FEBS Letters 367 (1995) 306–310 FEBS 15626

Alcohol dehydrogenase of class I: kiwi liver enzyme, parallel evolution in separate vertebrate lines, and correlation with 12S rRNA patterns

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Received 3 April 1995; revised version received 2 May 1995

Abstract Alcohol dehydrogenase class I from kiwi liver has been purified, analyzed, and compared with that of other alcohol dehydrogenases. The results show that several avian and mammalian forms of the enzyme exhibit parallel evolutionary patterns in two independent lineages of a single protein, establishing a pattern in common. Furthermore, the data correlate the enzyme evolutionary pattern with that of 12S rRNA. Biologically, the patterns complement those on ratite and other avian relationships. Functionally, the enzyme has a low K_m with ethanol and a branched-chain residue at position 141, like the mammalian enzymes but in contrast to the other characterized ratite enzyme (with Ala-141 and a higher K_m). This pattern of natural variability suggests a frequent but not fully complete correlation between a large residue size at position 141 and tight ethanol binding.

Key words: Alcohol dehydrogenase; Substrate-binding site; Amino acid substitutions; Kiwi protein; Molecular evolution; Protein/rRNA parallels

1. Introduction

Liver alcohol dehydrogenase of the ethanol-active class I type is one of the phylogenetically well studied enzyme systems, through known structures of the protein from 20-odd vertebrate species [1], representing all major lines from humans to bony fish, below which this form of the enzyme has not been found [2]. Within this spread, the alcohol dehydrogenase system is known in further multiplicity from a number of gene duplications during vertebrate evolution, to create now minimally six different classes, with later isozyme separations in several cases. Data are most extensive for class I (the classical liver alcohol dehydrogenase) but also extensive for class III (glutathione-dependent formaldehyde dehydrogenase [3]), which is concluded to be the ancestral form present in the first vertebrate branch [2], invertebrates [4,5] and down to prokaryotes [6,7]. Some evolutionary properties of class IV, expressed in epithelia, in particular stomach, are also known [8].

Molecular relationships have been of particular interest for avian phylogeny, where DNA hybridization has revolutionized bird taxonomy [9], DNA sequences of 12S rRNA have established separate colonizations by ratites (flightless birds) [10], cytochrome *b* genes have confirmed a close relationship between storks and new world vultures [11], and antiprotease domains have showed details for a large number of species [12–14].

We have now characterized class I alcohol dehydrogenase from the liver of brown kiwi, *Apteryx australis australis*. Kiwis are of particular interest since they have one of the largest egg to body weight ratios of any birds, a property which has been suggested to represent an evolutionary shrinkage of body weight rather than increase of egg size [15]. Biochemically, the present data on the alcohol dehydrogenase system complement those on rRNA, while biologically they allow further evaluations of avian interconnections.

2. Materials and methods

2.1. Protein purification

Liver from one individual of Brown Kiwi, *Apteryx australis australis*, was used. The individual was sacrificed with permission (because of a malformed pelvis) from the Department of Conservation, New Zealand (Jan. 19, 1993). The liver (35 g) was frozen immediately in liquid nitrogen and transported in dry ice for mRNA preparation from one sample (5 g) and purification of alcohol dehydrogenase protein from another (12 g).

Chromatographies for alcohol dehydrogenase purification utilized DEAE Fast flow, AMP Sepharose and Mono Q (all from Pharmacia) in steps similar to those for recent purifications of other alcohol dehydrogenases [2,5]. Protein amounts during purification were monitored with the Bradford method [16] while in pure state they were determined by amino acid analysis after hydrolysis. Enzyme activity with ethanol and other alcohols was monitored in 0.1 M glycine/NaOH, pH 10, with 0.5 mM NAD^+, while that with glutathione-conjugated formaldehyde was measured in 0.1 M sodium pyrophosphate, pH 8.0, with 1 mM glutathione/1.2 mM NAD^+. Purities were evaluated by SDS/polyacrylamide gel electrophoresis using a Phast system (Pharmacia) and Coomassie brilliant blue for protein staining, while activity staining was performed with nitro blue tetrazolium/phenazine methosulphate after isoelectric focusing [5].

2.2. Enzymology

Enzyme activities were determined at 25°C in buffers mentioned above, by measurements at 340 nm using a molar absorptivity of 6.22×10^3 cm^{-1} for NADH, and a weighted non-linear regression analysis program for calculation of kinetic constants [17].

2.3. Protein structure analysis

Peptides for amino acid sequence analysis were generated by digestions of the [^{14}C]-carboxymethylated protein with Lys-specific and Glu-specific proteases, followed by purifications by reverse phase HPLC as described for other alcohol dehydrogenases [2,5].

Total compositions were determined by amino acid analysis with a Pharmacia Alpha Plus analyzer after hydrolysis with 6 M HCl/0.5% phenol for 24 h in evacuated tubes at 110°C. Sequencer degradations were carried out in Milligen Prosequencers 6600 and 6625. Two

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SSDI 0014-5793(95)00554-4

peptides, including the acyl-blocked N-terminal peptide, were analyzed by a Lasermat or Lasermat 2000 mass spectrometer.

2.4. cDNA library, cloning and DNA sequence analysis

mRNA was prepared from the freshly frozen liver aliquot (5.1 g) by the guanidinium thiocyanate/CsCl method with subsequent oligo(dT)chromatography [18,19]. 2 μg of mRNA was used as a template for the construction of a cDNA library in phage λgt10 using a cDNA synthesis kit (Pharmacia). Sequence analysis was performed using T7 sequence kits (Pharmacia). Interpretations were assisted with the software package from the University of Wisconsin Genetics Computer Group [20].

3. Results

3.1. Major liver alcohol dehydrogenase

Liver from Brown Kiwi was postmortem immediately frozen in liquid nitrogen and used after thawing for purification of alcohol dehydrogenase. The major ethanol-active enzyme was purified after homogenization of the liver, and utilizing a 3-step chromatographic method, with ion exchange chromatography on DEAE Fast flow, affinity chromatography on AMP-Sepharose and FPLC on Mono Q. As expected from alcohol dehydrogenase patterns common to most vertebrates [1], separate enzymes, detectable by activity staining after isoelectric focusing, account for ethanol dehydrogenase activity (class I alcohol dehydrogenase) and glutathione-dependent formaldehyde dehydrogenase (class III).

The major ethanol-active enzyme was quite basic (pI around pH 9) and yielded more anodic subforms in lower amounts, resembling the pattern obtained also from other species [2] after isoelectric focusing and activity staining. The major fraction, contributing the most cathodic band, was obtained in a purification yield typical of class I liver alcohol dehydrogenase (Table 1). Consequently, this kiwi liver protein was analyzed for establishment of the enzyme relationships.

Enzymatic analysis with ethanol as substrate revealed that the K_m for ethanol was 0.9 mM, typical of the class I enzyme.

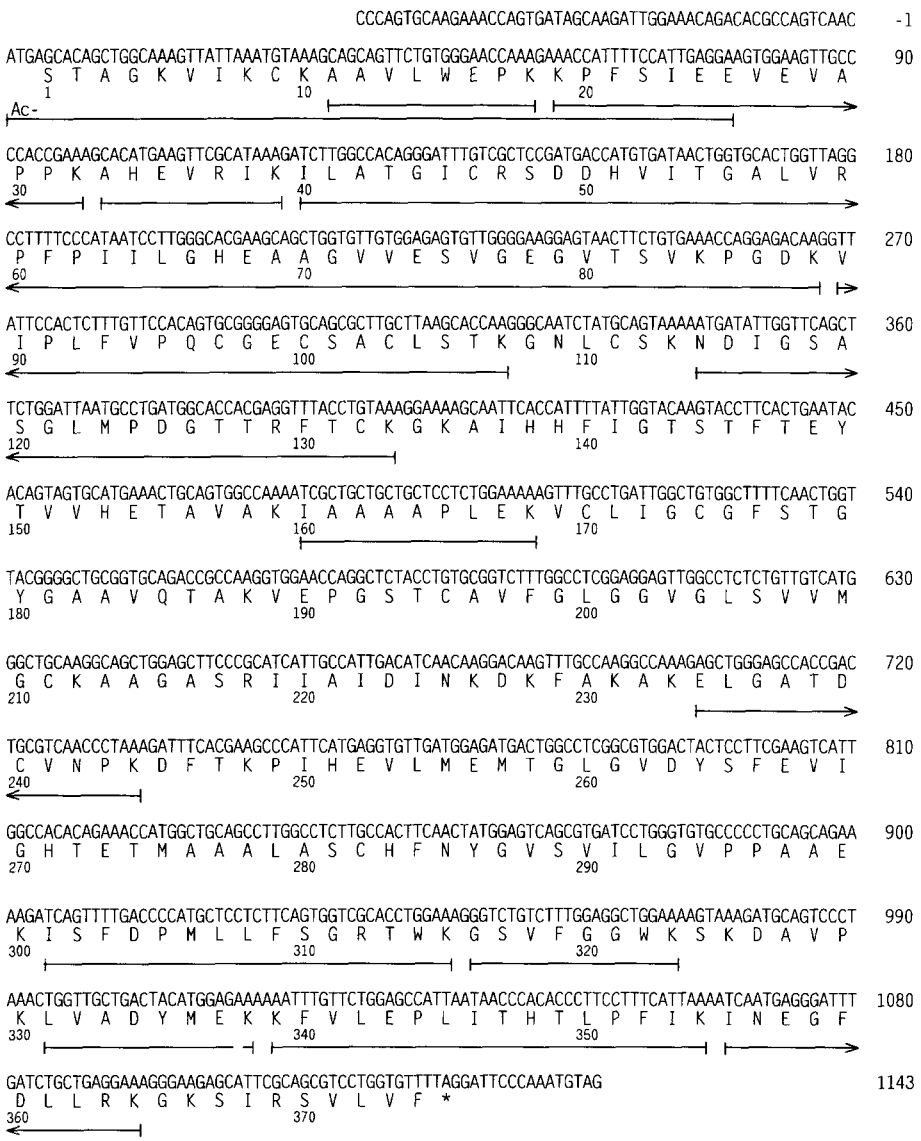


Fig. 1. cDNA and protein structures of kiwi liver class I alcohol dehydrogenase. The lines indicate the 204 positions obtained by sequence analysis of peptides, constituting the forms characterized from a Lys-specific cleavage, except for the acetyl-blocked N-terminal peptide which was analyzed by mass spectrometry, giving a total mass compatible with an acetylated serine residue corresponding to the codon after that for the initiator Met.

3.2. Protein and cDNA structure

The primary structure of the protein was determined by a combination of peptide and DNA sequence analysis. In principle, one major protease (Lys-specific) was used for peptide generation. Combined, the peptides gave over half of the entire amino acid sequence (204 of 374 residues; cf. Fig. 1). The N-terminal part of the peptide chain was prepared by Glu-specific digestion and used for mass spectrometry with a Lasermat instrument. The $(M + H)^+$ value obtained (2861.0 versus 2861.37 expected) confirmed the presence of an acetyl-blocked N-terminal structure and showed it to be acetyl-Ser. This corresponds to position 2 of the nascent protein as deduced from the cDNA (Fig. 1) also analyzed (below), showing that the initiator-methionine is removed during protein processing as in other alcohol dehydrogenases, and in agreement with the properties expected from the residue pattern in the proform structure [21].

Another aliquot of the frozen liver was used for preparation of a cDNA library, which was then screened with a probe from class I chicken alcohol dehydrogenase cDNA [22]. Over 100 clones gave positive screening signals and 12 were purified to homogeneity and analyzed for insert size. The two longest cDNAs were used for sequence analysis to obtain a 1196-nucleotide DNA sequence, coding for a 374-residue mature (excluding the initiator-Met) protein (Fig. 1). At all positions analyzed by both peptide and nucleotide data, results agree and confirm each other as shown.

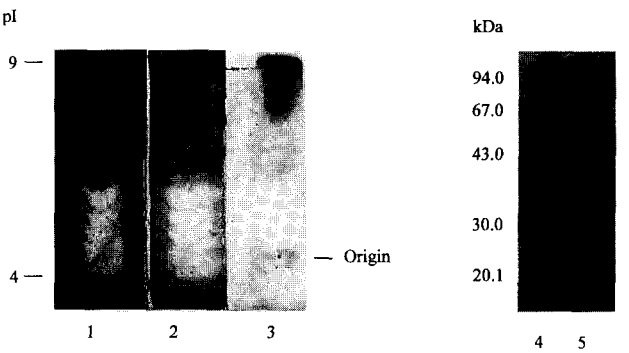
3.3. Evolutionary relationships

The amino acid sequence obtained is homologous with that of class I medium-chain alcohol dehydrogenases, allowing the conclusion that conformations are similar, as for even other classes of the enzyme [23]. Hence, direct comparisons of residues participating in binding interactions at the active site are meaningful, allowing correlation with differences in functional properties (below). As expected, relationships are closest with other avian forms, and with the alligator enzyme [24], also known in structure, from the reptilian line giving rise to the avian line. The knowledge of now many avian and mammalian forms permits comparison of the evolutionary rates of the same enzyme from these two different branches of the animal system. It further allows correlation with similar data for an rRNA [10], also known structurally from ratites and mammals.

The results show that branchings are similar in the mammalian and avian lines, starting at approximately similar times, and with a split between the ratite and non-ratite avian enzymes (Fig. 2). A rough estimate at evolutionary rates using the novel kiwi structure suggests values like 10 PAM per 100 MY for the relationships to both the human enzyme (mammalian line) and alligator enzyme (avian line), corresponding to a separation of the ratite lines roughly 60 MY ago. Of course, these values are tentative, assuming constant evolutionary changes, and based on the structures known, but anyway suggest a roughly parallel evolutionary rate of the avian and mammalian lines. This is also compatible with fossil data, with a distant origin of the kiwi (cf. [10]), and with a similar pattern from the same four species of avian 12S rRNA (Fig. 2). Notably, other systems, even those well established, like globins, cytochrome *c*, and lysozymes, are not yet known in similar detail for these species, making alcohol dehydrogenase and 12S rRNA uniquely useful for tracing these relationships.

Table 1
Purification of kiwi liver class I alcohol dehydrogenase

Purification (step)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Homogenate	9.8	459	0.02	1	100
DEAE	7.6	57	0.13	6	77
AMP-Sepharose	2.4	4	0.55	26	24
Mono Q	1.2	0.4	3.1	144	13



The values apply toward the recovery of the main form of this class only, as obtained from 12 g of tissue. The bottom insert shows the pattern upon activity staining (left) with ethanol (lane 1), pentanol (lane 2), and formaldehyde/glutathione (lane 3) after native isoelectric focusing and Coomassie staining (right) of standard proteins (lane 4) and pure alcohol dehydrogenase (lane 5) after SDS/polyacrylamide gel electrophoresis.

3.4. Alcohol dehydrogenase functional segments

Because of the strict homology between different forms of class I alcohol dehydrogenase, residues at positions of substrate binding interactions are meaningful to correlate. The structural analysis of the kiwi enzyme reveals that also its substrate interacting residues are largely conserved. However, comparisons (Table 2) show that the largely identical ostrich enzyme deviates in two properties: it has Ala-141 in the inner part of the substrate binding pocket, and it has a substantially higher K_m value with ethanol (Table 2). This Ala-141 replacement is unique among all alcohol dehydrogenases characterized and is concluded to contribute a substantial basis for the large K_m value with ethanol.

4. Discussion

Enzymatically, the kiwi enzyme exhibits a K_m for ethanol that is similar to those for most class I enzymes where it has been determined (Table 2; cf. [1,24]). Presence in the kiwi liver of also glutathione-dependent formaldehyde dehydrogenase activity shows that kiwi in addition has a classical class III enzyme, as expected from the pre-avian duplicatory origin of the class I enzyme form from class III [1]. Structurally, the kiwi liver class I alcohol dehydrogenase is an overall typical member of the vertebrate enzyme. Combined with the other mammalian and avian structures available, it demonstrates that functional differences in class I may be correlated with smaller, non-branched residues at position 141 (Table 2), giving extra space in this part of the pocket and less tight binding likely to explain the lower affinity of the ostrich enzyme for ethanol. However,

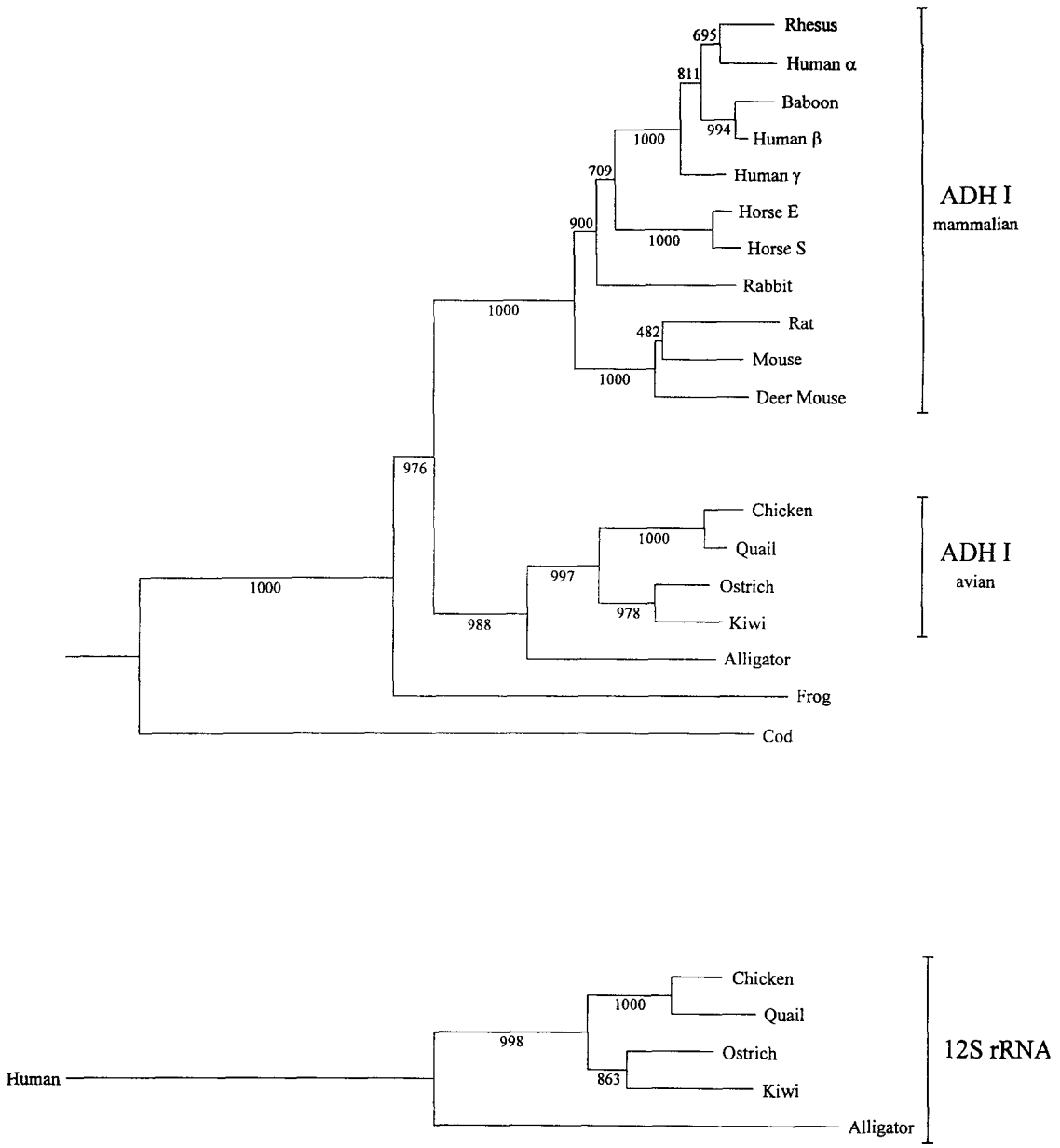


Fig. 2. Phylogenetic relationships among liver class I alcohol dehydrogenases characterized, emphasizing parallel patterns between two lines (avian and mammalian) of the enzyme, and correlation with a corresponding pattern of 12S rRNA from an identical set of avian line species (structures from EMBL data bank). Relationships shown are those obtained with the program CLUSTAL W [25]. Human class III alcohol dehydrogenase was used as outgroup for class I structures. Numbers indicate results from bootstrap analysis (1000 bootstrap replicates [26]).

correlation is not fully kept in other cases (quail enzyme, also with high K_m but Ile-141; cf. Table 2), demonstrating the influence also of other positions. In any event, the ratite enzymes constitute natural variants directly demonstrating structure–function correlations much like variants from site-directed mutagenesis corresponding to the position (residue 48) of another substrate-interaction [27].

This enzyme constitutes one of the few structures determined from kiwi, the only protein functionally characterized from kiwi, and the only protein with known functional differences in a kinetic constant between ratite forms. The results also allow comparisons of the evolutionary patterns for the two kiwi molecules structurally characterized. An enzyme (alcohol dehydrogenase; this work) and a structural RNA (12S rRNA segments [10]) correlated between the same set of species, as shown in Fig. 2. Patterns are highly similar for the two types of molecule, and demonstrate similar replacement speed between the avian and mammalian lines. Notably, among these two molecules, although both formally have about 370 positions, the protein has a larger potential variability (20 alternatives per position) than the RNA (4 per position). Hence, the support of previous rRNA data with the present protein data is significant, and both sets place the ratite separation (kiwi/ostrich) in between the chicken/quail separation (more recent) and the galliform/ratite separation (more distant), strengthening conclusions on repeated ratite colonizations in ancient days (cf. [10]).

Table 2
Residues at the inner, middle, and outer parts of the substrate binding pocket of avian and mammalian class I alcohol dehydrogenases, for which K_m values have been characterized for ethanol at pH 10.0

Protein forms	Inner (positions)				Middle (positions)					Outer (positions)			K_m (mM)
	48	93	140	141	57	115	116	294	318	110	306	309	
Kiwi	Ser	Phe	Phe	Ile	Leu	Asp	Ile	Val	Val	Leu	Met	Phe	0.9
Ostrich	Ser	Phe	Phe	**Ala**	Leu	Asp	Ile	Val	Val	Leu	Met	Phe	5.9
Quail	Ser	Phe	Phe	Ile	Leu	Asp	Leu	Val	Val	Leu	Met	Phe	8.1
Chicken	Ser	Phe	Phe	Val	Leu	Asp	Leu	Val	Val	Leu	Met	Phe	0.5
Rabbit	Ser	Phe	Phe	Ile	Ile	Asp	Leu	Val	Ile	Phe	Met	Leu	1.6
Rat	Ser	Phe	Phe	Leu	Leu	Asn	Leu	Val	Ile	Leu	Met	Leu	1.4
Mouse	Ser	Phe	Phe	Ile	Leu	Asp	Leu	Val	Ile	Phe	Met	Leu	0.15
Horse E	Ser	Phe	Phe	Leu	Leu	Asp	Leu	Val	Ile	Phe	Met	Leu	2.2
Horse S	Ser	Phe	Phe	Leu	Leu	Δ	Leu	Val	Ile	Leu	Met	Leu	7
Human γ	Ser	Phe	Phe	Val	Leu	Asp	Leu	Val	Ile	Tyr	Met	Leu	1.1
Human β	Thr	Phe	Phe	Leu	Leu	Asp	Leu	Val	Val	Tyr	Met	Leu	1.2
Human α	Thr	Ala	Phe	Leu	Met	Asp	Val	Val	Ile	Tyr	Met	Leu	1.5

Δ indicates a deletion. The substrate binding positions are those defined in [23]. Position 115 is formally not involved in the middle segment but listed there since that deletion when present (in the horse S form) influences binding. Sequence and K_m data from previous reports (cf. [1,24]). Bold face printing of Ala 141 indicates the unique replacement functionally discussed (cf. text).

Independent of these biological implications, the present results give clear biochemical conclusions for alcohol dehydrogenase, demonstrating accumulation of functional differences and unique structures in a short time. This is compatible with rapid evolutionary changes in class I alcohol dehydrogenase, distinguishing this form of the system from the more ancestral and 'constant' class III alcohol dehydrogenase.

Acknowledgements: We are grateful to Eric Fox, Otorohanga Kiwi House, for making the kiwi liver available, Carina Palmberg and Susanne Rothstein for skilful help, and to Finnigan Mat, Dr. Anita Keane for help with Lasermat mass spectrometry. This study was supported by grants from the Swedish Medical Research Foundation, and Peptech (Australia) Ltd.

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