Purification and substrate specificities of three human liver alcohol dehydrogenase isoenzymes

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Alcohol dehydrogenase *Human liver isoenzymes* *Substrate specificity*

1. INTRODUCTION

Human alcohol dehydrogenase (ADH, EC 1.1.1.1) occurs in multiple forms [1,2]. The isoenzymes are determined by 3 different gene loci, *ADH-1, ADH-2* and *ADH-3* which code for the different polypeptides α, β_1, γ_1, which randomly associate to the dimeric enzymes [3,4]. Alleles occur at the *ADH-2* and *ADH-3* locus, coding for β_2 (atypical ADH) and γ_2 [3–6].

ADH from phenotypically different tissues exhibit different substrate specificities [3,4,6,7]. However, little data is available concerning purified isoenzymes of known subunit ,composition. Pure ADH is obtained by double ternary complex affinity chromatography, but it still contains all pyrazole-sensitive isoenzymes [8]. Two preliminary reports on affinity purified isoenzymes [9,10] and a comparison of normal $\beta_1\beta_1$ with atypical $\beta_2\beta_2$ from Oriental individuals have been published [14].

Here, we report the purification of 2 homodimeric isoenzymes $\beta_1\beta_1$ and $\gamma_1\gamma_1$ and the corresponding heterodimeric form $\beta_1\gamma_1$ from human liver of normal ADH phenotype by double ternary complex affinity chromatography and CM-cellulose ion-exchange chromatography. These isoenzymes showed large differences in catalytic properties when ethanol, *n*-pentanol, cyclohexanol, and benzylalcohol were used as substrates. They exhibited marked substrate inhibition as well as non-linear Michaelis–Menten kinetics. The results support the suggestion that human liver ADH subunits do not seem to act independently of one another.

2. MATERIAL AND METHODS

2.1. *Chemicals*

NAD^+ (grade 1) was obtained from Boehringer (Mannheim) and ion-exchange celluloses from Whatman (Maidstone). 4-[3-(*N*-6-aminocaproyl)-aminopropyl]-Pyrazole–Sepharose was synthesized as in [12]. Starch for electrophoresis was from Electrostarch (Madison WI) and reagents for polyacrylamide gel electrophoresis were from Serva (Heidelberg).

2.2. *Purification of human liver ADH isoenzymes*

ADH was purified from human autopsy livers of normal ADH phenotype as in [8]. The resulting isoenzyme mixture was chromatographed on CM-cellulose CM-52 equilibrated with 50 mM Tris–HCl (pH 9). The isoenzymes were eluted from the gel with a linear gradient of 0–50 mM NaCl in equilibrating buffer. Fractions which exhibited only one band after starch gel electrophoresis were pooled and stored at 4°C.

2.3. *Electrophoresis*

SDS gel electrophoresis was performed on slabs of 12% polyacrylamide [12]. Starch gel electrophoresis was performed at pH 7.7 [2] and stained for ADH-activity [13].

2.4. *Identification of the isoenzymes*

Isoenzymes were identified by their mobilities on starch gel electrophoresis as in [3]. The subunit composition was verified by monomerization and

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hybridization with horse liver isoenzyme EE (purified as in [15]). Isoenzymes were monomerized by dialysis into sodium phosphate buffer (pH 7.5) followed by the addition of 50 mM NaCl and slow freezing at −20°C. The frozen enzyme solutions were then quickly thawed, the enzymatic activity was measured to determine the degree of reconstitution and electrophoresis was performed.

2.5. *Enzymatic assay*

ADH-activity was measured photometrically at 340 nm by monitoring the production of NADH at 25°C. Standard assay conditions in a total volume of 3 ml were 67 mM glycine/NaOH, (pH 10.5) or 67 mM sodium phosphate buffer (pH 7.0), containing 1.6 mM NAD^+ and 16.7 mM ethanol. The reaction was started by adding enzyme or in the case of crude samples by adding substrate after exhaustion of the blank reaction. Activities are expressed as International Units (IU) and represent μmol NADH produced/min. Specific activities are expressed as IU/mg protein, determined as in [16]. The substrate curves were determined in the standard assay buffers, containing various concentrations of the respective substrates.

3. RESULTS AND DISCUSSION

The results of a typical purification procedure are presented in table 1. Remarkable is the 5-times lower specific activity of $\beta_1\beta_1$ compared to the other two isoenzymes. This observation agrees with the qualitative very low k_{cat} of $\beta\beta$ towards ethanol [9]. Therefore it seems that the γ_1-chain stabilized the β_1-chain in the $\beta_1\gamma_1$ heterodimer, since this form has a specific activity towards ethanol similar to that of $\gamma_1\gamma_1$.

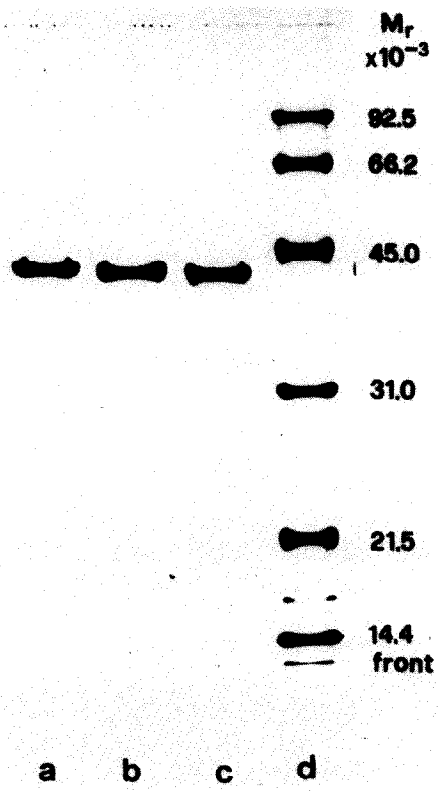

Fig.1. SDS gel electrophoresis of purified human liver ADH isoenzymes: (a) $\beta_1\beta_1$; (b) $\beta_1\gamma_1$; (c) $\gamma_1\gamma_1$; (d) Biorad Standard protein mixture; ~20 μg each isoenzyme was loaded.

Table 1

Purification of human liver alcohol dehydrogenase isoenzymes $\beta_1\beta_1$, $\beta_1\gamma_1$ and $\gamma_1\gamma_1$

	Total protein (mg)	Total activity (IU)	Spec. act. (IU/mg)	Yield (%)
Extract	120 050	7200	0.06	100
DEAE-pool	31 122	6240	0.20	87
CapGapp pool[a]	2579	3529	1.09	49
$\beta_1\beta_1$	97	34	0.35	0.5
$\beta_1\gamma_1$	147	275	1.87	3.8
$\gamma_1\gamma_1$	6.4	13	2.03	0.2

[a] Pool after 4-[3-(*N*-6-aminocaproyl)aminopropyl]-pyrazole-Sepharose chromatography

All 3 isolated isoenzymes showed a single band after SDS–polyacrylamide gel electrophoresis (fig.1). The app. M_r was 43 000 ± 1000, close to estimates on isoenzyme mixtures [8,17] and purified $\beta_1\beta_1$ [2].

The subunit composition of the isolated isoenzymes was determined by monomerization and hybridization with horse liver isoenzyme EE. Mixing EE with $\beta_1\beta_1$, $\beta_1\gamma_1$ or $\gamma_1\gamma_1$ always resulted in only 2 bands with electrophoretic mobilities characteristic for the respective forms (fig. 2b,d,3a). No con-

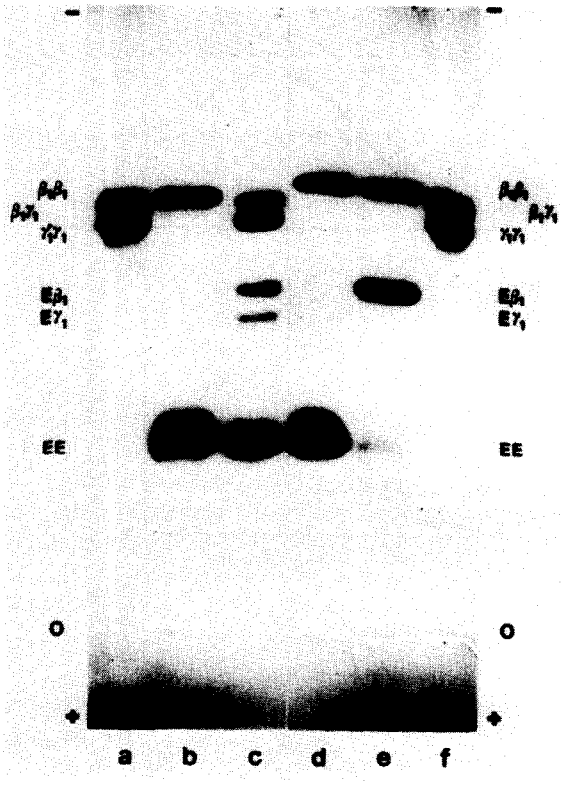

Fig.2. Starch gel electrophoresis of monomerized and hybridized horse liver isoenzyme EE and human liver isoenzymes $\beta_1\gamma_1$ and $\beta_1\beta_1$. Mixture of $\beta_1\beta_1$, $\beta_1\gamma_1$ and $\gamma_1\gamma_1$ without monomerization (lanes a,f). Mixture of EE and $\beta_1\gamma_1$ before monomerization (lane b) and after monomerization and hybridization (lane c). Mixture of EE and $\beta_1\beta_1$ before monomerization (lane d) and after monomerization and hybridization (lane e).

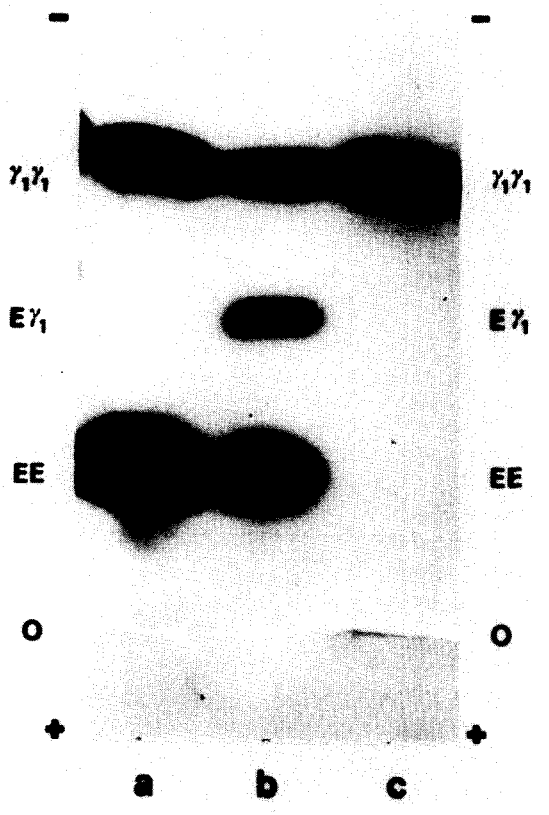

Fig.3. Starch gel electrophoresis of monomerized and hybridized horse liver isoenzyme EE with human liver isoenzyme $\gamma_1\gamma_1$. Purified $\gamma_1\gamma_1$ (lane c). Mixture of EE and $\gamma_1\gamma_1$ before monomerization (lane a) and after monomerization and hybridization (lane b).

taminating isoenzyme bands were detectable. Monomerization and hybridization of EE and $\beta_1\gamma_1$ resulted in 2 additional bands positioned between EE and $\gamma_1\gamma_1$, i.e., the hybrid enzymes Eβ_1 and Eγ_1 (fig.2c). $\beta_1\beta_1$ which also should have formed could not be detected, presumably because of its low specific activity. Hybridization of EE with $\beta_1\beta_1$ resulted in only one additional band with the mobility of the hybrid enzyme Eβ_1 (fig.2e). Hybridization of EE with $\gamma_1\gamma_1$ produced an additional band with the mobility of Eγ_1 (fig.3b).

All 3 isoenzymes exhibited pH enzyme activity curves similar to that reported for the mixture of normal human liver ADH isoenzymes [8,18] with a maximum at pH 10.5 and a shoulder around pH 7.0 (not shown). The pH 10.5:7.0 ratio of $\gamma_1\gamma_1$ and $\beta_1\gamma_1$ was 4 and 3.4, respectively, that of $\beta_1\beta_1$ only 1.6, reflecting the lower specific activity of this form at pH 10.5 (table 1, [11]).

Fig.4 shows the substrate rate profiles at pH 10.5 and 7.0 with ethanol, *n*-pentanol, cyclohexanol and benzylalcohol, representing linear, cyclic and aromatic alcohols. Ethanol, *n*-pentanol and cyclohexanol showed considerable substrate inhibition. Benzylalcohol showed no substrate inhibition and had by far the lowest concentration at apparent half-maximal velocity. Since the substrate rate profiles deviate substantially from linearity when plot-

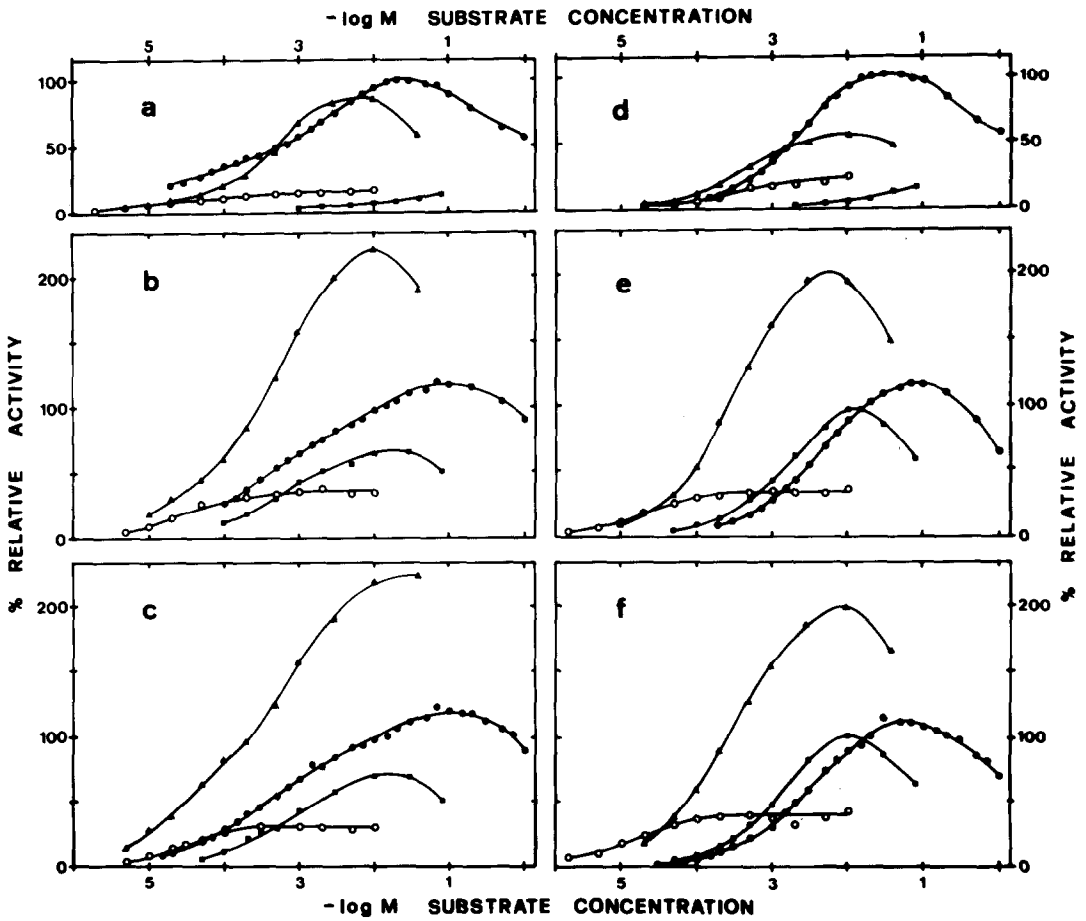

Fig.4. Substrate rate profiles of human liver isoenzymes $\beta_1\beta_1$ (a,d), $\beta_1\gamma_1$ (b,e) and $\gamma_1\gamma_1$ (c,f) with ethanol (•—•), *n*-pentanol (▲—▲), cyclohexanol (■—■) and benzylalcohol (○—○) at pH 7.0 (a,b,c) and pH 10.5 (d,e,f). All points are average values of 2–6 measurements. The activity is plotted relative to 16.7 mM ethanol at the respective pH-value.

ted according to Lineweaver-Burk, only the substrate concentrations at apparent half-maximal velocity are listed in table 2. Large differences in the capacity to oxidize these substrates as well as in pH-dependence were found. $\beta_1\beta_1$ could not be saturated with cyclohexanol within the range of solubility in accordance with a very high K_m for this isoenzyme/substrate pair [9]. Both γ_1-containing isoenzymes exhibited a higher apparent maximal velocity than $\beta_1\beta_1$ with *n*-pentanol in keeping with the observation that γ-containing isoenzymes showed faster activity staining with *n*-pentanol than with ethanol after starch gel electrophoresis [4,19]. $\beta_1\beta_1$ exhibits faster activity staining with ethanol than with *n*-pentanol [4,19] in accordance with the higher apparent maximal velocity of this isoenzyme at pH 10.5 (fig.4d).

Increasing hydrophobicity of the substrate resulted in a decrease of the substrate concentration at apparent half-maximal velocity (table 2) in agreement with the results obtained with the mixture of isoenzymes and various linear alcohols [8]. In addition, at pH 7.0 the concentrations of ethanol and cyclohexanol at apparent half-maximal velocity were lower than at pH 10.5 in contrast to the other, more hydrophobic substrates, which exhibited increased values.

Conventionally purified $\beta_1\beta_1$ does not obey Mi-

Table 2

Substrate concentration (mM) at apparent half-maximal velocity and of human liver alcohol dehydrogenase isoenzymes $\beta_1\beta_1$, $\beta_1\gamma_1$ and $\gamma_1\gamma_1$

pH	Isoenzyme	Ethanol	*n*-Pentanol	Cyclohexanol	Benzylalcohol
7.0	$\beta_1\beta_1$	0.55	0.39	[a]	0.032
	$\beta_1\gamma_1$	0.77	0.38	0.58	0.024
	$\gamma_1\gamma_1$	0.80	0.33	0.60	0.024
10.5	$\beta_1\beta_1$	1.70	0.38	[a]	0.027
	$\beta_1\gamma_1$	3.40	0.27	1.30	0.018
	$\gamma_1\gamma_1$	2.70	0.25	1.00	0.011

[a] Saturation of $\beta_1\beta_1$ with cyclohexanol could not be reached due to insolubility of the substrate. K_m-values estimated from Lineweaver-Burk plots over 20–80 mM were 60 mM at pH 7.0 and 86 mM at pH 10.5

chaelis–Menten kinetics when measured over a sufficiently wide ethanol concentration range [20]. We observed the same kinetic deviations not only with $\beta_1\beta_1$, but also with $\gamma_1\gamma_1$ and $\beta_1\gamma_1$.

It has been suggested that the kinetic properties of ADH heterodimers are not the simple average of the corresponding homodimers and that therefore subunit interaction has to be considered [9,21]. Our data obtained with $\beta_1\beta_1$, $\beta_1\gamma_1$ and $\gamma_1\gamma_1$ would support this interpretation.

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