Cloning and characterization of a novel rat alcohol dehydrogenase of class II type

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Abstract A class II type alcohol dehydrogenase from rat liver was characterized at the cDNA level after screening cDNA libraries in combination with PCR amplification of the 5′-part. The open reading frame translates into a polypeptide of 376 amino acid residues, which show 73% positional identity to the human class II enzyme. This suggests that the class II enzyme is the most variable form of the mammalian alcohol dehydrogenases. A deletion is apparent corresponding to position 294 of the human enzyme and amino acid residues unique to the rat protein of those interacting with the coenzyme NAD^+ are found at positions 47, 51, 178, and 271. Position 47 is occupied by Pro instead of Arg or His found in most mammalian alcohol dehydrogenases. This exchanged residue will not hydrogen bond to the pyrophosphate of the coenzyme and will change the local environment around position 47 to strictly hydrophobic.

Key words: Alcohol dehydrogenase; cDNA sequence; Amino acid substitution; Active site

1. Introduction

Rat is a widely used model species in studies of the metabolism and pharmacology of ethanol. Three distinct forms of rat alcohol dehydrogenase (ADH) have been defined by activity staining after starch gels electrophoresis, isolation [1], and structural characterization [2,3]. These three forms correspond to the ADHs of classes I, III and IV [2–4]. The rat class IV ADH exhibits enzymatic characteristics similar to some of those of class II [1], but so far the existence of a class II ADH in rat or other rodents has not been shown.

Human class II ADH, with π subunits, is relatively insensitive to inhibition by pyrazole [5], an effective inhibitor of class I ADH. Class II ADH shows higher K_m and k_{cat} values for ethanol than the class I isozymes [6]. It catalyzes the oxidation/reduction of a broad range of alcohols/aldehydes with K_m values ranging from 7 μM (hydrophobic substrates) to about 0.5 M (fairly hydrophilic substrates) [6,7]. The large variation in K_m values for different substrates reflects the importance of hydrophobic interactions in substrate binding. The primary structure has been determined for human class II ADH [8], which shows about 60% residue identity to those of the other classes of human ADH [9]. Other mammalian class II type ADHs have been isolated and characterized enzymatically from horse and monkey livers [10,11]. An ADH with class II type structural characteristics has also been isolated and characterized from ostrich liver [12].

This report describes the characterization of cDNA clones coding for a rat class II type enzyme, and defines the relationships to other mammalian ADHs.

2. Materials and methods

2.1. Screening of cDNA libraries

Two rat liver cDNA libraries in λgt11 with mRNA isolated from female and male rats, 5′-extended and oligo(dT) plus random primed (Clontech Inc., CA), were screened with a 350 bp cDNA fragment coding for human class II ADH [8]. The fragment was nick-translated with [α-^{32}P]dCTP (Amersham, UK) and DNA polymerase/DNase (International Biotechnology Inc., CT) prior to screening the cDNA libraries. Phage isolation was performed according to standard procedures [13]. cDNA inserts were size estimated by agarose gel electrophoresis after cleavage with *Eco*RI, were purified with glass beads (BIO 101, CA) and were subcloned into pEMBL vectors. Restriction mapping with endonucleases was performed, and selected fragments were further purified and subcloned into M13mp18 or mp19 vectors. Plasmids were purified with the Qiagen ion exchange procedure (Diagen, FRG).

2.2. Generation of full-length cDNA

mRNA was isolated from rat liver (1 g) with the guanidinium-thiocyanate method and further purified on oligo-dT cellulose spin columns (Pharmacia Biotech, Sweden). One μg mRNA was reverse transcribed with 2.5 U of avian myeloblastosis virus reverse transcriptase (Boerhinger-Mannheim, FRG) using a 30-mer oligonucleotide primer (p1; Fig. 1). The cDNA product obtained was purified on a Chroma-Spin 400 column (Clontech Inc., CA) and residual mRNA was hydrolyzed with 0.5 M NaOH. The single-stranded cDNA was ligated with an anchor 27-mer oligonucleotide, harbouring an *Eco*RI restriction site, using T4 RNA ligase (Amplifinder; Clontech Inc., CA). The cDNA obtained was PCR-amplified with *Taq* polymerase (AmpliTaq, Perkin-Elmer, CA) using a 5′-anchor primer (Clontech Inc., CA) complementary to the ligated 27-mer oligonucleotide, and a 3′-primer harbouring a *Hin*dIII restriction site (p2; Fig. 1). The protocol for PCR amplification used: 35 cycles of 94°C, 45 s; 56°C, 45 s; 72°C, 1 min. The PCR product was size fractionated on 1.5% agarose gel, purified with glass beads and digested with *Eco*RI and *Hin*dIII before ligation into digested pBluescript vector.

2.3. Sequence analysis

DNA sequence analysis was carried out with T7 DNA polymerase (Pharmacia Biotech, Sweden) and [α-^{35}S]dATP (Amersham, UK). The entire cDNA was determined by DNA sequence analysis on both strands and the PCR amplified 5′-end was analyzed in two independently cloned fragments. All sequences obtained were analyzed with the Wisconsin Package [13] and compared with the EMBL data bank.

3. Results

3.1. Isolation of cDNA clones

A total of 500,000 plaques derived from two independent rat liver cDNA libraries were screened with a cDNA fragment coding for human class II ADH. Ten faint positive signals were

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Note: The novel nucleotide sequence data reported here have been submitted to the EMBL/GenBank Data Banks.

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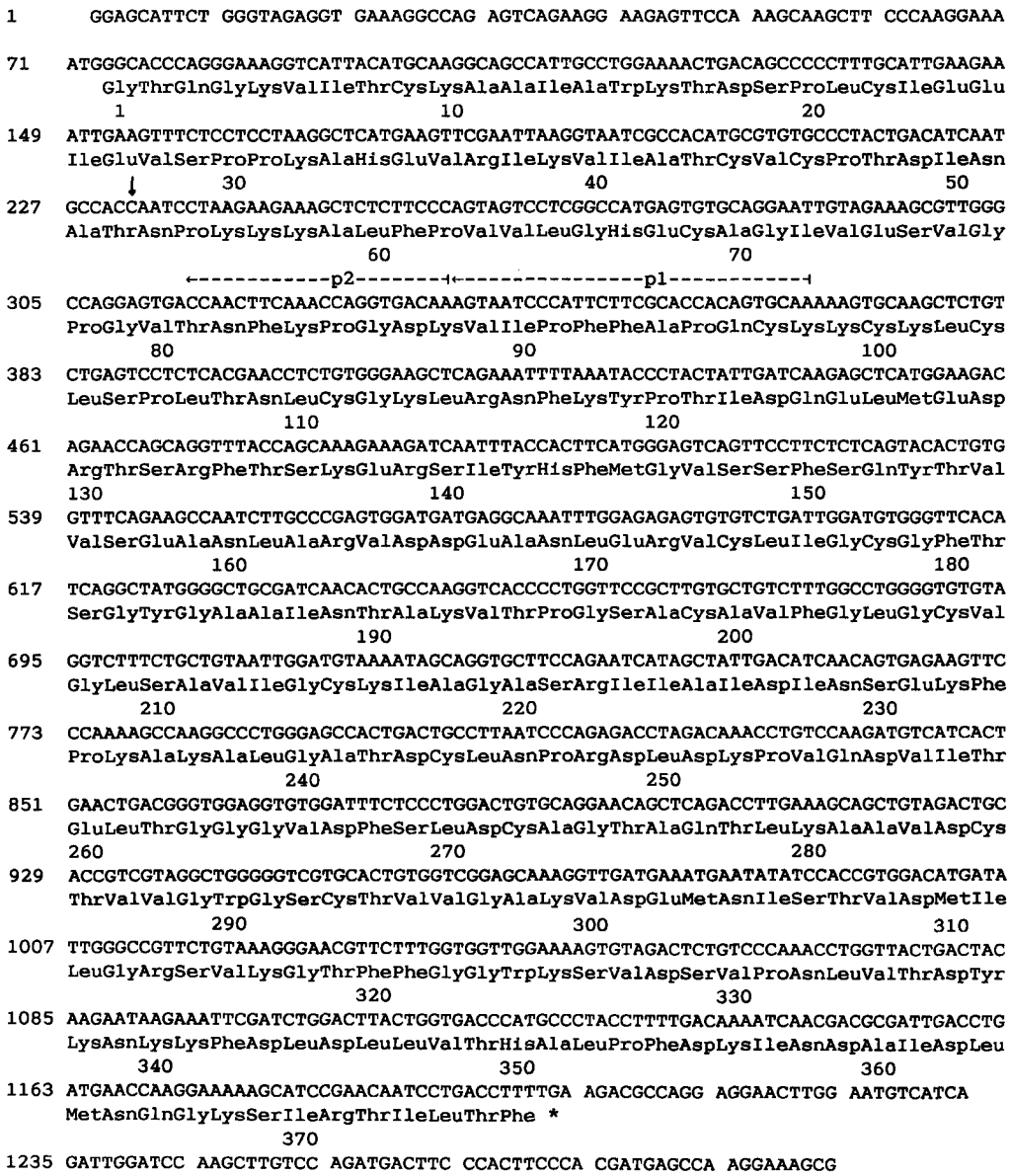

Fig. 1. Nucleotide and deduced amino acid sequences of rat class II type ADH. Nucleotides are numbered on the left-hand side and amino acid residues below the sequence. The numbering used in the running text and in Table II corresponds to the system for class I ADH [19]. The rat class II enzyme has a four-residue insertion at position 120 and a two-residue deletion at position 294. Arrows above the sequence outline the primers used for reverse transcription (p1) and PCR amplification (p2). The arrow at nucleotide 232 indicates the 5′-end of the cDNAs isolated.

obtained that gave strong signals after rescreening. The corresponding cDNA inserts were subcloned into pEMBL vectors. All cDNA clones harboured inserts of about 1100 bp and showed the same restriction pattern. One of the clones was subjected to DNA sequence analysis which resulted in a sequence of 1065 bp including a 89 bp 3′-noncoding region. This does not correspond to a full-length cDNA. Partial DNA sequence determination showed that all cDNAs are truncated at the same position. To obtain a full-length cDNA, a modified variant of rapid amplification of cDNA ends was used (RACE; [15]), including an anchor oligonucleotide and T4 RNA ligase. This resulted in a 300 bp fragment that was ligated into a pBluescript vector. The 300 bp fragment harboured the 5′-part of the cDNA and completed, together with the directly cloned cDNA the entire coding region. This generated a cDNA coding for a rat class II type ADH. The cDNA covers 1293 bp including the coding sequence of 1131 bp and a 5′-noncoding 70 bp segment (Fig. 1). The region around the ATG start codon shows large similarity to the human class II cDNA sequence and alignment of the whole cDNA sequence with that of the human class II enzyme reveals a positional identitiy of 79%.

3.2. Deduced amino acid sequence

The translated amino acid sequence results in a polypeptide chain of 376 amino acid residues (excluding the initiator Met presumably removed as in other ADHs [16]). Alignment of the

Table 1
Positional identities between alcohol dehydrogenases Top: amino acid residue identities between human and rat ADHs. Bottom: residue identities within the set of rat ADHs. Amino acid sequences and previously known positional identities from refs. [2,3,19,29]

Human versus rat.			
class I	class II	class III	class IV
82%	73%	94%	87%

Rat class II type versus rat classes I, III, and IV		
class I	class III	class IV
55%	57%	52%

rat amino acid sequence to other ADH sequences gives the highest identity to the human class II ADH, 73%, with two gaps. Comparisons of the other ADH classes from rat and human result in much higher values (Table 1). Within the set of rat ADHs, the class II type ADH shows positional identities with the other classes of 52%–57% (Table 1).

The deduced amino acid sequence shows an insertion of four residues around position 120, also found in the human class II amino acid sequence. The rat enzyme has two deletions, one at position 60 and one at positions 294–295 as compared to the human class II ADH. The regions around these two deletions show very low homology to the human class II ADH and the positions for the deletions are somewhat tentative. Amino acid residues lining the active site pocket differ widely from the ones of human class II, and at some positions the rat enzyme shows residues not found in other ADHs. These residues are Lys^{57} (recently found also in a deer-mouse variant [17]) at the middle part of the active-site pocket and Val^{306} at the outer part of the pocket (Table 2A). Amino acid residues lining the coenzyme-binding pocket but not found at corresponding positions in other ADHs are Pro^{47}, Asn^{51}, Ser^{178} and Thr^{271} (Table 2B).

4. Discussion

A class II type ADH from rat was isolated at the cDNA level after screening liver cDNA libraries. To obtain the entire cDNA, PCR amplification of the 5′-end was performed. The cDNA harbours an open reading frame of 1131 bp which translates into 376 amino acid residues. An insertion of four amino acid residues around position 120 is identified, as compared to other ADH classes, which seems to be typical for class II ADH [8,12]. Furthermore, the rat ADH has a two-residue deletion around position 294, which has not been found in any mammalian ADH. However, in a fish ADH (cod) a deletion is found at position 295, but is compensated for by a large residue at position 294 [18]. The position of the deletion in the rat enzyme is not unambiguously defined because of the low homology between class II ADH and other ADHs in the region around position 294. Position 294 is occupied by Val in most characterized mammalian ADHs and has been ascribed a structural function at the middle part of the active-site pocket [19]. In class I ADH, Val^{294} is further involved in the conformational change associated with NAD^+ binding and interacts with the nicotinamide moiety [20].

The amino acid residues lining the active site pocket in the rat enzyme differ in many respects from corresponding residues in other mammalian ADHs (Table 1A). This promotes changes

Table 2
Amino acid residues lining the substrate-binding cleft (A) and the coenzyme-binding cleft (B) in human and rat ADHs. The numbering corresponds to the system used for class I ADH [18]. Residues in italics are those determined in this report. Δ indicates a deletion. Data from refs. [2,3,17,19,29,30]

A.	inner part of binding cleft				middle and outhor part of binding cleft							
Position	48	93	140	141	57	110	115	116	294	306	318	
Human I α	Thr	Ala	Phe	Leu	Met	Tyr	Asp	Val	Val	Met	Leu	Ile
Human I β	Thr	Phe	Phe	Leu	Leu	Tyr	Asp	Leu	Val	Met	Leu	Val
Human I γ	Ser	Phe	Phe	Val	Leu	Tyr	Asp	Leu	Val	Met	Leu	Ile
Rat I	Ser	Phe	Phe	Leu	Leu	Tyr	Asn	Leu	Val	Met	Leu	Ile
Human II	Thr	Tyr	Phe	Phe	Phe	Phe	Ser	Asn	Val	Glu	Ile	Phe
Rat II	*Thr*	*Phe*	*Phe*	*Met*	*Lys*	*Leu*	*Arg*	*Asn*	*Δ**	*Val*	*Ile*	*Phe*
Human III	Thr	Tyr	Tyr	Met	Asp	Leu	Arg	Val	Val	Phe	Val	Ala
Rat III	Thr	Tyr	Phe	Met	Asp	Leu	Arg	Val	Val	Phe	Val	Ala
Human IV	Thr	Phe	Phe	Met	Met	Leu	Asp	Ile	Val	Met	Phe	Val
Rat IV	Thr	Phe	Phe	Met	Met	Leu	Asp	Leu	Ala	Met	Phe	Val
Human V	Thr	Phe	Phe	Gly	His	Phe	Lys	Gln	Val	Gln	Phe	Val

*The region around position 294 shows low homology between rat class II and the other ADHs which results in a somewhat tentative position for the deletion.

B.											
Position	47	48	51	178	203	223	224	228	269	271	369
Human I α	Gly	Thr	His	Thr	Val	Asp	Ile	Lys	Ile	Arg	Arg
Human I β^a	Arg	Thr	His	Thr	Val	Asp	Ile	Lys	Ile	Arg	Arg
Human I γ^b	Arg	Ser	His	Thr	Val	Asp	Ile	Lys	Ile	Arg	Arg
Rat I	Arg	Ser	His	Thr	Val	Asp	Ile	Lys	Ile	Arg	Arg
Human II	His	Thr	Thr	Thr	Val	Asp	Ile	Lys	Ala	Gly	Arg
Rat II	*Pro*	*Thr*	*Asn*	*Ser*	*Val*	*Asp*	*Ile*	*Lys*	*Ala*	*Thr*	*Arg*
Human III	His	Thr	Tyr	Thr	Val	Asp	Ile	Lys	Ile	Asn	Arg
Rat III	His	Thr	Tyr	Thr	Met	Asp	Ile	Lys	Ile	Asn	Arg
Human IV	Arg	Thr	His	Thr	Met	Asp	Leu	Lys	Ile	His	Arg
Rat IV	Gly	Thr	His	Thr	Met	Asp	Ile	Lys	Ile	Arg	Arg
Human V	Gly	Thr	His	Thr	Gly	Asp	Val	Lys	Ile	Asn	Arg

[a] The allelic variant β_2 has His at position 47 and the allelic variant β_3 has Cys at position 369.
[b] The allelic variant γ_2 has Gln at position 271.

in the shape of the active site pocket, first because of the deletion at position 294–295 (or in that loop region), and further because of the introduction of a proline residue at position 47. In most ADHs investigated, the latter position is occupied by Arg or His [19], residues that form hydrogen bonds to the pyrophosphate of the coenzyme [20,21]. In some ADHs, Gly occupies position 47 and this residue cannot yield hydrogen bonds to the pyrophosphate [9,21]. Two variants investigated, human class I α and rat class IV, show that Gly at position 47 results in highly altered kinetic parameters [3,22]. The same will probably be true for Pro, which also is unable to hydrogen bond to NAD^+. Moreover, the local environment around position 47 will also be changed by introduction of the strictly hydrophobic Pro. The rat class II type shows unique residues also at positions 51, 178, and 271, that likely will affect the coenzyme binding. The latter position, that of 271, differentiates the allelic γ_1 and γ_2 isozymes of human class I [23]. Furthermore, at the middle part of the substrate-binding pocket two positive charges are introduced as compared to the human enzyme. These are Arg^{115}, typical for class III ADH (Table 2A; [2,9]), and Lys^{57}, elsewhere found in a deer-mouse ADH [17]. Finally, Val^{306} is unique and probably will contribute to a slightly wider opening at the active-site pocket.

The results obtained from the characterized cDNA show that class II ADH is the most variable type of the mammalian class I–IV enzymes. Class III ADH, the origin of the medium-chain alcohol dehydrogenases, thus constitutes the far most conserved structure [9], and class II is shown to be another extreme with only 73% positional identity (Table I, cf. ref. [24]). The meaning of the large structural variance of the class II enzymes is not known, but in contrast to class III ADH/glutathione-dependent formaldehyde dehydrogenase [25], the function seems not to be strictly specific. The human class II enzyme can catalyze the conversion of a broad range of alcohols and aldehydes [5,6] including 4-hydroxyalkenals [7] and retinoic compounds [26]. Human class II ADH has been shown to be the best hepatic ADH in retinol/retinal metabolism, while class IV ADH has been shown to be the enzyme with corresponding properties in epithelial tissues [26]. The rat class II type now characterized has identical amino acid residues lining the inner part of the active-site pocket as class IV (Table 2A), and probably can catalyze the oxidation of retinol.

A class II ADH has never been observed in a rat organ at the protein level [1,27], but Northern blot analysis shows that mRNA coding for a rat class II type ADH is clearly present in liver, duodenum, kidney, stomach, and testis [28]. The rat class II type ADH can have a shifted substrate specificity as compared to other characterized mammalian ADHs and thereby not have been detected in the screening methods used. Class II type ADHs have been shown to be present in primates, horse and in ostrich [5,10–12], but rodents have not a class II ADH protein that has retained the structural and activity characteristics of the human enzyme.

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