Aldehyde dismutase activity of human liver alcohol dehydrogenase

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Received 24 June 1996; revised version received 19 August 1996

Abstract Human alcohol dehydrogenases of class I and class II but not class III catalyse NAD^+-dependent aldehyde oxidation in addition to the NADH-dependent aldehyde reduction. The two reactions are coupled, i.e. the enzymes display dismutase activity. Dismutase activity of recombinantly expressed human class I isozymes $\beta_1\beta_1$ and $\gamma_2\gamma_2$, class II and class III alcohol dehydrogenases was assayed with butanal as substrate by gas chromatographic-mass spectrometric quantitations of butanol and butyric acid. The class I $\gamma_2\gamma_2$ isozyme showed a pronounced dismutase activity with a high k_{cat}, 1300 min^{-1}, and a moderate K_m, 1.2 mM. The class I $\beta_1\beta_1$ isozyme and the class II alcohol dehydrogenase showed moderate catalytic efficiencies for dismutase activity with lower k_{cat} values, 60–75 min^{-1}. 4-Methylpyrazole, a potent class I ADH inhibitor, inhibited the class I dismutation completely, but cyanamide, an inhibitor of mitochondrial aldehyde dehydrogenase, did not affect the dismutation. The dismutase reaction might be important for metabolism of aldehydes during inhibition or lack of mitochondrial aldehyde dehydrogenase activity.

Key words: Alcohol dehydrogenase; Dismutation; Recombinant protein; Gas chromatography-mass spectrometry

1. Introduction

Mammalian alcohol dehydrogenase (ADH) represents a system of related enzymes, of which presently six classes are distinguished [1], capable of reducing a wide array of aldehydes [2–4], and is, together with aldehyde dehydrogenase, aldehyde reductase and glutathione *S*-transferase, one of the major aldehyde-metabolising enzymes [4]. The major organ for aldehyde detoxification in humans is the liver where three main classes of ADH have been characterised, classes I–III [5], and class I is further divided into isozymes derived from the presence of three subunit types (α, β, γ) [6].

Horse liver ADH of class I has been reported to exhibit an additional activity, oxidation of aldehydes [7–9]. Reevaluation of this reaction has shown remarkably fast kinetics for the aliphatic aldehydes studied: acetaldehyde, butanal and octanal [10,11]. Simultaneous oxidation and reduction of aldehydes occur when horse liver ADH is incubated with aldehyde and NAD^+. This results in an equimolar formation of the two products, alcohol and carboxylic acid (Fig. 1), and no net reduction of the coenzyme can be observed. In competition assays, the oxidation of aldehydes was as fast as the oxidation of the corresponding alcohols indicating a possible physiological role for ADH-catalysed aldehyde oxidation [11]. An important question is whether all ADHs possess this activity. *Drosophila* ADH, which is a form distinct from horse liver ADH (medium-chain dehydrogenases), displays aldehyde dehydrogenase properties [12], while the yeast ADH has been reported to lack this activity [13].

In this study, we have investigated the dismutase activity of human ADH (classes I–III) with the use of recombinant enzymes and butanal as substrate. For this purpose, a method based on gas chromatographic-mass spectrometric analysis was developed for determination of product formation.

2. Materials and methods

2.1. Construction of expression plasmids for class I γ_2 and class II ADH

cDNAs coding for class I γ_2 [14] and class II [15] were amplified by PCR using *Pfu* polymerase (Stratagene) under conditions recommended by the manufacturer. The cDNA coding for class I γ_2 was cloned into the unique *Nde*I and *Bam*HI restriction sites of the expression vector pET12b (Novagen), while class II cDNA was cloned into the *Nco*I and *Bam*HI sites of pET3d, in both cases yielding constructs that gave no additional amino acid residues in the translated protein. The two plasmid constructs pET12γ and pET3π were isolated and purified with the Qiagen ion-exchange method (Diagen). Both plasmids were verified by dideoxy sequence analysis [16].

2.2. ADH expression and isolation

Class I $\beta_1\beta_1$ and class III ADH were expressed and isolated as described [17,18] using expression plasmids based on the vector pKK223-3 (Pharmacia). The class I $\gamma_2\gamma_2$ and class II ADH were expressed in 0.5 litre cultures of *E. coli* strain BL21 (DE3) after induction with a burst of 0.5 mM isopropyl-thio-β-D-galactosidase at an OD_{595} ~1.0 and were harvested 8–14 h later. The isolation protocol for class I $\beta_1\beta_1$ was used also for class I $\gamma_2\gamma_2$. For isolation of class II ADH the cells were disrupted in 1 mM dithiothreitol, 1 mM benzamidine, 10 mM sodium phosphate, pH 7.5. Cells were lysed by sonication before centrifugation for 40 min at 48 000 × *g*. The supernatant, approximately 10 ml, was applied to a DEAE column (DE-52, Whatman; 150 ml). The void volume, containing class II ADH, was applied to a 5 ml HiTrap Blue column (Pharmacia) and eluted with 300 mM NaCl in 10 mM sodium phosphate, pH 7.5. The fraction with ADH activity was further purified by exclusion chromatography on a 25 ml Superose 12 column (Pharmacia) equilibrated with 100 mM NaCl, 50 mM sodium phosphate, pH 7.5. Proteins were concentrated (Microsep 30K cut off, Filtron) and analysed by SDS/polyacrylamide gel electrophoresis. Protein concentrations were determined with the Bio-Rad assay according to the method of Bradford [19] and complemented with amino acid analysis on a Pharmacia LKB AlphaPlus analyser.

2.3. Enzyme assays

The specific activities (units per mg protein, where one unit is defined as 1 μmol of NADH formed/min) of the recombinant class I isozymes and the class II enzyme were determined in 0.1 M glycine, pH 10.0 at 25°C with 33 mM ethanol and 2.4 mM NAD^+ (Sigma). Class III was assayed in 0.1 M sodium pyrophosphate, pH 8.0, with 1 mM each of glutathione (Sigma) and formaldehyde (Ladd Research). The reactions were monitored spectrophotometrically on a Pharmacia LKB Biochrom 4060 spectrophotometer by following the production of NADH (Sigma) as an increased absorbance at 340 nm. The production of NADH was calculated using a molar absorptivity of 6220 M^{-1} cm^{-1}. Dismutase assays were performed at 37°C in 0.1 M sodium phosphate, pH 7.5, 10.8 mM NAD^+ and with varied concentrations of butanal. Butanal (Aldrich) was distilled and stored under nitrogen at 4°C before use. Reactions were started by addition

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PII S0014-5793(96)00954-4

of butanal and stopped by the addition of half a volume of 12 M HCl to quench the enzymatic activity. Prior to addition, butanal was incubated in water for 10 min. Inhibition of the dismutation of butanal (5.5 mM) was tested for class I and class II ADH with the compounds 4-methylpyrazole (0.5–10 mM; Sigma) and cyanamide (2 mM; American Cyanamide).

2.4. Gas chromatography/mass spectrometry

Products were extracted from the reaction mixture with 150 μl diethylether and the ether phase was dried with anhydrous $CaSO_4$. Product concentrations were measured using a Hewlett Packard 5890A gas chromatograph on-line with a Hewlett Packard 5972 series mass selective detector. An aliquot (approximately 1 μl) was injected on-column on a Carbowax 20M column (25 m, 0.32 mm i.d., Quadrex Corporation) coated with a 1 μm crosslinked film. Helium was used as carrier gas and the column temperature was 80°C for 4 min and was raised to 230°C at 20°C/min. The internal standard, 2,6-di-*tert*-butyl-4-methylphenol, present as stabiliser in the ether, and the two products, butanol and butyric acid, were identified by their mass spectra as three dominating peaks in the chromatogram. The *m/z* peaks at 205, 56 and 60, respectively, were chosen for quantitations on the basis of intensity and lack of interference from contaminants. The quantitations were carried out by single ion mode, and solutions with known concentrations of butanol and butyric acid in 0.1 M sodium phosphate (pH 7.5) with 10.8 mM NAD^+ were used for calibration. The peak areas corresponding to butanol and butyric acid relative to that corresponding to the internal standard were found to be directly proportional to the concentrations. Standard curves were constructed by linear regression and used to determine the product concentrations in the reaction mixtures.

Weighted non-linear regression analysis was used to calculate kinetic parameters. Values of k_{cat} are based on a molecular mass of 80 kDa for all enzymes. All kinetic values given are the means of at least two different measurements with protein from separate preparations.

3. Results

3.1. Expression and isolation of ADH

Recombinant class I $\beta_1\beta_1$, class I $\gamma_2\gamma_2$, class II and class III ADH were expressed in *E. coli* and yields of isolated proteins ranged from 1 mg/litre culture medium of the class I $\beta_1\beta_1$ and 3 mg of the class III (expression plasmids based on pKK223 with *tac* promoter) to 20 mg of the class I $\gamma_2\gamma_2$ and class II (expression plasmids based on pET3/12 with T7 *lac* promoter). The specific activities of all recombinant ADH agreed with previously published values for ADH purified from human liver: class I $\beta_1\beta_1$, 0.2 units/mg of protein; class I $\gamma_2\gamma_2$, 0.8 units/mg of protein; class II, 1.1 units/mg of protein; class III, 3.4 units/mg of protein [2,20]. The values were determined with ethanol as substrate for classes I and II and with hydroxymethyl-glutathione as substrate for class III.

3.2. Steady-state dismutase kinetics

Dismutase reactions were carried out at 37°C in a phosphate buffer of physiological pH (7.5) with a NAD^+ concentration assuring saturation of the enzyme. A phosphate buffer was used since it does not react with aldehydes which is the

Table 1
Kinetic constants for elimination of butanal by dismutation

Human ADH	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})
Class I $\beta_1\beta_1$	75	2.1	36
Class I $\gamma_2\gamma_2$	1300	1.2	1100
Class II	60	13	5
Class III	no detectable dismutase activity		

Measurements were performed in 0.1 M sodium phosphate, pH 7.5, at 37°C.

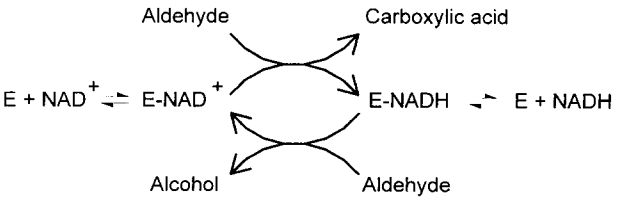

Fig. 1. Reaction scheme for aldehyde dismutation.

case with commonly used amino-group-containing buffers, such as glycine and Tris-HCl [12]. Reactions were started by the addition of butanal that had been incubated for 10 min in water to establish the equilibrium of free aldehyde and the hydrated *gem*-diol. Reaction rates, expressed as elimination of aldehyde, were determined by analysis of concentrations of products using gas chromatography-mass spectrometry. Reactions were quenched by lowering the pH below 1 and the reaction times were adjusted to yield 10–20% depletion of substrate. In the range of substrate concentration used, 0.32–22 mM, the reaction displayed Michaelis-Menten kinetics for class I and class II enzymes (Fig. 2). The rates of production of butyric acid and butanol were equal within experimental errors, as required by the stoichiometry of the reaction. No detectable dismutation could be observed with the class III enzyme. Here substrate concentrations up to 110 mM were used and reactions were run for 20 min with an enzyme concentration of 0.25 mg/ml. K_m values for the two class I isozymes are in the millimolar range and the k_{cat} values are 75 min^{-1} and 1300 min^{-1} for the $\beta_1\beta_1$ isozyme and the $\gamma_2\gamma_2$ isozyme, respectively. The K_m value for class II was 13 mM and k_{cat} was 60 min^{-1} (Table 1). Addition of 0.5 mM of the potent class I ADH inhibitor 4-methylpyrazole to the reaction mixtures inhibited the class I dismutation completely. Class II ADH activity was less sensitive to 4-methylpyrazole with 50% inhibition at a concentration of 1 mM and a total inhibition only at 10 mM. Cyanamide, a known inhibitor of mitochondrial aldehyde dehydrogenase, did not affect the dismutation.

4. Discussion

Aldehydes in mammals are mainly metabolised into the corresponding carboxylic acids by aldehyde dehydrogenase. The aldehydes can also be reduced to the corresponding alcohols either by ADH or by aldehyde reductase. In addition, horse class I ADH has dismutase activity which results in both the corresponding alcohol and carboxylic acid from the aldehyde substrate [7,10,11]. The aim of the present study was to establish whether dismutation of aldehydes is catalysed also by human ADHs. These were prepared as homogeneous homodimers of four enzymes expressed as recombinant proteins in *E. coli*. Thus, the enzymes used were not contaminated by aldehyde dehydrogenase or by ADH of other classes. A method to quantitate the products by gas chromatography-mass spectrometry was developed, since the dismutation cannot be followed by spectrophotometric determination of the coenzyme.

The results obtained show that particular human ADHs display clear dismutase activity. Kinetic constants indicate that class I $\gamma_2\gamma_2$ has a high catalytic efficiency for this reaction, while class I $\beta_1\beta_1$ and class II have more moderate catalytic

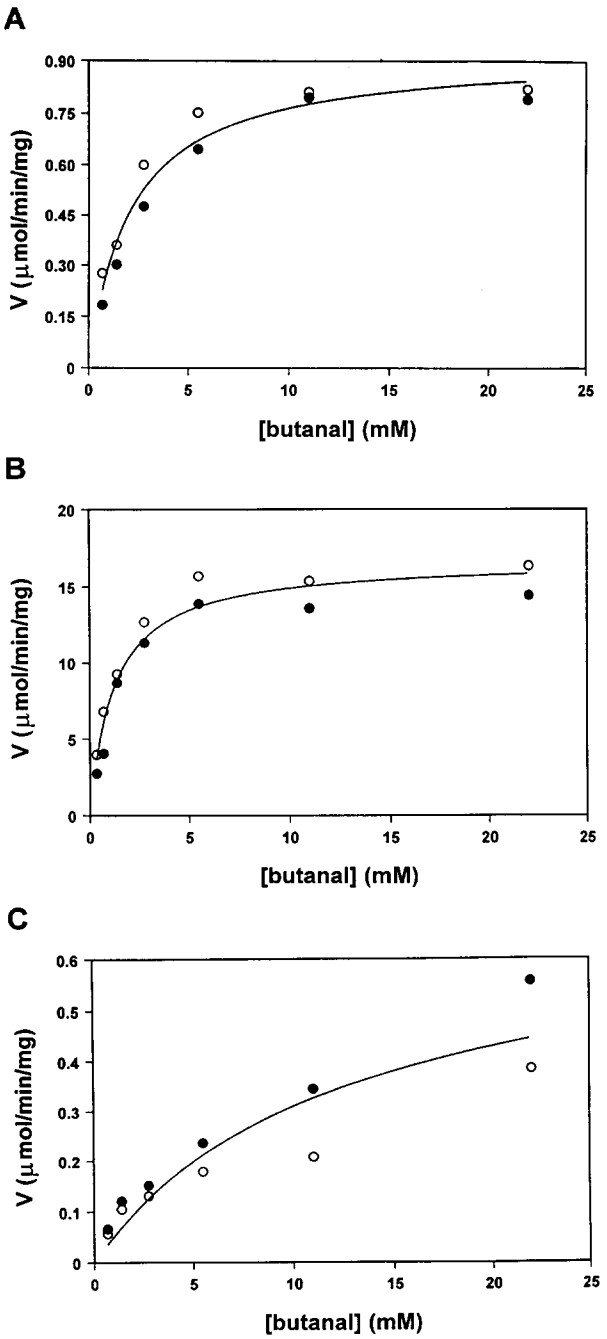

Fig. 2. Steady-state kinetics of human ADH. Rates of production of butanol (○) and butyric acid (●) are given as functions of butanal concentration for class I $\beta_1\beta_1$ (A), class I $\gamma_2\gamma_2$ (B) and class II (C) ADH. The reactions were catalysed in the presence of 10.8 mM NAD^+ and 0.1 M sodium phosphate, pH 7.5, at 37°C.

efficiencies (Table 1). However, these constants may not be compared directly with those of aldehyde reduction, since k_{cat}/K_m are composed of different sets of fundamental rate constants [11]. The variation in efficiency of these ADHs reflects the structural differences in the substrate-binding pocket [21]. The human class I isozymes show in many respects similar characteristics to those of class I ADHs from horse, where the human $\gamma\gamma$ isozyme is the variant with the greatest positional identity to the horse class I ADHs. The two isozymic forms of human class I ADH have been shown to differ in K_m and k_{cat} values for several substrates [2,3] and this is here shown in a 10-fold lower k_{cat} for $\beta_1\beta_1$ compared to $\gamma_2\gamma_2$. Class II ADH exhibits a dismutase activity comparable to that of the human $\beta_1\beta_1$ isozyme. The class III ADH, showing no dismutase activity, is the ADH that in many other respects shows different properties from the other ADH forms. This ADH cannot be saturated with ethanol and it is the only ADH with glutathione-dependent formaldehyde dehydrogenase activity [22]. Furthermore, this ADH has been shown to be the origin of the zinc-dependent ADH enzymes [23]. The results obtained in this study further strengthen that class III ADH differs in many functional respects, which can be explained by the wider substrate-binding pocket [21].

Most toxic aldehydes are found in low concentrations in living cells, and appear to be metabolised by low K_m enzymes such as mitochondrial aldehyde dehydrogenase. If ADH-catalysed oxidation has a physiological importance for some aldehydes these might accumulate during ethanol metabolism, when there is little NAD^+ bound to ADH [24]. Furthermore, the reaction may be important for aldehyde metabolism during conditions where no or limited aldehyde dehydrogenase activity is present, e.g. after treatment with disulfiram or cyanamide, or in individuals lacking an active mitochondrial aldehyde dehydrogenase.

Acknowledgements: This study was supported by grants from the Swedish Medical Research Council (Grants 2189 and 8639) and the Karolinska institute.

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