Atypical human liver alcohol dehydrogenase: the β_2-Bern subunit has an amino acid exchange that is identical to the one in the β_2-Oriental chain

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The 'atypical' human liver alcohol dehydrogenase dimer, homogeneous for β_2-Bern chains, was isolated from human liver of Caucasian individuals. It is derived from an allelic variant at the ADH_2 gene locus and exhibits a considerably higher specific activity and lower pH optimum than its 'typical' counterpart (isoenzyme $\beta_1\beta_1$) from the β_1-chain predominant in Caucasians. Peptides were prepared by trypsin or CNBr cleavage, and were purified by exclusion chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC). Structural analysis of the peptides showed that β_2-Bern differs at one position from β_1. Thus, Arg-47 in β_1 is substituted by His in β_2-Bern. This exchange, compatible with a one-base mutation, explains all functional differences by altered interactions with the pyrophosphate moiety of the coenzyme. The difference is also structurally identical to that found for another atypical β_2-subunit, the β_2-Oriental type of major Asian occurrence, linking these two atypical forms of human alcohol dehydrogenase.

Alcohol dehydrogenase *Human liver isoenzyme* *Primary structure* *Mutation*

1. INTRODUCTION

A large number of pyrazole-sensitive isoenzymes (class I isoenzymes [1]) have been demonstrated for human liver alcohol dehydrogenase (ADH, EC 1.1.1.1). The subunits α, β and γ are coded for by 3 different gene loci, ADH_1, ADH_2 and ADH_3, respectively, and combine to form the various dimeric enzymes [2]. Allelic variants are known both for the ADH_2 locus (coding for β_1 and possibly several β_2 polypeptides [2]); and for the ADH_3 locus (coding for the γ_1 and γ_2 subunits [2]). The 'atypical' human liver alcohol dehydrogenase of Caucasian origin [3] is such a variant from the ADH_2 locus [2] and the corresponding protein chain has been called the β_2-Bern subunit. Isoenzymes with β_2-Oriental subunits, isolated from livers of Oriental origin, have also been described [4,5], as well as forms with $\beta_{\text{Indianapolis}}$ chains from livers of African ancestry [6].

Catalytic and functional properties of β_2-Bern and β_2-Oriental are highly similar and 'atypical' in relation to the β_1-type [7–9]. Thus, alcohol dehydrogenase preparations containing either of these two atypical β_2-subunits distinctly differ in enzymatic properties from other preparations. The specific activity is considerably higher and the pH-optimum for ethanol oxidation is shifted from the normal pH around 10.5 down to about pH 8 [7,9]. Also, the susceptibility to inhibitors is significantly altered [3].

The β_2-Bern difference was earlier suggested to be an exchange of Ala-230 in β_1 for Pro [10]. Similarly, β_2-Oriental was later suggested unexpectedly to be the end result of changes of two residues, involving a one-position migration of the active-site zinc ligand Cys-46 [4]. Both the β_2-Bern [3] and β_2-Oriental [4] subunits are now known to

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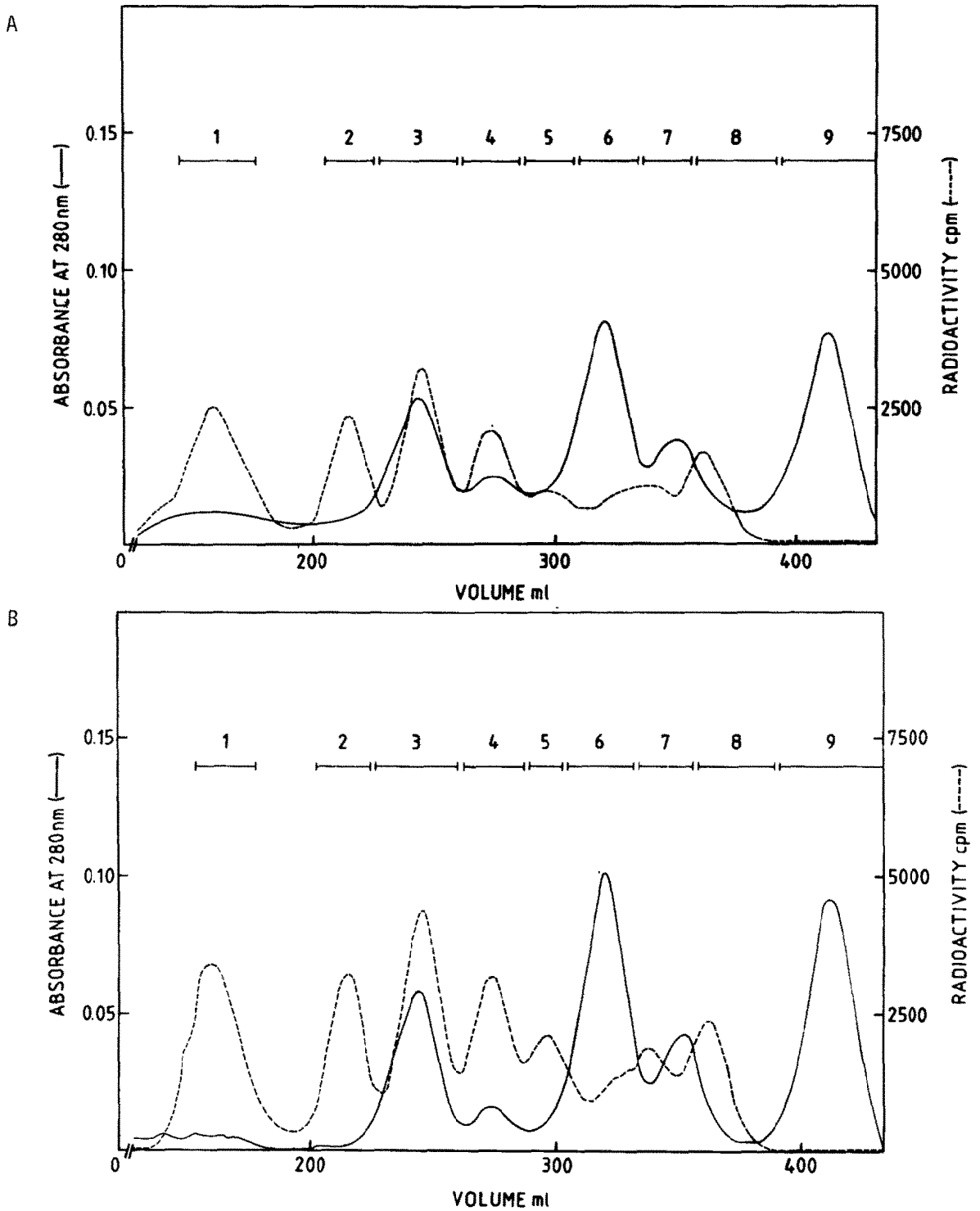

Fig.1. Sephadex G-50 chromatography of tryptic peptides of β_2-Bern (A) and β_1 (B). Horizontal bars show the fractions pooled for further purification by RP-HPLC. The β_1 digest is identical to that shown before in relation to β/γ differences [24] but is now compared to the β_2-Bern pattern instead of the γ pattern previously investigated [24]. A difference in relative ratios of radioactivity for pools 1 and 5 is noticed between the β_1 and β_2-Bern digests, and is explained by the different tryptic cleavages because of the Arg difference at position 47, giving an extra ^{14}C-labelled pool 5 tryptic peptide in β_1 relative to β_2-Bern.

have structures other than those initially suggested. Thus, β_2-Oriental has recently been shown to be derived from a single Arg/His exchange at position 47, which explains all deviating properties [5], and is compatible with the very first observations on the position of the active site cysteine residue in human alcohol dehydrogenase [11]. Similarly, the β_2-Bern difference is presently established, showing it to be different from the previously suggested Ala/Pro exchange [10] but instead identical to the exchange in β_2-Oriental [5]. It therefore appears that β_2-Bern and β_2-Oriental are identical subunits which have resulted from similar mutations or even the same mutational event, despite different gene frequencies in various populations [7,8], and despite isolation from different racial groups.

The results of the investigations of β_2-Bern subunits are now reported. Only one difference was found: Arg-47 in β_1 was substituted by His-47 in β_2-Bern. This is compatible with a single-base mutation. No evidence was found for the previously described substitution of Ala-230 in β_1 by Pro in β_2-Bern [10]. The Arg/His exchange affects the general anion binding site at position 47 that binds the pyrophosphate of the coenzyme [12–14]. NAD is less tightly bound to the His-containing anion binding site, explaining the higher turnover rate of the atypical enzyme, since dissociation of the reduced coenzyme is the rate-limiting step.

2. MATERIALS AND METHODS

2.1. *Purification of human liver alcohol dehydrogenases with β_2-Bern chains and of enzyme with β_1 chains*

Caucasian human livers of normal and atypical phenotype were obtained from autopsies, frozen 10–20 h after death, and stored at −20°C. The mixture of the pyrazole-sensitive class I [1] isoenzymes from livers with normal ADH phenotype was isolated by double ternary complex affinity chromatography as in [15,16], this step being performed at pH 8. When livers of atypical ADH phenotype were used as enzyme source, the affinity chromatography was performed at pH 9. Homogeneous isoenzymes were isolated by subsequent ion exchange chromatography on CM–cellulose CM-52 [16].

Purity of the isoenzymes was tested by SDS-gel electrophoresis and by starch gel electrophoresis [2]. Isoenzymes were also identified by their electrophoretic mobilities [2] and their subunit composition verified by monomerization and hybridization with the horse liver isoenzyme EE followed by starch gel electrophoresis [16,17].

2.2. *Peptide preparation*

After CM–cellulose chromatography, pools containing the dimeric isoenzymes $\beta_1\beta_1$ and $\beta_2\beta_2$-Bern were dialyzed extensively against distilled water and lyophilized. After reduction and ^{14}C-carboxymethylation [18], the enzyme proteins were cleaved with trypsin or CNBr. Peptides obtained were fractionated on Sephadex G-50 and further purified by RP-HPLC [19]. The isolated peptides were identified and checked for purity by analysis of total compositions and end groups.

2.3. *Structural analysis*

Manual sequence degradations were performed by the DABITC–Edman method [20] utilizing byproducts to assist the identifications [21]. Liquid phase sequencer degradataions were performed in a Beckman 890D sequencer with a 0.1 M Quadrol peptide program and with glycine-precycled polybrene as carrier [22]. Phenylthiohydantoin (PTH) derivatives were analyzed by RP-HPLC [23], supplemented at the positions corresponding to the exchanged residue by amino acid analysis after back hydrolysis in 6 M HCl, containing 0.1% $SnCl_2$, for independent residue confirmation. The presence of carboxymethylcysteine was also monitored by radioactivity measurements.

3. RESULTS

The ^{14}C-carboxymethylated protein chains β_2-Bern and β_1 were digested with trypsin. Resulting peptides were separated into different size-groups by chromatography on Sephadex G-50 in the same way as when the differences in the γ subunit were characterized [24]. Fig.1 shows the elution profiles of tryptic digests of the β_2-Bern (fig.1A) and β_1 (fig.1B) forms. Both are highly similar. However, the ratios of radioactivity between pools 1 and 5 were significantly different. Thus, pool 5 was considerably more radioactive in the β_1 digest and pool 1 slightly more in the β_2-Bern digest. Purification of the peptides and subsequent structural analysis revealed that pool 1

from β_2-Bern chains had a peptide covering positions 40–88. The corresponding peptide in pool 1 from β_1 chains was 8 residues shorter in the N-terminal part, covering only positions 48–88. The missing segment (positions 40–47) was recovered in pool 5 of the β_1 digest as a separate peptide absent in the β_2 digest. The amino acid compositions of all these deviating peptides are given in table 1 and the sequence data in table 2. The peptides are explained by one difference: Arg-47 of β_1 chains (residue 8 of the β_1 pool 1 tryptic peptide, table 2) is substituted by His in β_2-Bern chains. This leads to an additional tryptic fragment (residues 40–47) in the β_1 digest, because of cleavage at the arginine. All other investigated tryptic peptides of β_2-Bern (total compositions checked for all tryptic peptides, covering the entire protein chain; sequence tions 40–88. The corresponding peptide in pool 1 analysis performed for 28 of the tryptic peptides, covering a total of 256 positions) were identical to those of β_1.

Additional information on the substituted position was obtained from CNBr fragments. The largest CNBr fragments from both β_2-Bern and β_1 chains covered positions 41–208. The data for these fragments are also given in tables 1 and 2, further confirming the His/Arg difference. Thus, the residue in cycle 7 of the β_2-Bern CNBr fragment was His, corresponding to position 47 in the intact protein. The identification was further supported by amino acid analysis after back-hydrolysis of the PTH derivatives. It can be concluded that the atypical β_2-Bern subunit differs from the β_1 subunit at one position, Arg-47 in the β_1 chain is substituted by His-47 in the β_2-Bern form.

Table 1

^{sn}Amino acid compositions of peptides covering the position at which β_2-Bern differs from β_1

	Tryptic peptides			CNBr fragments	
Subunit	β_2-Bern	β_1		β_2-Bern	β_1
Position	40–88	40–47	48–88	41–208	41–208
Cys	0.4 (1)	0.9 (1)	– –	8.2 (9)	7.7 (9)
Asx	4.0 (4)	– –	3.3 (4)	16.1 (14)	14.3 (14)
Thr	3.7 (4)	– –	3.1 (4)	13.1 (14)	12.2 (14)
Ser	2.1 (2)	– –	2.3 (2)	9.3 (10)	8.5 (10)
Glx	3.1 (3)	– –	3.0 (3)	10.9 (9)	9.7 (9)
Pro	3.0 (3)	– –	3.2 (3)	10.4 (10)	9.8 (10)
Gly	7.8 (7)	1.1 (1)	6.1 (6)	22.4 (22)	21.5 (22)
Ala	3.0 (3)	1.0 (1)	1.8 (2)	12.7 (11)	11.2 (11)
Val	9.4 (10)	2.0 (2)	6.6 (8)	20.1 (22)	20.9 (22)
Met	0.7 (1)	0.9 (1)	– –	0.4 (1)	0.2 (1)
Ile	2.0 (3)	1.0 (1)	1.6 (2)	7.1 (7)	6.7 (7)
Leu	3.1 (3)	– –	3.0 (3)	11.5 (12)	11.8 (12)
Tyr	– –	– –	– –	3.2 (3)	3.1 (3)
Phe	– –	– –	– –	6.3 (6)	6.3 (6)
Lys	1.6 (2)	– –	1.7 (2)	9.4 (9)	8.7 (9)
His	1.7 (3)	– –	1.7 (2)	4.7 (5)	3.6 (4)
Arg	– –	1.0 (1)	– –	4.8 (4)	5.4 (5)
Sum	49	8	41	168	168

Values shown are molar ratios without corrections for impurities, slow release or destruction; and within parentheses, the values from sequence analysis. β_1 has an Arg at position 47 and therefore yields two tryptic peptides, as shown. Low recoveries of Val and Ile are due to the occurrence of Val–Val and Val–Ile structures slowly hydrolyzed. Met was determined as Hse in low yield in the CNBr fragments. Data for β_1 are from [19]

Table 2

Results of sequence analysis of the peptides containing the amino acid exchange in β_2-Bern

Position in β_2-Bern (from homology with β_1)	Cycle in degradation of tryptic peptide 40–88	Residue	Cycle in degradation of CNBr fragment 41–208	Residue
40	1	Met HT 12		
41	2	Val HT 9	1	Val HT 35
42	3	Ala HT 7	2	Ala HT 30
43	4	Val HT 9	3	Val HT 25
44	5	Gly HT 9	4	Gly HT 25
45	6	Ile HT 5	5	Ile HT 30
46	7	Cys HR 8	6	Cys HR 20
47	8	His H 5	7	His HB 20
48	9	Thr H 6	8	Thr HT 25
49	10	Asp H 5	9	Asp HT 20
50	11	Asp H 4	10	Asp HT 25
51	12	His H 6	11	His HB 15
52	13	Val H 3	12	Val HT 20
53	14	Val H 3	13	Val HT 20
54	15	Ser H 3	14	Ser HT 15
55	16	Gly H 1	15	Gly HT 10
56	17	Asn H 3	16	Asn HT 15
57	18	Leu H 2	17	Leu HT 15
58			18	Val HT 15
59			19	Thr HT 10
60			20	Pro H 10

Degradations were performed in a liquid phase sequencer. Residues were identified by HPLC (H), thin-layer chromatography (T), back hydrolysis with $HCl/SnCl_2$ (B), and radioactivity (R). Values show nmol recovered from degradataion of 40 nmol CNBr fragment and 20 nmol tryptic fragment. Positions in the whole protein chain are evident from homology with the horse enzyme and from independent analysis of the β_1 and γ_1 human enzymes. As shown, cysteine (identified as carboxymethylcysteine) is at position 46 and histidine at position 47

4. DISCUSSION

Structural differences between the human alcohol dehydrogenase subunit β_1 and the 'atypical' variant β_2 have been reported. A single-residue Pro/Ala exchange at position 230 was concluded from the apparent presence of the tripeptide Phe-Ala-Lys after tryptic digestion of the β_1 protein chain but Phe-Pro-Lys after digestion of the β_2-Bern form [10]. Another study [4] did not find this difference in the β_2-Oriental subunit but suggested a successive, two-step substitution involving two amino acids at the active site (an inversion of residues Cys-46––Arg-47 of β_1 to Arg-46––Cys-47, and an exchange of Arg by His in β_2-Oriental). However, such an inversion in the β_1 structure was incompatible with the known position of the active site cysteine residue [11], and recently it has been unambiguously shown that the β_2-Oriental difference only involves one amino acid exchange at the active site, i.e., Arg-47 in the β_1 protein chain is substituted by His-47 in the β_2-Oriental form [5]. We establish here that the β_2-Bern subunit also has His-47 and not Arg-47. Therefore, the β_2-Bern protein chain derived from livers of Caucasian origin is identical in primary structure to the β_2-Oriental chain from livers of Oriental origin but its occurrence is much less fre-

quent in Caucasian than in Oriental livers [7]. Based on the total compositions of all other tryptic peptides, we could not detect other differences. In particular, we did not find a tripeptide Phe-Pro-Lys (previously suggested for β_2-Bern [10]), but detected only the 'typical' tripeptide containing Ala-230, as in the β_1 form. It appears possible that the previously reported tripeptide [10], if significant, may be derived from still another related protein chain, not yet structurally characterized (like α or other alcohol dehydrogenase subunits).

The evolutionary implications of the Arg/His-47 exchange in the β_2-Oriental chain [5] therefore apply also to the β_2-Bern chain. Thus, two different lines of alcohol dehydrogenase appeared early in evolution, leading to the mammalian enzymes containing Arg-47, and the yeast enzymes containing His-47. Later, at least two types of mutations occurred independently: in man, of Arg-47 to His-47 in the β_2-Bern/β_2-Oriental forms, and in yeast of His-47 to Arg-47 in a mutant strain (S-AA-5) [5,25]. It appears likely that the mutation giving rise to β_2-Bern chains is a reflection of the same event as the one giving rise to β_2-Oriental chains and that the present relative differences in apparent β_2 protein amount only reflect different gene frequencies, apart from possibly different transcriptional control in the corresponding gene of different racial origin (Caucasian/Oriental). However, the possibility still exists, although it is considered less likely, that the two atypical β_2 chains reported are derived from independent mutations that similarly affect identical positions.

The Arg/His-47 substitution between β_1/β_2-Bern subunits is fully compatible with the observed differences in catalytic activities, including the pH optima [7,8]. Thus, the interaction of the pyrophosphate moiety of the coenzyme with the anion binding site [12–14] is expected to be much stronger with the Arg in the β_1 protein chain than with the His in the β_2-Bern form. Since the NADH dissociation from the enzyme is the rate-limiting step during catalysis [26], this would lead to a higher turnover number because of less tightly bound NADH, as is indeed observed both for β_2-Bern and β_2-Oriental forms [7,8]. The normal yeast enzymes with His also have a higher turnover number than the mutant yeast enzyme [27] with Arg, supporting the conclusions about the functional role of position 47. Similarly, the lower pH-optimum for the enzymatic activity of enzyme forms with β_2-Bern subunits relative to those with β_1 subunits [7,8] can be explained by the different pK_a values of the guanidine and imidazole groups. Thus, all properties are explained, and β_2-Bern and β_2-Oriental protein chains are shown to be structurally and functionally identical.

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