindström et al., 1986; Eklund et al., unpublished results). These and all other interactions with the coenzyme in the crystallographically investigated class I horse enzyme have been summarized (Eklund et al., 1984). In π, residue 47, which interacts with the coenzyme pyrophosphate, is histidine, an alternative identical with that in the β_2 subunit (Jörnvall et al., 1984a) but dissimilar to that in the other class I subunits (von Bahr-Lindström et al., 1986; Jörnvall et al., 1986). Position 48, which binds to the coenzyme NMN ribose is serine in the γ subunits and the horse liver enzyme, but it is threonine in the subunits of all other human alcohol dehydrogenases studied. Similarly, residue 51, which also forms a hydrogen bond with the NMN ribose, is ex-

Table III: Positions with Residue Differences between α, β, and γ Subunits of Class I and the π Subunit of Class II of Human Liver Alcohol Dehydrogenase[a]

position	α	β	γ	π	position	α	β	γ	π	position	α	β	γ	π
1	S	S	S	G	121a				S	272	L	L	L	S
3	A	A	A	K	121b				D	273	D	D	D	E
13	V	V	V	I	121c				Q	276	M	M	M	K
14	L	L	L	A	121d				Q	278	S	S	S	A
17	L	V	L	A	122	T	T	T	L	280	L	L	L	D
18	K	K	K	G	123	L	L	L	M	282	C	C	C	T
21	F	F	F	L	124	Q	Q	Q	E	283	H	H	H	T
22	S	S	S	C	126	G	G	G	K	284	E	E	E	A
25	E	D	E	E	128	S	R	R	S	285	A	A	A	G
34	H	Y	H	H	133	R	R	S	K	286	C	C	C	W
39	K	K	K	Q	134	R	G	G	G	288	T	T	T	S
40	M	M	M	I	137	I	I	I	V	289	S	S	S	C
41	V	V	V	I	138	H	H	H	Y	290	V	V	V	T
43	V	V	A	T	141	L	L	V	F	291	I	I	I	F
44	G	G	G	S	143	I	T	V	T	292	V	V	V	I
45	I	I	I	L	153	D	D	D	S	295	P	P	P	A
47	G	R/H	R	H	154	E	E	E	D	296	P	P	P	A
48	T	T	S	T	155	N	N	N	I	297	D	A	D	G
50	D	D	E	A	156	A	A	A	N	299	Q	Q	Q	K
51	H	H	H	S	157	V	V	V	L	300	N	N	N	G
53	V	V	V	I	162	A	A	A	D	302	S	S	S	T
54	S	S	S	D	163	A	A	A	D	303	M	I	I	I
55	G	G	G	S	164	S	S	S	A	304	N	N	N	F
56	T	N	N	K	165	P	P	P	N	306	M	M	M	E
57	M	L	L	F	168	K	K	K	R	307	L	L	L	E
58	V	V	V	E	172	I	I	I	L	309	L	L	L	I
59	T	T	T	G	182	S	S	S	A	310	T	T	T	I
60	P	P	P	L	184	V	V	V	I	314	W	W	W	I
60a				A	185	N	N	K	N	315	K	K	K	N
61	L	L	L	F	186	V	V	V	N	317	A	A	A	T
65	L	L	L	V	207	A	A	V	A	318	I	V	I	F
76	V	V	V	I	208	I	V	V	V	319	L	Y	F	F
78	E	E	E	P	217	A	A	A	S	322	F	F	F	W
82	T	T	T	N	221	A	A	A	G	325	K	K	K	V
93	A	F	F	Y	222	V	V	V	I	326	E	E	E	D
94	I	T	T	A	226	K	K	K	S	327	C	G	S	S
96	Q	Q	Q	L	227	D	D	D	E	328	V	I	V	I
98	G	G	G	R	230	A	A	A	V	333	A	A	A	T
101	R	R	R	K	234	E	E	E	A	335	F	F	F	Y
102	I	V	I	F	239	E	E	E	D	336	M	M	M	K
104	K	K	K	L	241	I	I	I	L	337	A	A	A	N
105	N	N	N	S	244	Q	Q	Q	R	341	S	S	S	N
107	E	E	E	L	246	Y	Y	Y	L	346	I	I	I	V
108	S	S	S	T	247	K	K	K	H	348	H	H	N	H
110	Y	Y	Y	L	254	L	L	L	I	349	V	V	I/V	T
112	L	L	L	G	255	K	K	K	I	353	E	E	E	D
114	N	N	N	I	257	M	M	M	L	356	N	N	N	S
115	D	D	D	S	259	D	D	D	K	358	G	G	G	A
116	V	L	L	N	265	S	S	S	A	362	L	L	L	M
117	S	G	G	L	266	F	F	F	L	363	H	H	R	N
118	N	N	N	K	267	E	E	E	D	364	S	S	S	Q
119	P	P	P	S	268	V	V	V	C	371	I	V	V	I
120	Q	R	R	P	269	I	I	I	A	373	M	T	T	I
121	G	G	G	A	271	R	R	R/Q	G					

[a] Underlined residues are positions where the residue in the π subunit is not identical with any of the residues in the class I subunits. At position 47 the β_1/β_2 difference is shown, and at positions 271 and 349 the γ_1/γ_2 differences are shown.

changed. In combination, these changes alter the coenzyme-binding properties of the π enzyme in relation to the class I enzymes. This may affect the catalytic properties, since dissociation of the reduced coenzyme is the rate-limiting step for the oxidation of ethanol in the horse class I enzyme (Theorell & Chance, 1951), and in the human class II enzyme with primary aliphatic alcohols and several other substrates (Bosron et al., 1979; Ditlow et al., 1984).

The residues liganding the catalytic Zn atom as well as all the ligands to the second Zn atom are conserved. The latter atom, however, is surrounded by a region in which there are multiple exchanges. That region is also very dissimilar in sorbitol dehydrogenase, which is homologous to alcohol dehydrogenase but lacks the second Zn atom (Jörnvall et al., 1984b; Jeffery et al., 1984b). Finally, residues at positions 265–328, including the area of the major subunit interactions, are largely replaced. The many differences of that region probably explain the lack of interclass hybrid dimers for alcohol dehydrogenase.

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