Class III Human Liver Alcohol Dehydrogenase: A Novel Structural Type Equidistantly Related to the Class I and Class II Enzymes†

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ABSTRACT: The primary structure of class III alcohol dehydrogenase (dimeric with χ subunits) from human liver has been determined by peptide analyses. The protein chain is a clearly distinct type of subunit distantly related to those of both human class I and class II alcohol dehydrogenases (with α, β, γ, and π subunits, respectively). Disregarding a few gaps, residue differences in the χ protein chain with respect to β_1 and π occur at 139 and 140 positions, respectively. Compared to class I, the 373-residue χ structure has an extra residue, Cys after position 60, and two missing ones, the first two residues relative to class I, although the N-terminus is acetylated like that for those enzymes. The χ subunit contains two more tryptophan residues than the class I subunits, accounting for the increased absorbance at 280 nm. There are also four additional acidic and two fewer basic side chains than in the class I β structure, compatible with the markedly different electrophoretic mobility of the class III enzyme. Residue differences between class III and the other classes occur with nearly equal frequency in the coenzyme-binding and catalytic domains. The similarity in the number of exchanges relative to that of the enzymes of the other two classes supports conclusions that the three classes of alcohol dehydrogenase reflect stages in the development of separate enzymes with distinct functional roles. In spite of the many exchanges, the residues critical to basic functional properties are either completely unchanged—all zinc ligands and space-restricted Gly residues—or partly unchanged—residues at the coenzyme-binding pocket. This suggests that the overall conformations are related and that some functional replacements account for the different enzymatic properties. Thus, several of the residues at the substrate-binding pocket are markedly changed in χ, including some substitutions for smaller residues, and have an increased polarity, with one charge. This is compatible with an altered substrate specificity for the class III enzyme. The corresponding isozyme from horse liver was also characterized in part, revealing that between the two species the structure of this enzyme class is more conserved than that of the class I enzymes. This further distinguishes the classes and suggests a strict functional importance for the class III alcohol dehydrogenase.

Mammalian alcohol dehydrogenase is a dimeric enzyme with a zinc atom at the active site. In man, three types of subunit, α, β, and γ, the primary structures of which are known [review in Jörnvall et al. (1987a)], constitute the polypeptides of the class I enzymes (Vallee & Bazzone, 1983). They are typical isozymes (Jörnvall et al., 1987b) of the most abundant forms of liver alcohol dehydrogenase, and different combinations of residue exchanges in the subunits affect a total of 35 of the 374 positions. The conformations of these subunits are clearly related to that of the well-studied horse enzyme [reviews in Brändén et al. (1975) and Eklund and Brändén (1987)]. On the basis of the tertiary structure of the latter as a model, the effects of all residue exchanges within the human class I enzymes have been interpreted (Eklund et al., 1987). Recently, the structure of the class II enzyme of human liver alcohol dehydrogenase, which is composed of π subunits, has also been determined (Höög et al., 1987). It differs significantly from that of the class I enzymes—with residue exchanges at no less than 147 positions relative to those of the class I β_1 subunit. This suggests that the interclass variations represent steps in the development of enzymes, rather than merely an isozyme variation as exemplified by the forms within a class (Jörnvall et al., 1987b). However, the structure of class III has not been analyzed before in any species, including man; this gap in knowledge has precluded an all-inclusive description of the mammalian alcohol dehydrogenase system.

The primary structure of the χ subunit of alcohol dehydrogenase from human liver has now been determined. The horse class III structure was partially analyzed in parallel, additionally allowing an assessment of the extent of species variation. The χ structure is about equidistantly related to both the class I and class II enzymes, proving that all three classes are distant entities. Given the 373 positions in the human χ subunit and disregarding a few gaps in relation to the β_1 subunit of the class I enzyme and in the π subunit of the class II enzyme from the same species, residue exchanges occur at 139 and 140 positions, respectively. Major effects of these exchanges are interpreted in terms of function.

Materials and Methods

Protein. The $\chi\chi$ enzyme of human liver alcohol dehydrogenase was purified as described (Parés & Vallee, 1981; Dafeldecker & Vallee, 1982; Wagner et al., 1984). The pure protein was dialyzed against distilled water to remove salts,

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lyophilized, and dissolved in 6 M guanidine hydrochloride, 0.4 M tris(hydroxymethyl)aminomethane (Tris), pH 8.1, and 2 mM ethylenediaminetetraacetic acid (EDTA). It was then reduced (dithiothreitol, 5 μmol/10 mg of protein, 37 °C, 2 h, under N_2), and carboxymethylated with neutralized ^{14}C-labeled iodoacetic acid (Amersham; diluted to ~2400 cpm/nmol; 1.5 μmol/mg of protein; 1.5 h, 37 °C, under N_2, in the dark). Reagents were removed by extensive dialysis against distilled water. The class III enzyme from horse liver was prepared and analyzed similarly.

Peptide Generation and Purification. Cleavages with CNBr were performed in 70% formic acid (100 mg of CNBr/mL) at room temperature for 30 h. Enzymatic cleavages were carried out at 37 °C for 4 h with trypsin, Lys-endoprotease from *Achromobacter lyticus* (Wako Chemicals, Neuss, FRG), chymotrypsin, and Glu-specific *Staphylococcus aureus* strain V8 extracellular protease at enzyme to protein ratios of 1:100–1:50 in 0.1 M ammonium bicarbonate, pH 8.0. Digests of the whole protein were purified in two steps, a prefractionation on Sephadex G-50 in 30% acetic acid, followed by reverse-phase high-performance liquid chromatography (HPLC) of each fraction on TSK ODS-120T (LKB, Stockholm, Sweden) with linear gradients of acetonitrile in 0.1% trifluoroacetic acid. Secondary peptides obtained by redigestion of large fragments were purified in one step by direct HPLC.

Structural Analysis. Total compositions were determined with a Beckman 121M amino acid analyzer after hydrolysis for 24 h at 110 °C in evacuated tubes with 6 M HCl/0.5% phenol. For high-sensitivity analysis of fragments present in low yield, HPLC of phenylthiocarbamyl derivatives was also performed (Bergman et al., 1986). Amino acid sequences were determined by gas-phase sequencer degradations, liquid-phase sequencer degradations, and manual dimethylaminoazobenzene isothiocyanate degradations, as described (Jeffery et al., 1984; Hallden et al., 1986). Phenylthiohydantoin derivatives were quantitated by reverse-phase HPLC (Hewlett-Packard 1090 instruments) on 5-μm Nucleosil C18, using an acetonitrile/sodium acetate system modified from Zimmerman et al. (1977) by an increase of the pH (to 4.75), a decrease of the molarity (to about 2 mM sodium acetate), and a change of the gradient (start with 26%, end with 46% at 8 min, isocratic until regeneration at 29 min). This system gives full separation of all derivatives; positions of the His and Arg derivatives are highly sensitive to molarity but are daily set to elute after Tyr and Leu, respectively.

Peptide Nomenclature. Throughout, peptides are named by letter–digit combinations. The letter indicates the method of origin for the peptide: generation by treatment with CNBr is indicated by B; generation with trypsin by T; with Lys-specific protease by K; and with Glu-specific protease by E. In each case, the subsequent digit gives the relative order in the original protein chain of the peptides purified. For peptides derived by redigestions of a primary peptide, the second proteolytic treatment is shown in the name of the final peptide by a second letter–digit combination. Some T peptides formally relate to the protein from horse as explained in the legend of Figure 3.

Structural Comparisons. Alignments with the human isozymes of class I alcohol dehydrogenase (Jörnvall et al., 1987a) and with the human enzyme of class II (Höög et al., 1987) allow comparisons of all three classes of alcohol dehydrogenase in man.

RESULTS

Primary Structure. The structural determination was largely based on analysis of CNBr fragments of the ^{14}C-carboxymethylated class III human enzyme. Digestions of a sample with the Lys-specific protease produced peptides overlapping the primary CNBr fragments, and redigestions of the latter with trypsin, the Lys-specific protease, and the Glu-specific protease gave additional material of internal segments. Work in parallel with the χ-chain of the horse liver enzyme, purified in a similar manner, carboxymethylated, and cleaved directly with trypsin, resulted in still more material of closely related tryptic peptides. The peptides from the two species were either identical or differed only at single positions. Due to this close similarity the horse tryptic peptides were initially of help to establish overlaps between the human CNBr fragments. Simultaneously, the analyses in parallel gave an estimate of the extent of species variation, proving this to be quite limited. The major steps of peptide purification are summarized in Figure 1. In total, all positions of the human enzyme were checked with CNBr fragments or peptides from secondary digestions and most also with enzymatic peptides, giving the entire structure of the enzyme, while many positions were checked with tryptic peptides of the horse enzyme, tentatively indicating a 96% identity between the two species (10 differences among 225 positions checked).

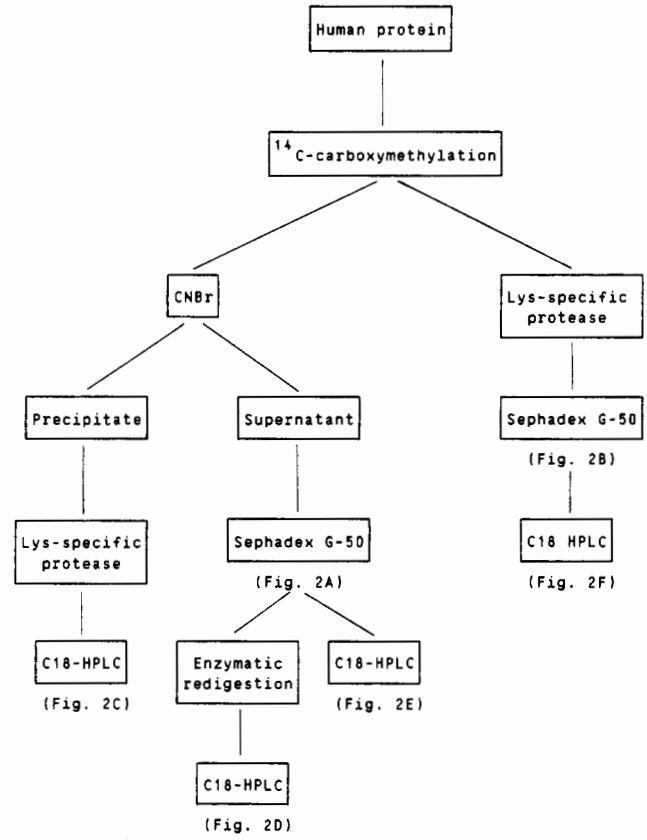

FIGURE 1: Flow scheme to show generation of the major peptide sets that form the basis of the structural analyses.

The digests obtained with CNBr and the Lys-specific protease were first prefractionated by chromatography on Sephadex G-50 in 30% acetic acid (Figure 2A,B). In the case of the CNBr digest, not all fragments were soluble, and the most hydrophobic one (B7), which was contaminated with large peptides because of incomplete stoichiometry of cleavage at different methionine residues, was separated as a precipitate (Figure 1) before Sephadex chromatography of the supernatant. The precipitate was solubilized in 9 M urea and digested in separate batches with *Achromobacter* Lys-specific protease and chymotrypsin after dilution with 0.1 M ammonium bicarbonate to a final concentration of 0.9 M urea. The peptides thus produced were separated directly by HPLC (Figure 2C).

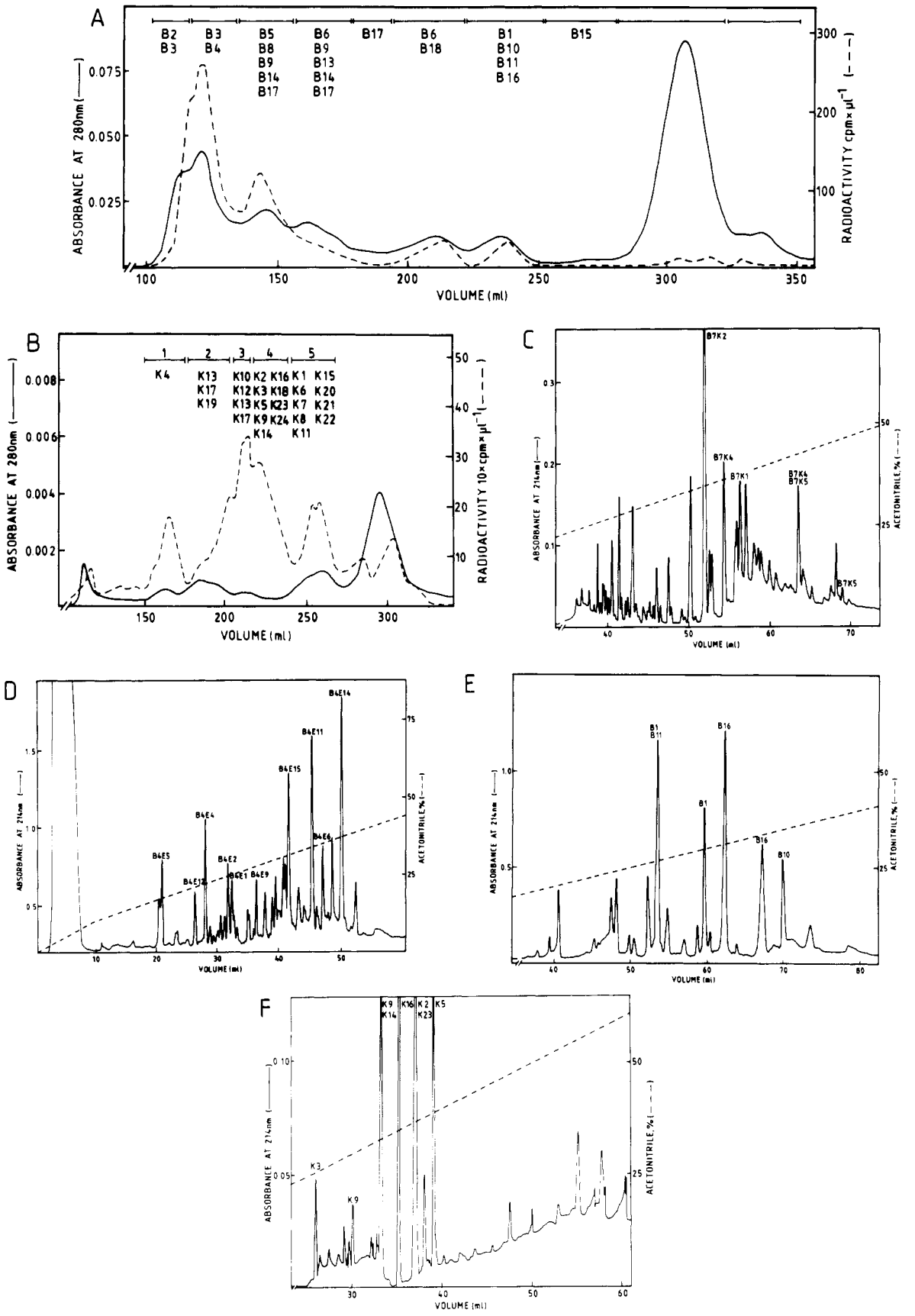

FIGURE 2: Purification of the peptides analyzed. The elution positions of all peptides identified are shown by the peptide designations, which correspond to those in Figure 3. (A, B) Prefractionation on Sephadex G-50 (1.9 × 180 cm) of the soluble part of the CNBr digest (A) and the digest with the Lys-specific protease (B) of the human protein. Numbered bars indicate fractions pooled. (C) Purification of the precipitate from the CNBr digest before the Sephadex step in (A) and after redigestion with the Lys-specific protease to generate soluble peptides. Reverse-phase HPLC on Ultropac TSK ODS-120T in 0.1% trifluoroacetic acid with a linear gradient (dashed line) of acetonitrile. (D) Purification of fraction 2 from (A) after redigeston with Glu-specific protease. HPLC as in (C). (E) Purification of fraction 7 from (A). Reverse-phase HPLC as in (C). (F) Purification of fraction 4 from (B) by HPLC as in (C).

Table I: Total Composition of the χ Subunit of Human Liver Alcohol Dehydrogenase[a]

residue	acid hydrolysis (mol/mol)	sum of sequence (mol/mol)	
Cys[b]	13.9	15	
Asp	24.2	23	14
Asn			9
Thr	23.9	23	
Ser	23.8	21	
Glu	32.0	32	26
Gln			6
Pro	17.0	16	
Gly	40.5	40	
Ala	33.6	34	
Val	34.6	39	
Met	8.0	7	
Ile	24.8	28	
Leu	21.9	22	
Tyr	8.1	7	
Phe	14.4	14	
Trp	3.5	4	
Lys	32.9	32	
His	7.7	7	
Arg	10.1	9	
sum		373	

[a] Analytical values show molar ratios as obtained by acid hydrolysis for 24 h of the χχ enzyme preparations investigated, without corrections for slow release, destruction, or impurities. Values within parentheses show the integers from the sum of the sequence analysis now reported. [b] Analyzed as Cys(Cm).

In all pools from the prefractionation, peptides were purified further by reverse-phase HPLC on Ultropac TSK ODS-120T in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile (Figure 2). The largest CNBr fragment from the prefractionation was digested separately with the staphylococcal Glu-specific protease and the Lys-specific protease to produce smaller fragments for analysis; they were separated directly by reverse-phase HPLC in the same system as above (Figure 2D for the Glu-specific digest).

Peptides obtained were monitored by absorbances at 214 and 280 nm, by ^{14}C radioactivity, by total composition, and by N-terminal groups. Relevant peptides were submitted to gas-phase sequencer degradations. Combined, different peptides from all regions of the molecule were recovered and analyzed to give the primary structure of the human χ-chain (Figure 3). Much of that of the horse χ-chain was also obtained (legend to Figure 3).

The analyses demonstrate that the χ subunit of human liver alcohol dehydrogenase consists of 373 residues. Compared to the 374 residues of the human and horse class I subunits (Jörnvall et al., 1987a), the χ polypeptide lacks the first two residues and has an insertion of a cysteine residue after position 60 in the numbering system of the class I subunit (cf. Figure 4, below). The N-terminus is blocked and was recovered from the human protein in two CNBr fragments (B1 and B2), a peptide from the redigestion of B2 with the Glu-specific protease (B2E1), and a tryptic peptide from digestion with the Lys-specific protease (K1). The identity of the C-terminus was proven by the recovery of C-terminal Ile in the CNBr fragment B18. Furthermore, a tryptic peptide from the horse enzyme (which contains a C-terminal Leu) ends at the same position.

The total composition of the human class III enzyme with χ subunits as determined by acid hydrolysis is in agreement with the results of the sequence analysis (Table I). Total compositions of all peptides purified also agree with the sequence data as shown for relevant fragments in Table II. Two features of these analyses presented special problems and are dealt with further below. They concern the blocked N-ter-

Table II: Total Compositions of Peptides Purified[a]

	B2E2	B4E1	B4E4	B4E7	B4E11	B4E12	B4E13	B4E14	B4E15	B6	B7K1	B7K3	B7C1	B7C2	B9	B10	B13	B15	B16	B17	B18
Cys	0.8 (1)			0.7 (1)	0.8 (1)			0.6 (1)	2.6 (3)	0.8 (1)		1.6 (2)	0.6 (1)		1.5 (2)	0.3 (1)	0.6 (1)			4.0 (4)	
Asx		0.2 (–)		1.9 (2)				1.2 (1)	2.2 (2)	1.1 (1)	1.0 (1)	3.0 (3)			5.0 (5)	2.7 (3)					
Thr				2.8 (3)				1.9 (2)	2.0 (2)	2.9 (3)	2.7 (3)	2.0 (2)	1.2 (1)		1.4 (1)	0.9 (1)	2.9 (3)	1.0 (1)		1.1 (1)	0.9 (1)
Ser	0.4 (–)	0.9 (1)		1.0 (1)			0.9 (1)			1.1 (1)	3.2 (3)	1.3 (1)	1.3 (1)		2.2 (2)	1.4 (1)	2.2 (2)		2.7 (3)	2.1 (2)	2.1 (2)
Glx	1.3 (1)	3.0 (3)	2.2 (2)	1.1 (1)	1.1 (1)	1.1 (1)	1.1 (1)	2.0 (2)	2.2 (2)		1.0 (1)		1.2 (1)		5.9 (6)	1.3 (1)	3.8 (4)		2.0 (2)	3.0 (3)	
Pro		1.1 (1)	1.9 (2)	1.1 (1)	1.0 (1)			2.0 (2)	1.1 (1)	1.0 (1)		2.0 (2)	1.0 (1)		1.9 (2)		1.3 (1)		1.0 (1)		
Gly	0.4 (–)	1.1 (1)		1.2 (1)	2.2 (2)	2.2 (2)	1.0 (1)	2.9 (3)	2.2 (2)	2.2 (2)	1.2 (1)	4.2 (4)	1.2 (1)	4.0 (4)	3.9 (4)	2.7 (3)	5.2 (5)	3.0 (3)		0.4 (–)	1.1 (1)
Ala	2.9 (3)	1.0 (1)	2.1 (2)	3.6 (4)		1.0 (1)		1.2 (1)			1.9 (2)	3.9 (4)	1.7 (2)		4.9 (5)		5.6 (6)	1.0 (1)		1.2 (1)	
Val	1.5 (2)		1.3 (1)	2.1 (2)	0.9 (1)	0.9 (1)	1.0 (1)	1.9 (2)	1.3 (1)		2.2 (3)	2.1 (2)	1.8 (2)	1.1 (1)	3.2 (3)	3.1 (3)	5.5 (6)		3.0 (3)	2.1 (2)	1.7 (2)
Met									0.3 (1)	0.3 (1)					0.3 (1)	0.3 (1)			0.3 (1)	0.3 (1)	
Ile	0.5 (1)	0.9 (1)	1.1 (1)	2.6 (3)	0.8 (1)	0.9 (1)		2.0 (2)	1.1 (1)	1.0 (1)	0.9 (1)	2.1 (2)			4.5 (6)	0.9 (1)	1.1 (1)			2.0 (2)	2.1 (2)
Leu		1.0 (1)		1.2 (1)	1.1 (1)			2.0 (2)	2.8 (3)	1.0 (1)		3.7 (4)	1.0 (1)	1.9 (2)	1.4 (1)		1.7 (2)		0.9 (1)	2.0 (2)	
Tyr				0.9 (1)				0.9 (1)		0.2 (1)	0.7 (1)	1.0 (1)				0.5 (1)			0.2 (1)		
Phe					1.0 (1)				0.9 (1)	1.1 (1)	1.0 (1)		1.1 (1)		2.9 (3)	1.0 (1)	1.3 (1)	1.0 (1)		3.0 (3)	
Trp	0.7 (1)																+ (2)	+ (1)			
Lys	1.9 (2)	1.0 (1)	1.3 (1)	1.2 (1)				2.0 (2)	3.8 (4)	2.0 (2)	0.9 (1)	2.0 (2)	0.9 (1)		5.1 (5)	1.1 (1)	1.2 (1)	1.0 (1)	2.0 (2)	4.1 (4)	2.0 (2)
His			0.9 (1)	0.9 (1)	1.0 (1)					1.0 (1)							0.8 (1)			1.1 (1)	1.1 (1)
Arg				1.1 (1)					1.0 (1)	1.0 (1)					1.9 (2)		2.6 (3)				1.0 (1)
sum	11	10	10	24	10	6	4	21	24	18	18	29	13	7	48	18	39	8	14	26	12

[a] Values are given in Table I. T and K peptides were analyzed at smallest possible scale, frequently forcing acceptance of some cross-contaminations. After correction for the known contaminants, these compositions are relevant but not listed, since values shown are uncorrected.

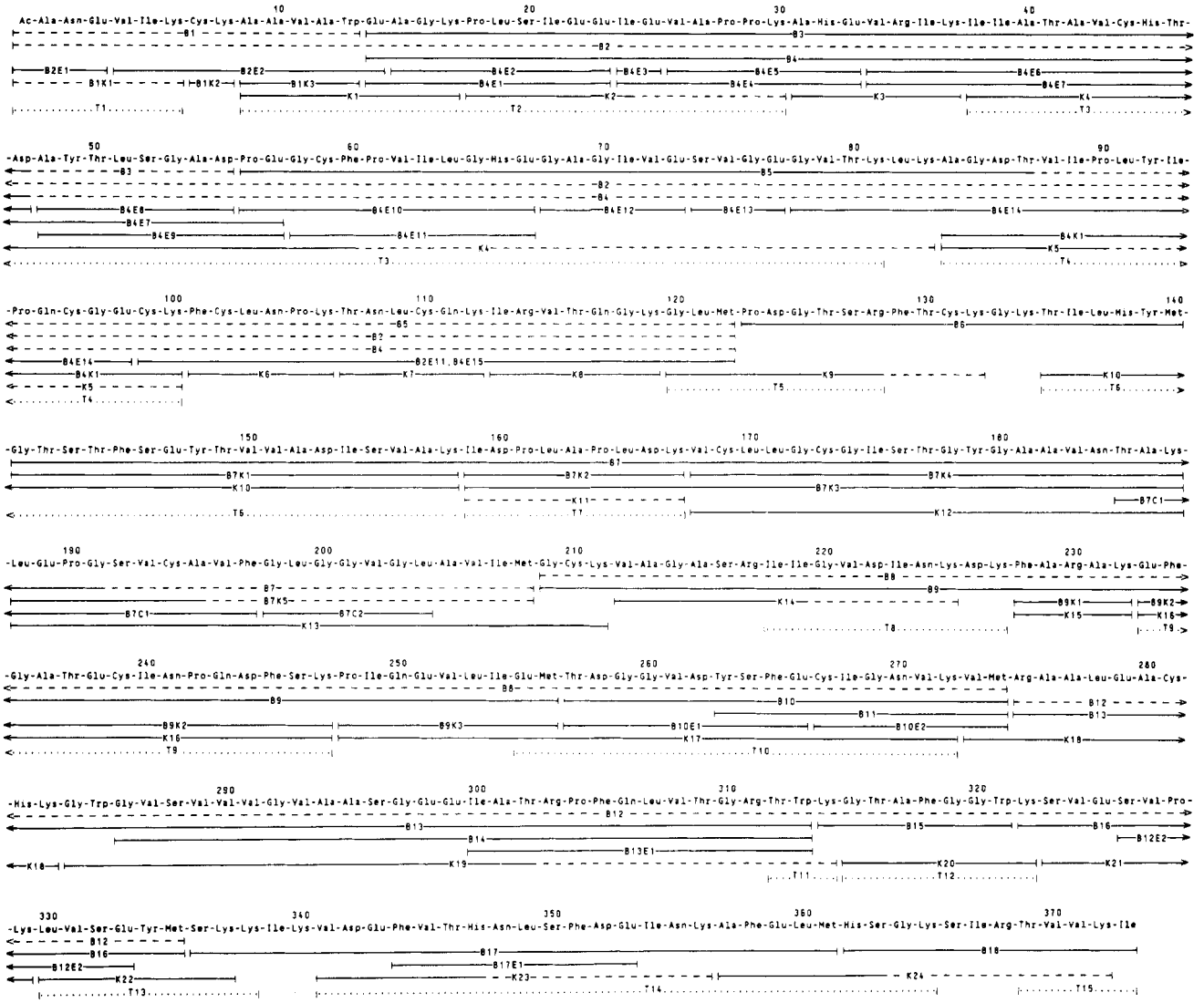

FIGURE 3: Primary structure of the χ subunit of human liver alcohol dehydrogenase. Peptides analyzed to establish the structure of the human protein are indicated by solid lines (—) for those parts determined by sequence degradations and by dashed lines (- -) for those regions analyzed from compositions only. The residue numbering system shows the absolute position of each residue. The structures of the N-terminal peptides B2E1 and B1K1 were determined by mass spectrometry and by use of synthetic peptides as reported separately (Fairwell et al., 1987). Peptide nomenclature as in Figure 2, with B indicating generation by CNBr, E generation by the Glu-specific protease, and K generation by the Lys-specific protease. The human protein has been recovered in peptides from all regions, as shown. In addition, the horse protein was analyzed partly by recovery of many of its tryptic peptides (stippled and indicated by T, covering 225 positions). At all but 11 of these positions, the horse and human structures are identical. These positions, where the horse protein now is known to deviate from the human enzyme structure shown, are positions 1, 2, 19, 24, 59, 237, 348, 353, 356, 363, and 373.

minus and the presence of unexpected proteolytic cleavages and/or poorly soluble peptides.

Blocked N-Terminus. Attempts at direct sequencer degradation of the intact protein did not yield any degradation, indicating the lack of a free α-amino group. Peptide B1 is the shortest CNBr fragment that lacks an α-amino group and derives from the blocked N-terminal segment of the protein. Because of nonquantitative cleavage after Trp-13, there is a second blocked CNBr fragment, terminating at Met-122 (cf. B2, Figure 3). Redigestion of these fragments with the Glu-specific protease confirmed the assignment by giving rise to all the peptides expected from the N-terminal region. The blocked N-terminus itself was recovered in a tripeptide (blocked-(Ala,Asx)-Glu, Figure 3) after digestion with the Glu-specific protease and a hexapeptide (blocked-(Ala,-Asx)-Glu-Val-Ile-Lys) in the digest with the Lys-specific protease. The C-terminal end of the latter peptide was proven by sequence analysis of B2E2 (Figure 3). The nature of the blocking group and the sequence of the first three residues were established by mass spectrometry of peptides from the rat, human, and horse χ structures and by comparisons of chromatographic properties with those of a set of synthetic peptides made to correspond to all the alternatives, as reported separately (Fairwell et al., 1987). From these results, the N-terminal structure of the human form was deduced to be acetyl-Ala-Asn-Glu-Val-Ile-Lys (Figure 3). Both the horse and rat forms differed at two of these positions (Fairwell et al., 1987; Juliá et al., 1988).

Proteolytic Cleavages. With a few exceptions, all enzymatic and CNBr cleavages encountered were as anticipated. However, in the CNBr digest, a Met–Ser bond (positions 335–336) and a Met–Thr bond (positions 256–257), frequently expected to result in low-yield cleavage (Jeffrey et al., 1984; Schroeder et al., 1969), were hydrolyzed in appreciable yield. Recovery of B17, starting at Ser-336, was about equally as high as that of other CNBr fragments. However, the Met–Thr bond was also recovered uncleaved, yielding a large CNBr fragment (B8, Figure 3) and the two constituent fragments (B9, B10, Figure 3). The cleavage was rendered stoichiometric by treatment of B8 in fraction 3 of the Sephadex fractionation (Figure 2A)

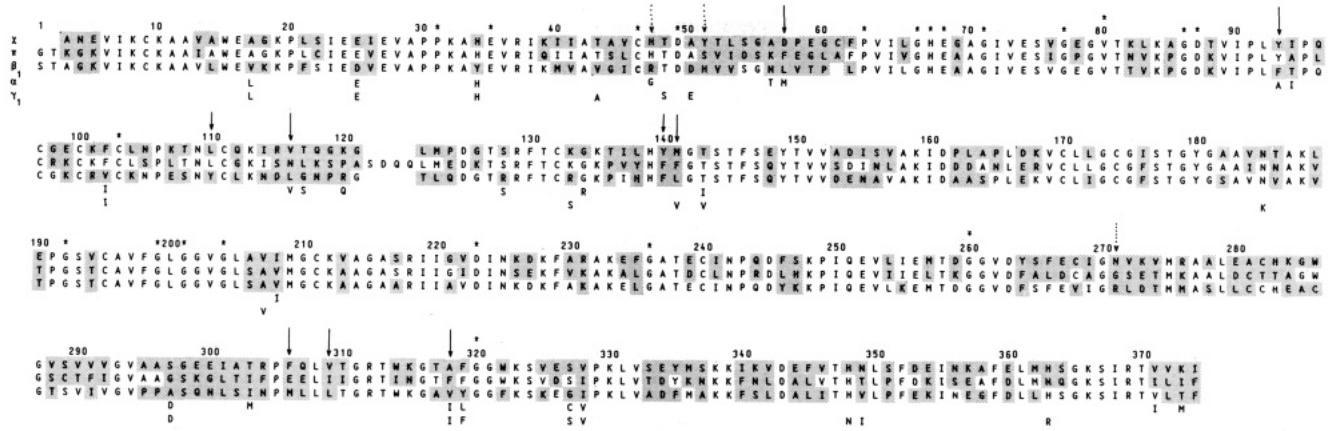

FIGURE 4: Structural comparison of the subunits of all enzyme classes from human liver alcohol dehydrogenase and positions of residue exchanges in class III relative to class I that are likely to affect substrate binding and coenzyme binding. The top line shows the χ structure corresponding to the continuous line in Figure 3. The second line illustrates the π subunit (Höög et al., 1987) with a few positions of residue differences between protein and cDNA data given by the alternative indicated as most likely in Höög et al. (1987). The bottom continuous line constitutes the β subunit, with residue alternatives for α and γ given at those positions only where these subunits differ from β (Jörnvall et al., 1987a). Spaces denote gaps. For the β and γ subunits, only the alternatives from one of the alleles (β_1 and γ_1, respectively) are given [β_2 differs at at least one position from β_1 and γ_2 at two from γ_1, as shown in Jörnvall et al. (1987a)]. Residues in substrate-binding and coenzyme-binding contacts as determined for the horse liver alcohol dehydrogenase EE enzyme (Eklund & Brändén, 1987; Eklund et al., 1982) and known to apply also to the human class I type of enzyme (Eklund et al., 1987) are indicated by arrows (solid for substrate binding and dotted for coenzyme binding) where different in the χ and β subunits. Of residues regarded as substrate binding or coenzyme binding in the β subunit (Eklund et al., 1987), three and eight, respectively, are unchanged in χ, i.e., those at positions 48, 67, and 294, and those at 48, 178, 203, 223, 224, 228, 269, and 369, respectively. Asterisks indicate 22 positions strictly conserved in 17 different alcohol dehydrogenases characterized previously (Jörnvall et al., 1987c); they are all conserved also in the χ strucure. In order to allow comparisons with the class I enzymes, the numbering system follows that of the E subunit of the horse liver enzyme, which is also that of the α, β, and γ subunits of the class I human liver enzyme (Jörnvall et al., 1987a). Hence, the residue numbering system of this figure differs from that of Figure 3 because of two deletions (at the N-terminal end) and one insertion (of Cys after position 60) in χ versus β. Stippled residues in the χ line show all positions where χ differs from at least one of the other two forms. This corresponds to 191 positions with any differences among the enzymes, giving the encircled number in Figure 5, right. Stippled positions in the π and β lines show residues where π and β, respectively, differ from the χ structure, giving numbers 139 and 140 in Figure 5.

with 0.1 M ammonium bicarbonate for 4 h. The Trp positions also gave rise to extensive cleavages with CNBr. The strong conditions used (30 h, room temperature, 0.1 g of CNBr/10 mg of protein) probably account for both the cleavages at Trp and the high extent of Met–Ser cleavage. The nonstoichiometric Trp cleavages increased the number of peptides and reduced their yields. The Asp–Pro bond (positions 55–56) was sensitive to cleavage as expected, but this was also nonquantitative, yielding additional partly overlapping peptides.

The presence of insoluble peptides also complicated the patterns and further reduced the yields. As shown in Figure 1, one CNBr fragment precipitated and was redigested separately with a protease, as was the largest soluble CNBr fragment. Especially the segment at positions 190–208 presented analytical problems because of low recovery, and positions 205–208 were only reached in one degradation. For all other peptides from the redigestions, yields were acceptable (Figure 2), and these separate treatments therefore resolved the difficulties with the hydrophobic and large CNBr fragments.

Relationship of the Class III Structure to the Class I and II Structures from the Same Species. The class III structure now determined is aligned in Figure 4 with those of the class I and class II enzymes, all from human liver. The three enzyme classes are clearly different—and disregarding a few gaps—distinguished by 139–147 amino acid substitutions in all combinations of pairwise comparisons between β_1, π, and χ as representing each of the three classes (Figure 5). Consequently, the classes constitute separate entities that have diverged from one another to approximately similar extents. This conclusion is encouraged further when the intrahuman variations (139–147 differences) are related to those between yet additional alcohol dehydrogenases. Thus, the same three human enzyme subunit classes each differ from the maize type

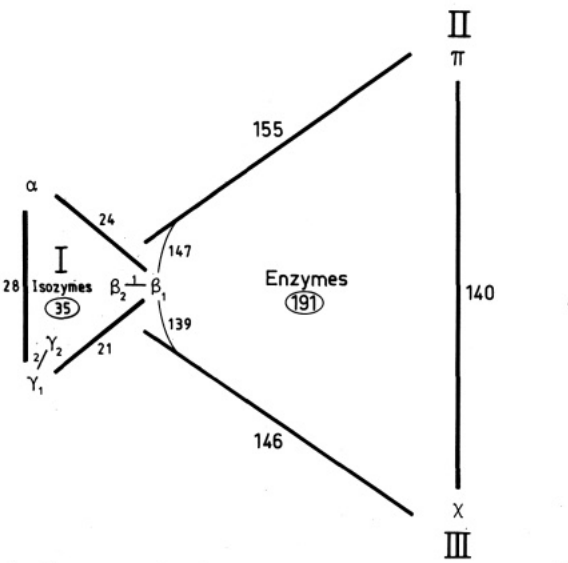

FIGURE 5: Schematic representation of residue differences among all major forms of human alcohol dehydrogenase subunits characterized. The major triangle to the right separates the different enymes of classes I, II, and III with α, β, γ, and π and χ subunits, respectively. The minor triangle to the left separates the different isozymes within class I, with allelic variants ($\gamma_1 - \gamma_2$ and $\beta_1 - \beta_2$) denoted internally. Values show the number of residue exchanges [disregarding a few gap positions (cf. Figure 4) and including a tentative value of 1 for the β_1/β_2 difference, which is a minimal difference based on limited analyses of β_2]. The circled values in the center of each triangle show total number of positions affected by residue exchanges among the isozymes (left) and enzymes (right).

I subunit (Dennis et al., 1984) to only a somewhat greater extent, 169–192 differences, i.e., 169 for the maize subunit compared with χ, 187 compared with β_1, and 192 compared with π. Consequently, the comparison with the plant enzyme

illustrates the wide separation between the forms of the three human classes. Interestingly, though, the χ structure now determined relates marginally more to the plant enzyme than the subunits of the other two human classes do. This may imply that of the three human enzyme classes class III could be the most ancient form, on the basis of particular similarities with the plant enzyme, or the form with the most stringent function, on the basis of extensive conservation.

In addition to the separate enzyme types between the classes, an intraclass variability is evident, reflected in closely related structures that in each case constitute typical isozymes (Figure 5). This type of variation has been established directly for the α-, β-, and γ-chains of the class I group (Jörnvall et al., 1987a), appears possible for the class II group, where differences among protein-analyzed structures and DNA-analyzed structures could indicate the presence of π_1 and π_2 subunits (Höög et al., 1987), and has also been suggested for the class III group, on the basis of electrophoretic differences of subforms, suggesting the presence of χ_1 and χ_2 structures (Parês & Vallee, 1981; Adinolfi et al., 1984), although the latter multiplicity might also derive from secondary chemical modification (Adinolfi et al., 1984).

Conserved residues in all three classes of human liver alcohol dehydrogenase constitute only 181 of all the residues in the χ subunit, i.e., less than half (Figure 4). Significantly, these include all 22 residues that are strictly conserved in the 17 enzyme proteins characterized previously (Jörnvall et al., 1987c) and that are therefore considered to be typical for alcohol dehydrogenases, as shown by the asterisks in Figure 4. Even residues at variable positions vary within observed limits, adding futher strength to previous consensus constructions at 13 additional positions (Jörnvall et al., 1987c). In summary, the class III structure, although not included in previous comparisons, supports available observations on generally conserved structures and does not change the major conclusions drawn.

Discussion

Structure Deduced and Subunit Properties. The structure is based on recovery of peptides from all parts of the polypeptide chain. The composition of all peptides (Table II) and of the whole protein (Table I) fit the structure deduced (Figure 3). Furthermore, it is distantly homologous to those of other alcohol dehydrogenases (Figure 4). Consequently, the amino acid sequence is concluded to represent a χ subunit of human liver alcohol dehydrogenase of class III.

In order to obtain overlapping fragments for some regions before a second batch of the human enzyme was available for direct characterization with the Lys-specific protease, a tryptic digest of the corresponding horse enzyme was also investigated. That digest further allows an estimate of the extent of species variation in the class III enzyme. It is thus evident that the class III structure is highly conserved. Tentatively, in the horse enzyme only 11 of 225 assigned positions differ (legend to Figure 3). This extent of species variation is smaller than that between the class I enzymes. Thus, the horse E-chain of class I differs from the human α-, β-, and γ-chains of class I at 47, 48, and 44 positions, respectively. Obviously, the class III structure of alcohol dehydrogenases is subject to more strict functional requirements, limiting its structural variability.

In relation to the χ structure at large, the species variation is negligible. Thus, as shown in Figure 5 (circled right) and Figure 4 (stippled residues in χ line), in total the χ structure differs from either the β or π alternatives at no less than 191 positions. Furthermore, species variation affects positions variable also among the classes (Figure 4). Consequently, conclusions on the general properties of the χ subunit can be based on the present structures, and variations within the class III group largely appear to affect minor properties only.

Electrophoretic separations have revealed the presence of different types of χ forms, with doublets composed mainly of a more basic major form and a less basic minor form, both anodal upon starch gel electrophoresis at pH 7.7–8.2 (Parês & Vallee, 1981; Adinolfi et al., 1984). However, those forms appear to co-vary, and the minor form has been suggested to be derived from secondary chemical modification of a single primary translation product (Adinolfi et al., 1984). Direct proof for microheterogeneities that can be ascribed to χ_1 and χ_2 structures was not now detected. However, the present starting material was an unresolved $\chi\chi$ preparation and therefore consisted largely of χ_1 chains. Although differences at single positions cannot be excluded, it appears likely that, if present, any isozyme variation within class III is quite limited. The class III doublet chains may represent chemically modified forms of a single translation product (Adinolfi et al., 1984). The situation for the class III enzymes would then resemble that for the class I enzymes, where, especially in the horse (Pietruszko & Theorell, 1969) and the rat (Jörnvall, 1973), chemically modified forms ("prime-fractions") have been found, apart from the translationally different subunits with proven residue differences.

Alcohol dehydrogenases in mammalian species thus reveal three types of multiplicity: separate enzymes derived from distantly related subunit types of different classes; isozymes derived from the presence of closely related subunit types within one class [α, β, γ of class I (Jörnvall et al., 1987a)]; and forms probably due to chemical modifications (Pietruszko & Theorell, 1969; Jörnvall, 1973). The differences between classes I, II, and III (Figures 4, 5) are considerably more extensive (affecting ~51% of all positions) than the isozyme differences within class I (about 9% of all positions or less). This is compatible with the conclusion that there are separate levels of duplication in the evolution of these forms and with distinctions of the differences between isozymes within a class and enzymes between the classes (Jörnvall et al., 1987b, and Figure 5). It is apparent that human liver alcohol dehydrogenase represents a complex system with multiplicities of forms both at functional and structural levels.

Functional Conclusions. The class III enzyme consisting of χ subunits differs from the class I and II enzymes in regard to substrate specificity, most characteristically by its extremely high K_m for ethanol (>3 M) (Parês & Vallee, 1981; Wagner et al., 1984; Juliá et al., 1987). The extent of species variation is one further distinction with functional implications between the class I and class III enzymes. As indicated here for the horse χ subunit and shown for the rat $\chi\chi$ enzyme (Juliá et al., 1988), species variations of χ (horse/man; 4% differences in characterized regions; rat/man, 9% differences) are considerably less than the same species variation within class I, i.e., rat/man class I, 18% differences (Jörnvall, 1974; Crabb & Edenberg, 1986). Consequently, functional constraints on the χ subunit appear to be larger than those on the class I subunits. This conclusion suggests that the classes of alcohol dehydrogenases fulfill different functional roles.

Distribution of the residue exchanges between the β and χ subunits of classes I and III, respectively, of human alcohol dehydrogenases also shows that the relationships are distant; not only are exchanges frequent, but they are distributed similarly between the two domains (Table III). This is a pattern typical for differences between enzymes in contrast to the pattern for intraclass isozymes (Jörnvall et al., 1987b,c).

Table III: Distribution of Residue Differences between the Two Domains When the Human χ Subunit Is Compared with the Human β Subunit[a]

structural segment	extent of residue differences between χ and β	
	no. of residues	% of total residues
catalytic domain	91	40
coenzyme-binding domain	48	33
total subunit	139	37

[a] Gaps are disregarded.

Consequently, the class III enzyme is a separate entity with distinctive properties but fitting those expected from other known alcohol dehydrogenase enzymes of other origins. The unique structure of $\chi\chi$ alcohol dehydrogenase is also consistent with the distribution of this enzyme class, which is apparently the only one present in mammalian brain (Beisswenger et al., 1985); placenta (Parés et al., 1984), and perhaps testis (Dafeldecker & Vallee, 1986), and with an embryological occurrence separate from those of the two other human alcohol dehydrogenase classes (Adinolfi et al., 1984).

The structural patterns observed can account for the altered properties between the class I and class III enzymes. Enzymatically, the $\chi\chi$ enzyme is hardly active toward ethanol (Parés & Vallee, 1981; Wagner et al., 1984; Adinolfi et al., 1984; Juliá et al., 1987b), a circumstance that is also consistent with the suggestion that the three classes of alcohol dehydrogenase represent evolutionary steps toward the development of new enzymes with separate functions (Jörnvall et al., 1987b). Electrophoretically, the $\chi\chi$ enzyme separates from those of the other classes by opposite migration at pH 8.2, i.e., anodic versus cathodic. This is compatible with the several exchanges of charged residues leading to a less positive χ subunit; there are four additional acidic and two fewer basic residues as compared to the β subunit.

Regarding residues of particular structural and functional importance, all ligands to both the catalytic and the noncatalytic zinc atom are conserved: Cys-46, His-67, and Cys-174 and Cys-97, Cys-100, Cys-103, and Cys-111, respectively. Structurally important glycine residues are also conserved [cf. Jörnvall et al. (1987c)]. These facts suggest that spatial relationships, overall conformation, and enzymatic mechanisms in the χ subunit and the subunits of the alcohol dehydrogenases analyzed previously are generally similar. Thus, coenzyme-binding and substrate-binding pockets of the $\chi\chi$ enzyme would also be expected to be formed by residues at positions similar to those of the class I type horse liver alcohol dehydrogenase that was analyzed crystallographically (Eklund & Brändén, 1987). However, differences in details are noted, as shown in Figure 4. Thus, regarding coenzyme-binding positions in the model enzyme (Eklund et al., 1984), the human χ alcohol dehydrogenase subunit has a histidine at position 47 (interacting with the coenzyme pyrophosphate) and a tyrosine at position 51 (where there is His in many other alcohol dehydrogenases) that participates in a hydrogen bond to the coenzyme (Eklund et al., 1984). However, these deviating χ residues, although different from those of the model horse EE enzyme, are also present in other alcohol dehydrogenases. Thus, His-47 is present in the π subunit of the human class II enzyme (Höög et al., 1987) and, together with a Tyr-51, further occurs in the plant alcohol dehydrogenases that have been characterized [Dennis et al., 1985; Chang & Meyerowitz, 1986; cf. Jörnvall et al. (1987c)]. The rat enzyme of class III also has both these alternatives (Juliá et al., 1988), but no other mammalian alcohol dehydrogenase characterized so far has both His-47 and Tyr-51. In summary, the combination of residues in the coenzyme-binding pocket of the χ subunit differs from that of the model enzyme, forming a unique pattern, although the exchanges in the coenzyme-binding pocket occur individually in some of the enzymes previously characterized (Jörnvall et al., 1987c). Thus, the $\chi\chi$ enzyme would be expected to utilize the features of coenzyme binding known from other alcohol dehydrogenases but with a new combination at single positions, suggesting a related coenzyme binding with minor deviations.

Many of the residues known to be involved in substrate interactions in the horse enzyme investigated crystallographically (Eklund et al., 1982; Eklund & Brändén, 1987) and the related class I human alcohol dehydrogenases (Eklund et al., 1987) are exchanged in the χ subunit, as shown by the arrows in Figure 4. In fact, only three of the residues of the substrate pocket (Eklund & Brändén, 1987) are unchanged: positions 48 (Ser), 67 (His), and 294 (Val) (Figure 4). Furthermore, several of the exchanges are not conservative but increase the polarity in the pocket by adding a charge (position 57) or hydroxyl groups (positions 93, 140), as shown by the residues at the arrows in Figure 4. Finally, some of the exchanges yield smaller residues in χ than in β (cf. Figure 4). All these exchanges are consistent with the fact that the $\chi\chi$ enzyme exhibits a different substrate specificity, capable of acting on some large substrates but being only minimally active toward ethanol. More detailed analysis of combined effects requires simultaneous consideration of all residues exchanged. Computer graphics in relation to the crystallographic structure of the horse enzyme might be effective in a manner similar to that for the class I enzymes (Eklund et al., 1987) or sorbitol dehydrogenase (Eklund et al., 1985). However, in view of the many alcohol dehydrogenase structures now known (Jörnvall et al., 1987c), it would seem imperative to obtain direct X-ray data also on a second type of the enzyme. Considering the unique properties of the χ subunit and the evolutionary patterns of mammalian alcohol dehydrogenases, with duplications at separate levels (Jörnvall et al., 1987b), the human enzymes are of particular interest for this purpose. However, even now the comparisons show the extent and general type of structural differences underlying the altered properties.

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REFERENCES

Adinolfi, A., Adinolfi, M., & Hopkinson, D. A. (1984) *Ann. Hum. Genet. 48*, 1–10.

Beisswenger, T. B., Holmquist, B., & Vallee, B. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8369–8373.

Bergman, T., Carlquist, M., & Jörnvall, H. (1986) *Adv. Methods Protein Microsequence Anal. 1986*, 45–55.

Brändén, C.-I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes (3rd Ed.) 19*, 103–190.

Chang, C., & Meyerowitz, E. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1408–1412.

Crabb, D. W., & Edenberg, H. J. (1986) *Gene 48*, 287–291.

Dafeldecker, W. P., & Vallee, B. L. (1982) *J. Protein Chem. 1*, 59–69.

Dafeldecker, W. P., & Vallee, B. L. (1986) *Biochem. Biophys. Res. Commun. 134*, 1056–1063.

Dennis, E. S., Gerlach, W. L., Pryor, A. J., Bennetzen, J. L., Inglis, A., Llewellyn, D., Sachs, M. M., & Peacock, N. M. (1984) *Nucleic Acids Res. 12*, 3983–4000.

Dennis, E. S., Sachs, M. M., Gerlach, N. L., Finnegan, E. J., & Peacock, W. J. (1985) *Nucleic Acids Res. 13*, 727–743.
Eklund, H., & Brändén, C.-I. (1987) *Biol. Macromol. Assem. 3*, 74–142.
Eklund, H., Plapp, B., Samama, J.-P., & Brändén, C.-I. (1982) *J. Biol. Chem. 257*, 14349–14358.
Eklund, H., Samama, J.-P., & Jones, T. A. (1984) *Biochemistry 23*, 5982–5996.
Eklund, H., Horjales, E., Jörnvall, H., Brändén, C.-I., & Jeffery, J. (1985) *Biochemistry 24*, 8005–8012.
Eklund, H., Horjales, E., Vallee, B. L., & Jörnvall, H. (1987) *Eur. J. Biochem. 167*, 185–193.
Fairwell, T., Juliâ, P., Kaiser, R., Parês, X., Vallee, B. L., & Jörnvall, H. (1987) *FEBS Lett. 222*, 99–103.
Halldén, G., Gafvelin, G., Mutt, V., & Jörnvall, H. (1986) *Arch. Biochem. Biophys. 247*, 20–27.
Höög, J.-O., von Bahr-Lindström, H., Hedén, L.-O., Holmquist, B., Larsson, K., Hempel, J., Vallee, B. L., & Jörnvall, H. (1987) *Biochemistry 26*, 1926–1932.
Jeffery, J., Cederlund, E., & Jörnvall, H. (1984) *Eur. J. Biochem. 140*, 7–16.
Jörnvall, H. (1973) *Biochem. Biophys. Res. Commun. 53*, 1096–1101.
Jörnvall, H. (1974) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Yonetani, T., Williamson, J. R., & Chance, B., Eds.) pp 23–32, Academic, New York.
Jörnvall, H., Hempel, J., & Vallee, B. L. (1987a) *Enzyme 37*, 5–18.
Jörnvall, H., Höög, J.-O., von Bahr-Lindström, H., & Vallee, B. L. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2580–2584.
Jörnvall, H., Persson, B., & Jeffery, J. (1987c) *Eur. J. Biochem. 167*, 195–201.
Juliâ, P., Farrês, J., & Parês, X. (1987) *Eur. J. Biochem. 162*, 179–189.
Juliâ, P., Parês, X., & Jörnvall, H. (1988) *Eur. J. Biochem.* (in press).
Parês, X., & Vallee, B. L. (1981) *Biochem. Biophys. Res. Commun. 98*, 122–130.
Parês, X., & Farrês, J., & Vallee, B. L. (1984) *Biochem. Biophys. Res. Commun. 119*, 1047–1055.
Pietruszko, R., & Theorell, H. (1969) *Arch. Biochem. Biophys. 131*, 288–298.
Schroeder, W. A., Shelton, J. B., & Shelton, J. R. (1969) *Arch. Biochem. Biophys. 130*, 551–555.
Vallee, B. L., & Bazzone, T. J. (1983) *Isozymes: Curr. Top. Biol. Med. Res. 8*, 219–244.
Wagner, F. W., Parês, X., Holmquist, B., & Vallee, B. L. (1984) *Biochemistry 23*, 2193–2199.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem. 77*, 569–573.

Effect of Secondary Substrate Binding in Penicillopepsin: Contributions of Subsites S_3 and S_2' to k_{cat}†

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ABSTRACT: The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were determined at 25 °C and pH 4.5, 5.5, and 6.0 for the series of penicillopepsin substrates Ac-Ala$_m$-Lys-(NO$_2$)Phe-Ala$_n$-amide, where (NO$_2$)Phe is *p*-nitrophenylalanine and *m* and *n* equal 0–3. K_M values at pH 6.0 were the same for all 12 peptides and averaged 0.088 ± 0.02 mM but increased to different degrees at lower pH. In contrast, k_{cat} values increased with increasing chain length. At pH 6 and at the pH optimum of k_{cat}, the largest increases (about 37-fold on average) were obtained when alanine residues were added in positions P_2' and P_3. Only 1–2-fold increases were observed for positions P_2, P_3', P_4, and P_4'. These results show that occupation of subsites S_2' and S_3 is largely responsible for the rate enhancements caused by secondary substrate interactions with this series of peptides. Additional support for an important role of subsite S_3 comes from the observation that the two peptides where $m = 1$ and $n = 1$ or 2, respectively, are cleaved not only between lysine and *p*-nitrophenylalanine but also between the latter and alanine, suggesting that occupation of subsite S_3 by the N-terminal alanine overcomes the unfavorable interaction of alanine in subsite P_1'. Subsite S_3 is also important in the binding of pepstatin analogues and in transpeptidation reactions. It is proposed that the roles of subsites S_3 and S_2' are to facilitate the conversion of the first enzyme–substrate complex into a productive complex and to assist in the distortion of the scissile bond. Sequence comparisons suggest that the effects observed for penicillopepsin may be common to most aspartic proteinases.

Penicillopepsin is a well-characterized member of the family of aspartic proteinases. Its three-dimensional structure, which has been determined at high resolution (James & Sielecki, 1983), is very similar to that of pig pepsin (Andreeva et al., 1984) and to those of the aspartic proteinases from *Rhizopus chinensis* (Suguna et al., 1987a) and *Endothia parasitica* (Pearl & Blundell, 1984). Other representatives of this family of enzymes, the mammalian renins, gastricsins, and cathepsins D (Tang, 1987) and the fungal proteinases from *Mucor miehei* (Bech & Foltmann, 1981; Gray et al., 1986), *Mucor pusillus* (Tonouchi et al., 1986), *Saccharomyces cerevisiae* (Dreyer

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