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# Acetylated N-terminal structures of class III alcohol dehydrogenases

## Differences among the three enzyme classes

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The protein chains of mammalian alcohol dehydrogenases typically lack free α-amino groups. The blocked N-terminal regions of the class III type of the rat (ADH-2), human (χχ) and horse enzymes were isolated by digestions with proteases, and characterized by mass-spectrometry supplemented with chemical analysis of the peptides and their redigestion fragments. Results were confirmed by synthesis of the corresponding peptides, followed by chromatographic comparisons of the native and synthetic products. The N-terminal regions of the three class III alcohol dehydrogenase subunits are homologous but differ from the class I and II enzymes in both the exact start position and the amino acid sequence, which suggests that different N-terminal structures are typical for each of the three classes.

Alcohol dehydrogenase; Isozyme; Acetylation; Sequence homology; Amino acid sequence; N-terminal analysis

## 1. INTRODUCTION

Most alcohol dehydrogenases characterized so far have acetyl-blocked N-terminal residues [1]. This chemical modification is a feature common to many different types of protein [2], including several dehydrogenases [3]. Among the mammalian alcohol dehydrogenases which have been characterized, those of class I in liver have an *N*-acetylated serine residue [2]. However, the human liver contains three different classes of alcohol dehydrogenase, I, II, and III [4], and recent determination of the class II structure suggests that it may deviate from that of the class I pattern by virtue of acetyl-Gly [5].

The class III enzyme [6-9] has now been examined from human (χχ enzyme), horse, and rat liver (ADH-2). The N-terminal structures differ from those of both the class I and II enzymes characterized previously. The class III enzymes start at another position, have other constituent residues, and in two cases a different acetylated residue. Chromatographic properties of several synthetic peptides with structures related to the N-terminal region of the class III enzymes have also been compared.

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## 2. MATERIALS AND METHODS

Rat alcohol dehydrogenase ADH-2 [9], and

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human [6,7] and horse [8] $\chi\chi$ alcohol dehydrogenases were reduced, carboxymethylated and cleaved in different batches with *Achromobacter* Lys-specific protease, staphylococcal Glu-specific protease, trypsin and CNBr [10,11]. The peptides from the original N-termini were identified from compositions, lack of free $\alpha$-amino groups, and chromatographic properties [10,11]. They were sub-digested, as given in section 3, and the blocked peptides were purified by reverse-phase high-performance liquid chromatography on an Ultropac TSK ODS 120-T column (4.6 × 250 mm) utilizing a linear gradient of acetonitrile in 0.1% trifluoroacetic acid.

Total compositions were determined with a Beckman 121M analyzer after hydrolysis for 24 h at 110°C with 6 M HCl/0.5% phenol. Primary structures of non-blocked peptides were analyzed by degradations with an Applied Biosystems 470A gas-phase sequencer and reverse-phase high-performance liquid chromatography of phenylthiohydantoin derivatives in a Hewlett Packard 1090 instrument with a 5 $\mu$m Nucleosil C18 column and gradients of acetonitrile in 2 mM sodium acetate [10]. Mass-spectrometric analysis was performed as in [3]. Peptide replicates were synthesized chemically by the Merrifield solid-phase method [12], using a Beckman 990B peptide synthesizer. After cleavage and deprotection, the peptides were purified by high-performance liquid chromatography as for the native fragments. The correct structure of the peptides was verified by determination of their total compositions and chromatographic properties.

## 3. RESULTS

Human and horse liver $\chi\chi$ alcohol dehydrogenases of class III and the rat liver enzyme of the same class (ADH-2) were purified, carboxymethylated, and submitted to proteolytic treatment as described [10,11]. The blocked peptides containing the original N-termini were identified from chromatographic positions, total compositions, and lack of free $\alpha$-amino groups. In this manner, a total of eight different blocked peptides from the three alcohol dehydrogenases were purified (four from the rat protein, three from the human protein, and one from the horse protein, generated by the various proteolytic treatments).

A tryptic, blocked hexapeptide was purified from each of the three proteins, Hor-T1 from the horse protein, Rat-T1 from the rat protein and Hum-K1 from the human protein (K for generation of the tryptic cleavage with the Lys-specific protease rather than with trypsin itself). These peptides were submitted to structural analysis by electron impact mass-spectrometry of the methyl ester derivatives utilizing the solid inlet probe [3]. The total compositions of the peptides, together with that of a blocked redigestion fragment, are listed in table 1. The final structures deduced are given in table 2 (bottom).

The mass-spectrometric analysis of the blocked tryptic hexapeptide from horse (Hor-T1) produced fragments at *m/z* values of 58 (Ac-NH), 84 (Ac-Ser ($-H_2O$)), 130 (Ac-Ser), 159 (Lys-COOMe), 185 (Ac-Ser-Ala ($-H_2O$)) and 314 (Ac-Ser-Ala-Glu), identifying the N-terminal acetyl group, the blocked residue, and the next two residues, thus establishing the N-terminal structure of Hor-T1 to be acetyl-Ser-Ala-Glu- in agreement with the composition. Also present in the spectrum were peaks at *m/z* 258 (Ile-Lys-COOMe) and 357 (Val-Ile-Lys-COOMe), representing the C-terminal amino acid sequence of the peptide. Similar analysis of the blocked hexapeptide from both rat (Rat-T1) and human (Hum-K1) proteins failed to produce iden-

Table 1

Total compositions of the blocked tryptic peptides from the N-terminus of horse, rat and human liver alcohol dehydrogenase class III

| Peptide | Hor-T1 | Rat-T1 | Hum-K1 | Hum-E1 |
|---|---|---|---|---|
| Asx | – – | 1.1 (1) | 1.1 (1) | 0.9 (1) |
| Ser | 1.0 (1) | – – | – – | – – |
| Glx | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) |
| Ala | 1.1 (1) | 1.1 (1) | 1.1 (1) | 0.9 (1) |
| Val | 0.9 (1) | 0.8 (1) | 0.7 (1) | – – |
| Ile | 1.0 (1) | 0.7 (1) | 0.9 (1) | – – |
| Lys | 1.0 (1) | – – | 1.1 (1) | – – |
| Arg | – – | 1.3 (1) | – – | – – |
| Sum | – 6 | – 6 | – 6 | – 3 |

The peptides are Hor-T1 from the horse protein, Rat-T1 from the rat protein, and Hum-K1 from the human protein for which the blocked fragment obtained by redigestion with Glu-specific protease (Hum-E1) is also shown. Analytical values give molar ratios from acid hydrolysis, while the integers within parentheses show the values from the structure deduced (table 2)

Table 2

N-terminal structures of mammalian alcohol dehydrogenases

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Class I | Horse E | Ac | S | T | A | G | K | V | I | K |
| | Rat ADH-3 | Ac | S | T | A | G | K | V | I | K |
| | Human $\alpha$ | Ac | S | T | A | G | K | V | I | K |
| | Human $\beta$ | Ac | S | T | A | G | K | V | I | K |
| | Human $\gamma$ | Ac | S | T | A | G | K | V | I | K |
| Class II | Human $\pi$ | (Ac) | G | T | K | G | K | V | I | K |
| Class III | Horse $\alpha$ | | | Ac | S | A | E | V | I | K |
| | Human $\alpha$ | | | Ac | A | N | E | V | I | K |
| | Rat ADH-2 | | | Ac | A | N | Q | V | I | R |

All class I, II, and III polypeptide structures characterized at the protein level are included, class I and II from [5,13,14] and class III (bottom) from the present analyses. In each case, the protein chain has been shown to lack a free $\alpha$-amino group. In the $\pi$-subunit, the acetyl group (Ac) has been indirectly analyzed and is therefore given within parentheses

tifiable fragments except one at $m/z$ 114 (interpreted as Ac-Ala-), showing the acetyl group and suggesting the N-terminal residue to be Ala. This fragment was clearest from the rat peptide since the amount of the human peptide was very small (< 5 nmol). Presumably a low volatility of Rat-T1 and Hum-K1, because of the presence of amide groups (table 2), explains the lack of recovery of further fragments.

Attempts at digestion of Rat-T1 with the Glu-specific protease did not result in cleavage, whereas Hum-K1 was cleaved, showing that in the rat peptide the Glx residue in the total compositions (table 1) is likely to be Gln, while it is Glu in the human one. Lack of pyro-Glu as the N-terminal blocking group was confirmed by tests with pyroglutamic aminopeptidase (Boehringer-Mannheim, FRG), which failed to cleave either one of the native peptides, but hydrolyzed pyroglutamic acid blocked synthetic peptides. The remaining structures of the blocked hexapeptides were assigned tentatively, based on isolation of the fragments from cleavage of the human peptide with the Glu-specific protease. Fragment Hum-E1 (table 1) then contains the original residues 1–3 and is blocked, while another fragment starts with Val at position 4 and was analyzed by sequencer degradations proving the order of residues 4–6 of the human protein to be as given in table 2.

Combined, the mass-spectrometric and chemical analyses suggest that the structure of the horse blocked hexapeptide from the original N-terminus is acetyl-Ser-Ala-Glu-Val-Ile-Lys, that of the human blocked hexapeptide acetyl-Ala-Asx-Glu-Val-Ile-Lys, and that of the corresponding rat peptide acetyl-Ala-(Asx,Gln,Val,Ile)-Arg, where the orders within the parentheses are likely to be as given, if the rat, human and horse peptides are maximally homologous.

In order to prove these three structures, ten blocked hexapeptides corresponding to the possible alternatives with these compositions were synthesized chemically (structures in fig.1) by the Merrifield solid-phase method [12]. The synthetic peptides were submitted to reverse-phase high-performance liquid chromatography in three systems. Two of them utilized Ultropac TSK ODS 120-T and acetonitrile gradients in 0.1% trifluoroacetic acid (fig.1), the third a Dupont Bioseries PEP-RPI column with an acetronitrile gradient in 0.1% trifluoroacetic acid/0.1% triethylamine. The ten synthetic peptides eluted at different positions.

Chromatography of the native peptides demonstrated that they coincided in elution position with one synthetic peptide each. The native peptide from the rat enzyme (Rat-T1) eluted at the position of the synthetic peptide acetyl-Ala-Asn-Gln-Val-Ile-Arg (fig.1A), that from the human enzyme (Hum-K1) at the position of the synthetic peptide acetyl-Ala-Asn-Glu-Val-Ile-Lys (fig.1B), and that from the horse enzyme (Hor-T1) at the

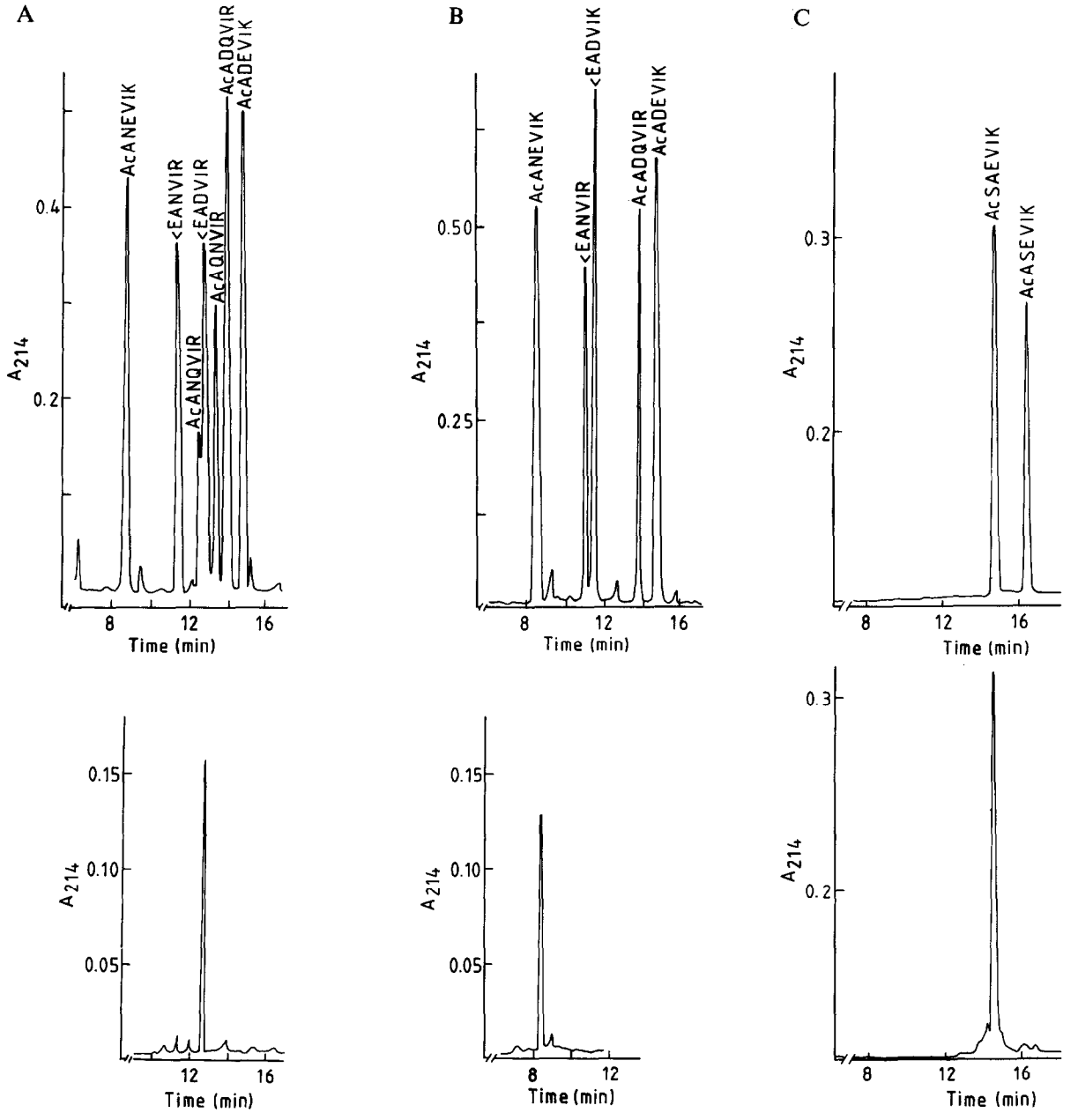


Fig.1. Chromatographic properties of synthetic peptides (top) and the native (bottom), tryptic, blocked N-terminal peptide of rat (A), human (B), and horse (C) alcohol dehydrogenases of class III. <E indicates a residue of pyroglutamic acid. Column: Ultropac TSK ODS 120-T. Elution: 0.1% trifluoroacetic acid with a gradient of acetonitrile (15-25% in 25 min for A, and 15-25% in 15 min for B and C). In each case, the elution position of the native peptide corresponds to the position of one of the synthetic peptides.

position of the synthetic peptide acetyl-Ser-Ala-Glu-Val-Ile-Lys (fig.1C). The native peptides are concluded to correspond to these structures, in agreement with the tentative assignments above from the mass-spectrometric and chemical analyses. The structure explain why the Glu-specific protease cleaves the human peptide (with Glu) but not the rat peptide (without Glu). The in-

dependent identification of the horse, human, and rat peptides, co-eluting with one synthetic peptide each, proves that Hor-T1, Hum-K1, and Rat-T1 are homologous, starting with blocked residues at equivalent positions (table 2).

## 4. DISCUSSION

Analysis by mass-spectrometry, total composition, and sensitivity to proteolytic enzymes show that the subunits of the class III alcohol dehydrogenases from human, horse, and rat liver are acetylated and have related N-terminal structures (table 2).

The N-terminal structures are now known for all three classes of alcohol dehydrogenase in mammalian liver. Thus, enzymes of class I have been analyzed from horse, rat, and human liver [13,14], class II enzymes from human liver [5], and now class III enzymes from human, horse and rat liver. In all instances, the N-terminal structures characterized are blocked by N-terminal acetylation. Thus, the mammalian alcohol dehydrogenases in general can be inferred to belong to the large group of N-terminally acetylated proteins [2], a native modification known to be common in several other dehydrogenases [3].

In spite of the common feature of acetylation, the patterns of the three classes of alcohol dehydrogenase differ in two ways (table 2). First, Ser is the residue acetylated in all class I enzymes characterized, while Gly, Ala or Ser are the corresponding residues in the class II and III enzymes. Moreover, alignment of the structures shows that the N-terminal residue of class III enzymes is at a postion corresponding to residue 3 of the class I and II forms. Thus, the N-terminal patterns of the three classes of alcohol dehydrogenase in mammalian liver differ typically compatible with the suggestion that the three classes have evolved into separate enzymes rather than merely into isozymes [15].

Finally, it is noted that all three classes of enzyme, although with different N-terminal patterns, still have acetylated segments typical of those of proteins in general. Thus, Ser, Gly and Ala are commonly encountered in other acetylated proteins [2], strengthening the conclusion of some common features [16] of N-terminal modifications of protein structures.

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## REFERENCES

[1] Jörnvall, H., Persson, B. and Jeffery, J. (1987) Eur. J. Biochem., in press.
[2] Persson, B., Flinta, C., Von Heijne, G. and Jörnvall, H. (1985) Eur. J. Biochem. 152, 523–527.
[3] Fairwell, T., Krutzsch, H., Hempel, J. Jeffery, J. and Jörnvall, H. (1984) FEBS Lett. 170, 281–289.
[4] Vallee, B.L. and Bazzone, T.J. (1983) Isoenzymes: Curr. Top. Biol. Med. Res. 8, 219–244.
[5] Höög, J.-O., von Bahr-Lindström, H., Hedén, L.-O., Holmquist, B., Larsson, K., Hempel, J., Vallee, B.L. and Jörnvall, H. (1987) Biochemistry 26, 1926–1932.
[6] Parés, X. and Vallee, B.L. (1981) Biochem. Biophys. Res. Commun. 98, 122–130.
[7] Wagner, F.W., Parés, X., Holmquist, B. and Vallee, B.L. (1984) Biochemistry 23, 2193–2199.
[8] Dafeldecker, W.P. and Vallee, B.L. (1982) J. Protein Chem. 1, 59–69.
[9] Julià, P., Farrés, J. and Parés, X. (1987) Eur. J. Biochem. 162, 179–189.
[10] Kaiser, R., Holmquist, B., Hempel, J., Vallee, B.L. and Jörnvall, H. (1987) Biochemistry, in press.
[11] Julià, P., Parés, X. and Jörnvall, H. (1987) Eur. J. Biochem., in press.
[12] Merrifield, R.B. (1969) Adv. Enzymol. 32, 221–296.
[13] Brändén, C.-I., Jörnvall, H., Eklund, H. and Furugren, B. (1975) in: The Enzymes, 3rd edn. (Boyer, P. ed.) vol. 11, pp. 103–190, Academic Press, New York.
[14] Jörnvall, H., Hempel, J. and Vallee, B.L. (1987) Enzyme 37, 5–18.
[15] Jörnvall, H., Höög, J.-O., von Bahr-Lindström, H. and Vallee, B.L. (1987) Proc. Natl. Acad. Sci. USA 84, 2580–2584.
[16] Flinta, C., Persson, B., Jörnvall, H. and Von Heijne, G. (1986) Eur. J. Biochem. 154, 193–196.