PURIFICATION AND CHARACTERIZATION OF CHICKEN LIVER ALCOHOL DEHYDROGENASE

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1. Introduction

Liver alcohol dehydrogenase from several species has been isolated and thoroughly studied [1]. The structures of corresponding yeast [2], *Drosophila* [3] and bacillar [4] enzymes have also been investigated. All enzymes appear related [3–7], but differences are extensive and affect functionally important residues, subunit interactions and other structural characteristics [5,7]. In general, the most conserved regions involve areas around the active site, including the ligands to the catalytic zinc atom. The region around the 2nd zinc atom, however, is more variable [5,7] and its ligands may even be missing [3], or involved in transitions between different sub-forms of the enzyme [8].

The aim of the present work was to purify chicken liver alcohol dehydrogenase, and to study the relationship of this previously uncharacterized protein to other alcohol dehydrogenases. To prepare the enzyme, general ligand affinity chromatography on a Sepharose-bound AMP-analogue was used [9,10]. The structures around the cysteine residues are of special importance, since cysteine residues contribute 6 of the 7 zinc ligands in the horse enzyme [11] and occur in both the relatively constant and the more variable regions [7]. Therefore, the chicken enzyme was purified, its cysteine residues were radioactively labelled by carboxymethylation, and after tryptic digestion all small and medium-sized radioactive peptides were analyzed. It was found that the enzyme is distinctly similar to that from mammalian livers, and that it fits the general evolutionary pattern and functional properties of alcohol dehydrogenases suggested from studies of these proteins fron non-avian sources.

2. Materials and methods

Chicken livers (75 g) were homogenized (Ultra Turrax) in 160 ml, 0.1 M sodium phosphate buffer, pH 7.5. After centrifugation (39 000 × *g*, 1.5 h) and removal of the lipid layer, the crude extract (212 ml) was applied to a column (1.5 × 19 cm) of 5′-AMP–Sepharose 4B (Pharmacia, Uppsala), pre-equilibrated with the phosphate buffer. The column was washed, 9 ml/h flow rate, with the same buffer (400 ml) and the enzyme subsequently eluted (90 ml) with 2.5 mM NAD^+ in the buffer. After chromatography on Sephadex G-100 (Pharmacia, Uppsala), the preparation was re-applied to the affinity column, which had been regenerated with 10 ml, 10 mM NADH and re-equilibrated with the buffer. After washing the adsorbed enzyme with buffer alone (120 ml), and buffer plus 0.25 mM NAD^+ (112 ml), it was eluted with 72 ml buffer plus 0.25 mM NAD^+ and 5 mM pyrazole, and subsequently dialyzed against the phosphate buffer. All purification steps were performed at 4°C, ethanol dehydrogenase activity was measured in 0.1 M glycine–NaOH, pH 10 [12], and protein was determined as in [13]. Slab-gel electrophoresis in 10% polyacrylamide and 0.1% SDS was performed as in [14].

The enzyme was carboxymethylated, after reduction with dithiothreitol (0.08 mg/ml), by treatment with iodo[2-^{14}C]acetate (0.3 mg/ml protein) in

buffered 8 M urea, pH 8.1 [2]. It was then digested with TPCK-trypsin (1% by wt, Worthington Biochemical Corp.), after which peptides obtained were purified on paper (Whatman 3 MM) by high-voltage electrophoresis and chromatography [2]. Radioactivity was localized by autoradiography on Fuji Rx Medical film. Total compositions were determined on a Beckman 121 M amino acid analyzer after hydrolysis in evacuated tubes with 6 M HCl/0.5% phenol at 110°C for 24 h (peptides) and for 20 h, 48 h and 72 h (the protein). In the latter case, values for serine and threonine were extrapolated to zero time, and the amounts of valine and isoleucine were taken as the 72 h values. End-groups and amino acid sequences were determined by the dansyl-Edman technique [2].

3. Results

3.1. *Purification*

Alcohol dehydrogenase from crude extracts of chicken livers was purified using 2 steps of AMP–Sepharose chromatography. The affinity-adsorbed enzyme was eluted by complex formation with NAD^+ or with NAD^+ plus pyrazole, using a technique found to be efficient for the horse enzyme [9]. Table 1 summarizes the purification. In the second affinity step the adsorbed alcohol dehydrogenase was washed with a low concentration of NAD^+, 0.25 mM, to remove other weakly-bound pyridine nucleotide-dependent proteins. A significant amount of alcohol dehydrogenase was co-eluted during this step, explaining the comparatively low yield in the subsequent elution with NAD^+ plus pyrazole. SDS–polyacrylamide slab gel electrophoresis revealed that the final preparation contained only one band with a mobility close to that of horse liver alcohol dehydrogenase, as shown in fig.1. The specific activity with ethanol as substrate was found to be about 1/10 of that for the horse enzyme assayed under identical conditions.

3.2. *Structural characteristics*

Repeated attempts to determine the N-terminal amino acid of the whole protein by the dansyl method in 8 M urea [15] gave negative results, suggesting the absence of a free α-amino group in chicken liver alcohol dehydrogenase. The total composition of the carboxymethylated enzyme is shown in table 2, and is compared with the known compositions of other alcohol dehydrogenases. The no. residues/subunit of the chicken enzyme is calculated for mol. wt 40 000 in agreement with the size determination from the SDS–polyacrylamide gel electrophoresis.

Mapping of the tryptic peptides of [^{14}C]carboxymethylated chicken-liver alcohol dehydrogenase, by electrophoresis and paper chromatography [16] followed by ninhydrin staining, revealed 42 major spots, which is close to the number expected from the total amount of lysine and arginine (table 2). Autoradiography showed 10 peptides to be strongly radioactive (5 neutral, 5 acidic), while 2 others (also acidic) were less radioactive and may be minor fragments due to peptide bonds only partially sensitive to tryptic digestion.

Another tryptic digest sample (20 mg) of the carboxymethylated enzyme was submitted to preparative peptide purification by direct application on paper. After multidimensional electrophoresis and chromatography [2], the small and medium-sized peptides were obtained pure, including all the neutral radioactive

Table 1
Purification of chicken liver alcohol dehydrogenase from a crude extract by affinity chromatography on AMP–Sepharose

Step	Enzyme act. (units)	Protein (mg)	Spec. act. (units/mg)	Purification (-fold)	Yield (%)
Crude extract	103	9770	0.010	1	100
First affinity chromatography	50	94	0.53	53	49
Second affinity chromatography	14	22	0.64	64	14

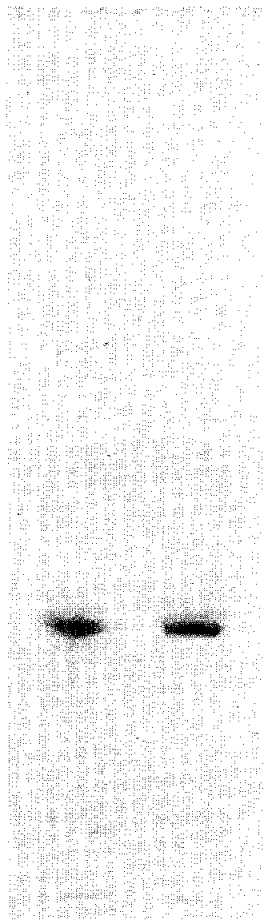

Fig.1. SDS–Polyacrylamide slab gel electrophoresis of chicken liver (right) and horse liver (left) alcohol dehydrogenases. Origin at top margin. Faint line at bottom corresponds to colour marker.

peptides together with one acidic ^{14}C-labelled peptide. Their structures determined by the dansyl-Edman method are shown in fig.2, and are in agreement with the total compositions obtained after acid hydrolysis.

4. Discussion

The chicken liver alcohol dehydrogenase preparation is pure as judged by SDS–polyacrylamide gel electrophoresis (fig.1). The absence of free α-amino groups and the agreement between peptide mapping and total compositions also support this conclusion. The subunit size of the enzyme is about 40 000, or close to that for mammalian liver alcohol dehydrogenases [1]. The lack of a free N-terminus in the protein is not surprising since all known alcohol dehydrogenases except a bacterial form [4] appear to be acetylated. The total composition of chicken liver alcohol dehydrogenase is more similar to the composition of mammlian alcohol dehydrogenases than to that of the corresponding yeast (table 2) or *Drosophila* enzymes [17], but the cysteine content appears lower. The structures around 5 of the 6 cysteine residues determined show clear homology with other alcohol dehydrogenases (fig.2) but in the 6th case (peptide pair 3) there are no obvious similarities. The latter alignment in the figure must therefore be considered tentative and alternative alignments cannot be excluded, especially with cysteine residues around position 100, which is the loop region around the 2nd zinc atom in the horse enzyme [11]. The structure analyzed contains about 10% chicken enzyme subunit, and exhibits an identity of 68% with corresponding regions of the horse enzyme. Interest-

Table 2
Amino acid composition of the subunit of carboxymethylated chicken-liver alcohol dehydrogenase

Residue	Chicken	Horse	Man	Rat	Yeast
		(mol/subunit)			
Cys (Cm)	11.7	14	14	16	8
Asp	24.1	25	29	27	27
Thr	27.9	24	24	23	15
Ser	27.9	26	21	26	21
Glu	26.6	29	27	26	29
Pro	17.6	20	21	22	13
Gly	38.8	38	40	35	44
Ala	37.4	28	34	34	35
Val	38.1	39	41	35	36
Met	7.9	9	8	5	6
Ile	24.1	24	21	24	20
Leu	27.9	25	29	30	24
Tyr	4.5	4	5	4	14
Phe	16.5	18	17	17	8
Lys	33.4	30	30	32	24
His	8.8	7	6	9	10
Arg	9.0	12	9	10	8
Trp	not det.				

Corresponding values for the horse, man, rat and yeast enzymes [1,2] are shown for comparison

Peptide pair	Sequence
1	Cys-Lys. 9 Cys-Lys
2	Phe-Thr-Cys-Lys. 132 Phe-Thr-Cys-Arg Ser
3	Ser-Cys-Leu-Ser-Thr-Lys. 282 Cys-Cys-Gln-Glu-Ala-Tyr Ser His Ser Cys
4	Gly-Asn-Leu-Cys-Ile-Lys. 111 Gly-Asn-Phe-Cys-Leu-Lys Ser Leu Cys Gln
5	Met-Val-Ala-Thr-Gly-Ile-Cys-Arg 46 Met-Val-Ala-Thr-Gly-Ile-Cys-Arg Val Ser Ala
6	Glx-Leu-Gly-Ala-Thr-Glx-Cys-Pro-Ile-Pro-Asx-Lys. 240 Glu-Val-Gly-Ala-Thr-Glu-Cys-Val-Asn-Pro-Gln-Asp Leu Asp Ile Glx Asx Glx

Fig.2. Amino acid sequences of all small and medium-sized radioactive tryptic peptides from [^{14}C]carboxymethylated chicken liver alcohol dehydrogenase (top row in each pair) compared to similar structures in the corresponding horse enzyme (bottom row, with positional numbers and with known alternative residues in the human or rat enzymes [1]).

ingly, peptide pair 5 (containing cysteine-46 at the active site of the horse enzyme) is identical in the two enzymes, peptide pair 3 (close to position 280, or, alternatively, in the loop around the 2nd zinc atom) shows large differences, and peptide pair 6 (containing strand βC in the β-pleated sheet of the horse enzyme [11]) lies between these extremes. This is exactly the same type of variability between different regions of the protein as has been found when only yeast and mammalian enzymes could be judged [5].

In summary, the present study gives for the first time a general characterization of the structure of an avian alcohol dehydrogenase. The results also show that the enzyme from this order shows regions of identity, similarity and difference with the horse liver enzyme that are remarkably like those seen among other alcohol dehydrogenases. This gives further support to the evolutionary and functional conclusions for alcohol dehydrogenases in general [7].

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References

[1] Brändén, C.-I., Jörnvall, H., Eklund, H. and Furugren, B. (1975) in: The Enzymes (Boyer, P. ed) 3rd edn, vol. 11, pp. 103–190, Academic Press, New York.
[2] Jörnvall, H. (1977) Eur. J. Biochem. 72, 425–442.
[3] Schwartz, M. F. and Jörnvall, H. (1976) Eur. J. Biochem. 68, 159–168.
[4] Bridgen, J., Kolb, E. and Harris, J. I. (1973) FEBS Lett. 33, 1–3.
[5] Jörnvall, H. (1977) Eur. J. Biochem. 72, 443–452.
[6] Eklund, H., Brändén, C.-I. and Jörnvall, H. (1976) J. Mol. Biol. 102, 61–73.
[7] Jörnvall, H. (1977) in: Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H. ed) pp. 62–71, Walter de Gruyter, Berlin, New York.
[8] Jörnvall, H. (1973) Biochem. Biophys. Res. Commun. 53, 1096–1101.
[9] Andersson, L., Jörnvall, H., Åkeson, Å. and Mosbach, K. (1974) Biochim. Biophys. Acta 364, 1–8.
[10] Andersson, L., Jörnvall, H. and Mosbach, K. (1975) Anal. Biochem. 69, 401–409.
[11] Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B.-O., Tapia, O., Brändén, C.-I. and Åkeson, Å. (1976) J. Mol. Biol. 102, 27–59.
[12] Dalziel, K. (1957) Acta Chem. Scand. 11, 397–398.
[13] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[14] Maizel, J. V., Jr. (1971) in: Methods in Virology (Maramorosh, K. and Koprowski, H. eds) vol. 5, pp. 179–246, Academic Press, New York.
[15] Bruton, C. J. and Hartley, B. S. (1968) Biochem. J. 108, 281–288.
[16] Eriksson, U., Lindberg, A. A., Jörnvall, H. and von Bahr-Lindström, H. (1976) FEBS Lett. 72, 15–18.
[17] Schwartz, M., Gerace, L., O'Donnell, J. and Sofer, W. (1975) in: Isozymes (Markert, C. ed) vol. 1, pp. 725–751, Academic Press, New York, London.