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Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase

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Formaldehyde dehydrogenase (EC 1.2.1.1) is a widely occurring enzyme which catalyzes the oxidation of *S*-hydroxymethylglutathione, formed from formaldehyde and glutathione, into *S*-formylglutathione in the presence of NAD. We determined the amino acid sequences for 5 tryptic peptides (containing altogether 57 amino acids) from electrophoretically homogeneous rat liver formaldehyde dehydrogenase and found that they all were exactly homologous to the sequence of rat liver class III alcohol dehydrogenase (ADH-2). Formaldehyde dehydrogenase was found to be able at high pH values to catalyze the NAD-dependent oxidation of long-chain aliphatic alcohols like *n*-octanol and 12-hydroxydodecanoate but ethanol was used only at very high substrate concentrations and pyrazole was not inhibitory. The amino acid sequence homology and identical structural and kinetic properties indicate that formaldehyde dehydrogenase and the mammalian class III alcohol dehydrogenases are identical enzymes.

Formaldehyde dehydrogenase; Alcohol dehydrogenase, class III; Sequence homology; Amino acid sequence

1. INTRODUCTION

Formaldehyde dehydrogenase (EC 1.2.1.1) was first described in 1955 by Strittmatter and Ball from beef and chicken livers [1]. It was purified to homogeneity and characterized from human liver by Uotila and Koivusalo in 1974 [2] and has later also been purified from some other sources [3]. A comprehensive review on formaldehyde dehydrogenase has appeared recently [3]. It is a ubiquitous cytosolic enzyme which needs both GSH and NAD to function and catalyzes the following reversible reaction:

$$\text{Formaldehyde} + \text{GSH} + \text{NAD}^+ \leftrightarrow \text{S-formylglutathione} + \text{NADH} + \text{H}^+$$

The reaction product, a glutathione thiol ester, is irreversibly hydrolyzed to GSH and formate in the reaction catalyzed by a second specific enzyme, *S*-formylglutathione hydrolase (EC 3.1.2.12), also purified to homogeneity and characterized from human liver [4] and other sources [5].

Here we report evidence that formaldehyde dehydrogenase belongs structurally to the family of alcohol dehydrogenases and is actually homologous with the class III alcohol dehydrogenases. We will further show that formaldehyde dehydrogenase is capable of catalyzing the NAD-dependent oxidation of long-chain aliphatic alcohols; glutathione is not needed in this reaction. The structural and functional features described for mammalian class III alcohol dehydrogenases were also found in purified rat liver formaldehyde dehydrogenase. These results strongly suggest that class III alcohol dehydrogenases are identical with formaldehyde dehydrogenase.

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2. MATERIALS AND METHODS

2.1. *Materials*

Female rats of Wistar strain weighing around 200 g were used as experimental animals. 5'-AMP Sepharose 4B and the materials for chromatofocusing were obtained from Pharmacia, Uppsala, Sweden. NAD, GSH, pyrazole, 12-hydroxydodecanoic acid and trypsin-TPCK were purchased from Sigma Chemical Co., St. Louis, MO, USA. The chemicals as well as other materials needed for the sequencing and high-performance liquid chromatography were of Protein Sequencing grade and were obtained from Applied Biosystems Ltd, Warrington, England.

2.2. *Purification of formaldehyde dehydrogenase*

Formaldehyde dehydrogenase was purified from the cytosolic frac-

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tion of rat liver by a combination of 5′-AMP-Sepharose affinity chromatography and chromatofocusing in a pH gradient 8–5 as described previously [6,7]. In the affinity chromatography step the enzyme was eluted by a linear gradient from 0 to 40 μM NAD. The purified enzyme was homogeneous in SDS-gel electrophoresis giving only one protein band with silver staining [8]. The slab gel electrophoreses were performed according to Laemmli [9] in a Mini-Protean II slab gel (Bio-Rad). The isoelectric focusing experiments were made on polyacrylamide gels pH 3.5–9.5 (PagPlate, LKB) in a Multiphor apparatus (LKB). Formaldehyde dehydrogenase activity staining on the gel was performed as described previously [10].

2.3. *Assay methods*

The assay mixture for formaldehyde dehydrogenase activity contained 1 mM formaldehyde, 1 mM GSH, 1.2 mM NAD and enzyme in 0.1 M sodium pyrophosphate buffer pH 8.0 [3], and that for class III alcohol dehydrogenase activity contained 1 mM *n*-octanol, 1.2 mM NAD and enzyme in 0.1 M NaOH-glycine buffer pH 9.6 when not stated otherwise. The reduction of NAD was monitored at 340 nm and 25°C, either on a Shimadzu UV-240 spectrophotometer or a nine-channel FP-9 Analyzing System (Labsystems Oy, Helsinki) connected to an Olivetti M 20 computer. One unit of activity equals to 1 μmol of NADH produced per min. The absorption coefficient of 6220 $M^{-1} \cdot cm^{-1}$ was used for NADH at 340 nm. The protein concentrations were determined by the method of Lowry et al. [11] or by a modification of the method of Bradford [12] with bovine serum albumin as the standard. Formaldehyde was assayed by the chromotropic acid method [13].

2.4. *Amino acid sequence analysis*

Tryptic digestion of formaldehyde dehydrogenase was carried out in 1% ammonium bicarbonate. A sample of 60 μg of protein was incubated with 3% (w/w) of trypsin for 2 h at 37°C. Then another 3% of the protease was added and the incubation continued for 2 h at 37°C. The released peptides were separated by reverse-phase high-performance liquid chromatography using a Vydac 218 TP 54 (0.46 × 25 cm) column equilibrated with 0.1% trifluoroacetic acid in water, with a linear gradient of acetonitrile (0–70% in 60 min) in the equilibration solution. Separated peptides were collected and used directly for the amino acid analysis.

The amino acid analysis was performed by automated Edman degradation using an Applied Biosystems 477A/120A on line pulsed liquid phase/gas phase sequencer in the gas phase mode. The sequencer has been modified to run in addition to the gas phase sequencing system in the same analysis (Baumann, M.H., submitted).

3. RESULTS

We have determined the amino acid sequences for 5 tryptic peptides obtained from homogeneous rat liver formaldehyde dehydrogenase, containing altogether 57 amino acids (fig.1). The N-terminal amino acid was blocked. All the sequences determined were exactly identical with the recently reported primary sequence of rat liver class III alcohol dehydrogenase (ADH-2) [14]. There were only minor differences from the sequence reported for human liver class III alcohol dehydrogenase (χ-ADH) [15] and about 50–60% sequence homology with the sequences reported for class I and II alcohol dehydrogenases [15]. These findings indicate that formaldehyde dehydrogenase is closely related to

11-19 AAVAWEAGK

17-27 AGKPLSIEEIE

85-101 AGDTVJPLYJPQXGEXK

189-199 VEPGSTXAVFG

358-366 AFDLMHSGN

Fig.1. Amino acid sequences for 5 tryptic peptides obtained from rat liver formaldehyde dehydrogenase. The numbering refers to the total sequence recently reported for rat liver class III alcohol dehydrogenase (ADH-2) [14]. X means a residue not identified.

alcohol dehydrogenases and especially to class III enzymes.

We investigated the NAD-dependent oxidation of alcohols by the purified rat liver formaldehyde dehydrogenase (table 1). Short-chain aliphatic alcohols from methanol to propanol at a concentration of 30 mM were not used as substrates. There was some activity for ethanol and propanol at high alcohol concentrations but the enzyme could not be saturated with up to 2 M ethanol. Aliphatic alcohols with 5–8 carbons as

Table 1

Oxidation of alcohols by the purified formaldehyde dehydrogenase from rat liver

Substrate (concentration)	Relative activity
n-Octanol (1 mM)	1.00
n-Heptanol (1 mM)	0.67
n-Hexanol (1 mM)	0.27
n-Pentanol (1 mM)	0.05
n-Butanol (1 mM)	no activity
n-Butanol (30 mM)	0.13
n-Propanol (30 mM)	no activity
Ethanol (30 mM)	no activity
Methanol (30 mM)	no activity
12-Hydroxydodecanoate (1 mM)	1.75
n-Octanol (1 mM) + 10 mM pyrazole	0.95
n-Octanol (1 mM) + 1 mM GSH	1.13

The activities were assayed for NADH formation in the mixture containing 0.1 M NaOH-glycine, pH 9.6, 1.2 mM NAD, the alcohol as given above, and the enzyme. The relative activity with 1 mM *n*-octanol as the substrate has been set to 1.00. This value corresponded to 0.81 ± 0.33 U/mg protein (mean ± SD) for the 3 preparations used. The relative activity in the standard formaldehyde dehydrogenase assay (with formaldehyde, GSH and NAD as the substrates at pH 8.0) was 3.68

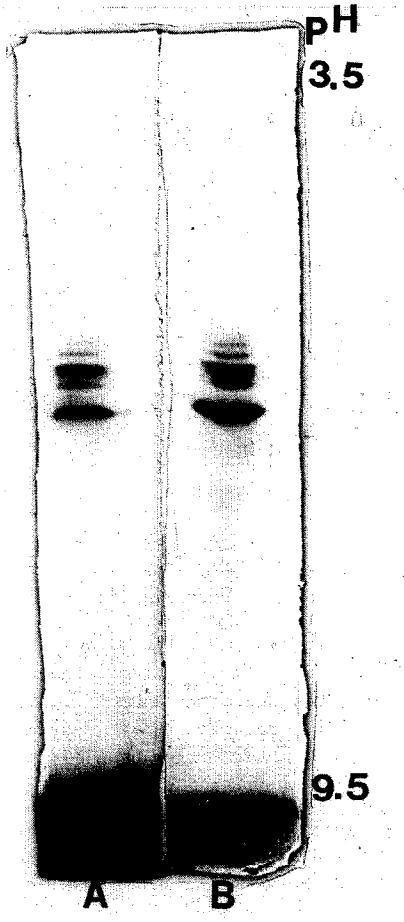

Fig.2. Activity staining of purified formaldehyde dehydrogenase from rat liver after isoelectric focusing on polyacrylamide gel (pH gradient 3.5–9.5). (A) Stained for formaldehyde dehydrogenase activity at pH 7.5 with formaldehyde, GSH and NAD as the substrates [10]. (B) Stained for alcohol dehydrogenase activity at pH 8.0 with *n*-octanol and NAD as the substrates.

well as 12-hydroxydodecanoate were active as substrates (table 1). The apparent K_m values for *n*-octanol, *n*-heptanol and 12-hydroxydodecanoate were 0.50 mM, 1.30 mM and 0.10 mM, respectively, at pH 9.6 in the presence of 1.2 mM NAD. The activities with the alcohols were not inhibited by even 10 mM pyrazole or 4-methylpyrazole. In contrast to the oxidation of formaldehyde, glutathione was not needed in the reaction with the alcohol substrates, and the latter reactions were only marginally activated by the addition of GSH to the assay mixture (table 1). This substrate specificity with the alcohols and the K_m values determined accord well with the values reported earlier for rat liver ADH-2 [16] and for the class III alcohol dehydrogenases from other sources [17,18]. The specific activity of purified rat liver formaldehyde dehydrogenase was 0.95 U/mg protein when 1 mM *n*-octanol was used as the substrate (1.2 mM NAD, 0.1 M NaOH-glycine pH 10.0), which also corresponds well to the value reported earlier for rat liver ADH-2, 1.1 U/mg protein [16]. The specific activity with formaldehyde and glutathione as the substrates is about 3 U/mg protein (pH 8.0).

When rat liver formaldehyde dehydrogenase was studied by isoelectric focusing (fig.2), the same 1–3 main bands at pH 5.8–6.4 were obtained when the gels were stained either for formaldehyde dehydrogenase activity with formaldehyde, GSH and NAD as the reactants or with *n*-octanol and NAD with or without pyrazole. Additional minor bands were also observed. These have earlier been reported for rat liver ADH-2 and have been proposed to arise from deamidation products of Cys-oxidation states [14]. In the column chromatographies used for isolation of formaldehyde dehydrogenase, 5′-AMP-Sepharose and chromatofocusing, the activity with formaldehyde, glutathione and NAD as well as the pyrazole-insensitive activity with *n*-octanol and NAD co-purified in our experiments exactly into the same fractions (data not shown).

4. DISCUSSION

The human alcohol dehydrogenases have been divided into 3 classes on the basis of their electrophoretic mobilities, kinetic properties and inhibition by pyrazole [19]. Analogous enzyme types have also been described in rat [16,18], mouse [20] and in several other mammals [18]. Among alcohol dehydrogenases, the class III enzymes are distinguished as the most anodic species in electrophoresis. In addition they are, in contrast to the enzymes from classes I and II, virtually insensitive to inhibition by pyrazole and have a very low affinity for ethanol but can catalyze the oxidation of long-chain aliphatic alcohols like octanol [18,19]. The class III enzymes are structurally distinct from class I and II enzymes. Enzymes from the separate classes differ from each other, e.g. in human liver by 139–147 amino acid residues in the 373–374-residue polypeptide chain [15]. Antibodies against human class III alcohol dehydrogenase do not cross-react with class I and II enzymes [21].

The tryptic peptides prepared in the present experiments from formaldehyde dehydrogenase had identical sequences with that reported for rat liver class III alcohol dehydrogenase [14] and we found that the homogeneous rat liver formaldehyde dehydrogenase could use long-chain aliphatic alcohols as substrates. The substrate specificity of formaldehyde dehydrogenase with alcohol substrates as well as the K_m and V_{max} values for those compounds were remarkably similar to the values reported in literature for class III alcohol dehydrogenase [15–20]. In addition, isoelectric focusing experiments gave identical activity staining results when either formaldehyde and GSH or *n*-octanol was used as the substrate. These results can on-

ly be explained so that formaldehyde dehydrogenase and class III alcohol dehydrogenase represent the activities of the same enzyme. The identity of these enzymes is further corroborated by their identical molecular weights (80000) and subunit molecular weights (40000) [3,15–20]. Both activities have been localized into cell cytosol and both have a very wide and identical tissue distribution in mammals [3,16,22]. The gene for the human formaldehyde dehydrogenase has been located to chromosome 4q21-25 [23] which is the same site found for human class III [24] and class I [25] alcohol dehydrogenases.

The physiological substrates and the function of the class III alcohol dehydrogenases have been obscure. They do not participate in the metabolism of ethanol. It has been suggested that they would have a function in the metabolism of endogenous long-chain alcohols and aldehydes, e.g. with intermediates of lipid metabolism [16]. The most apparent function of formaldehyde dehydrogenase is in the oxidation of the formaldehyde formed in oxidative demethylations and in some other metabolic reactions [3]. Other functions, e.g. in formyl transfer are also possible [3]. The true substrate for formaldehyde dehydrogenase is not free formaldehyde but the hemithioacetal formed nonenzymically from formaldehyde and glutathione, namely *S*-hydroxymethylglutathione [26]. Since the latter is oxidized to *S*-formylglutathione, the reaction is formally oxidation of an alcoholic hydroxyl group. Formaldehyde has been reported to occur physiologically in significant concentrations in animal cells, apparently in large part as the adduct with glutathione [27] which is present at a high concentration in most cells. We suggest that *S*-hydroxymethylglutathione is the previously unknown true substrate of class III alcohol dehydrogenases. It appears that the site on the enzyme which binds glutathione can in alternative substrates also bind a long hydrophobic carbon-carbon chain which does not contain sulfur. The enzyme can, however, catalyze the oxidation of *S*-hydroxymethylglutathione much more effectively than that of the alcohol substrates. The K_m value for *S*-hydroxymethylglutathione is low being 0.92 μM for the rat liver formaldehyde dehydrogenase [7] and the corresponding specificity constant k_{cat}/K_m is 235000 $mmol^{-1} \cdot min^{-1}$ [7]. The best alcohol substrate, 12-hydroxydodecanoate is bound to the enzyme at least 100-fold less effectively and the specificity constant [16] is also at least 100-fold lower for 12-hydroxydodecanoate than for the adduct of formaldehyde and glutathione, even when measured at high pH. At physiological pH the enzyme uses even the long-chain alcohol substrates very poorly [16] but the reaction with *S*-hydroxymethylglutathione is close to its pH optimum [7].

In summary the sequence homology and the identical structural and kinetic properties reported indicate that the formaldehyde dehydrogenase and the mammalian class III alcohol dehydrogenases are in fact identical enzymes.

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