Class III alcohol dehydrogenase from *Saccharomyces cerevisiae*: Structural and enzymatic features differ toward the human/mammalian forms in a manner consistent with functional needs in formaldehyde detoxication

M. Rosario Fernández[a], Josep A. Biosca[a], Annika Norin[b], Hans Jörnvall[b], Xavier Parés[a,*]

[a]*Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain*
[b]*Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden*

Received 1 June 1995

Abstract Alcohol dehydrogenase class III (glutathione-dependent formaldehyde dehydrogenase) from *Saccharomyces cerevisiae* was purified and analyzed structurally and enzymatically. The corresponding gene was also analyzed after cloning from a yeast genome library by screening with a probe prepared through PCR amplification. As with class III alcohol dehydrogenase from other sources, the yeast protein was obtained in two active forms, deduced to reflect different adducts/modifications. Protein analysis established N-terminal and C-terminal positions, showing different and specific patterns in protein start positions between the human/mammalian, yeast, and prokaryotic forms. K_m values with formaldehyde differ consistently, being about 10-fold higher in the yeast than the human/mammalian enzymes, but compensated for by similar changes in k_{cat} values. This is compatible with the different functional needs, emphasizing low formaldehyde concentration in the animal cells but efficient formaldehyde elimination in the microorganisms. This supports a general role of the enzyme in formaldehyde detoxication rather than in long-chain alcohol turnover.

Key words: Alcohol dehydrogenase; Substrate turnover; Formaldehyde elimination; N-terminal positions; Yeast protein

1. Introduction

Alcohol dehydrogenase (ADH, EC 1.1.1.1) constitutes a complex enzyme system, with class III forms present in many cells. This form has a distant origin [1], presumably reflecting the ancestor (cf [2]) of all the six classes of mammalian alcohol dehydrogenase presently known [3], and has a physiological role in the oxidation of formaldehyde, acting as a glutathione-dependent formaldehyde dehydrogenase [4]. This activity seems essential for normal life development. In contrast to the ethanol-active class I enzyme, which is absent in some animals [5], including lower vertebrates [6] and invertebrates [1,7], class III is found in all organisms from bacteria [8,9] to human tissues [10,11]. The class III enzyme has also been detected in several yeast species [12–15] and has been structurally analyzed via the corresponding DNA [14,15]. However, direct analysis of terminal structures of the protein or characterization of its enzymatic properties in relation to the different sources has not been performed.

We have now purified and analyzed the *Saccharomyces cerevisiae* enzyme, re-cloned and re-sequenced its gene, and correlated the enzymatic properties of the protein with those of the enzyme from higher organisms. The properties differ somewhat from those of other class III alcohol dehydrogenases, but in a consistent manner, and can be correlated with the different but specific needs for formaldehyde elimination in human/animals versus yeasts, compatible with the functional role of the class III form of alcohol dehydrogenase as part of a detoxication system.

2. Materials and methods

2.1. Cloning of yeast class III alcohol dehydrogenase genomic DNA

This study was initiated before a known DNA structure was available, and although therefore requiring more work than apparent, it is only briefly described since another report now exists [14]. Briefly, genomic DNA from X2180 *Saccharomyces cerevisiae* (ATCC 26109) prepared as described [16], was subjected to PCR amplification using two primers based on the degenerated DNA sequences of human and *Candida maltosa* class III alcohol dehydrogenase [15,17], 5′-A(AG)TG(CT)GGIGA(AG)TG(CT)AA(AG)TT(CT)TG-3′ (corresponding to nucleotides 284–305 of human cDNA) and 5′-CCCCAICC(TC)TT(AG)TG(AG)CAIGC(TC)TC-3′ (corresponding to nucleotides 835–857 of the human cDNA). The PCR product (100 to 25 ng), labelled with 50 μCi [α-^{32}P]dCTP (Amersham) was used (2×10^8 cpm/μg) in a plaque hybridization procedure to screen the X2180 *Saccharomyces cerevisiae* genomic library in λgt11 (Clontech Laboratories). After three rounds of screening, one positive clone was isolated. The insert was subcloned into pBluescript II SK (+) for double-strand DNA sequence analysis [18].

2.2. Purification of yeast class III alcohol dehydrogenase

Frozen bakers' yeast (l'Hirondelle, S.I., Lesaffre, Paris) was homogenized in a bead-beater (Biospec Products) with 0.5 mm diameter beads, at 4°C for 1 min (four times) in 10 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.5, and centrifuged at $29,000 \times g$ for 1 h at 4°C. After dialysis against 10 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.5, the material was applied to DEAE-Sepharose CL-6B (2.6×94 cm) in the same buffer. After washing, a linear gradient of increasing NaCl concentration (0–0.18 M in 1200 ml) eluted the glutathione-dependent formaldehyde dehydrogenase activity. Active fractions were concentrated to 60 ml (Amicon with a Diaflo PM 10 membrane), dialyzed against 10 mM KH_2PO_4, 0.5 mM DTT, pH 6.8, and applied to hydroxylapatite (2×16 cm). After isocratic elution at 36 ml/h (250 ml buffer) a linear gradient of 10–400 mM KH_2PO_4 in 1200 ml 0.5 mM dithiothreitol, pH 6.8, was applied. Active fractions were pooled and concentrated to 30 ml, dialyzed against 10 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.5, and applied to Blue-Sepharose (1×25 cm). After washing, a linear gradient of NADH (0–750 μM) in 600 ml 10 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.5, eluted two peaks of active material. The corresponding fractions were concentrated and dialyzed, for removal of NADH, against 100 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.5, and stored at −80°C.

*Corresponding author. Fax: (34) (3) 581 1264.

SSDI 0014-5793(95)00788-1

The purification was monitored by SDS-polyacrylamide gel electrophoresis and subsequent silver staining, and by isoelectric focusing in a pH 3–9 gradient (PhastSystem IEF 3–9, Pharmacia Biotech), developed with activity staining using 4.8 mM formaldehyde and 1 mM glutathione.

2.3. Enzymology

Enzyme activities were determined at 25°C by spectrophotometric measurements at 340 nm. Alcohol oxidation was measured with 4 mM NAD^+ in 0.1 M glycine-NaOH, pH 10; formaldehyde dehydrogenase activity in 0.1 M sodium phosphate, pH 8.0, with *S*-hydroxymethylglutathione (formed by mixing formaldehyde and glutathione) [4]. Concentrations of hydroxymethylglutathione were calculated from the dissociation constant of this compound [4], keeping free glutathione constant (1 mM) and changing the formaldehyde concentration. Formaldehyde concentrations were determined enzymatically by the NAD^+ to NADH conversion in the presence of *Pseudomonas putida* formaldehyde dehydrogenase. One unit of activity (U) corresponds to 1 μmol NADH formed per minute. For each kinetic experiment, at least five substrate concentrations were assayed in duplicate. With substrates that saturated the enzyme, the general concentration range was from 0.3 to 10 K_m. Because of low solubility, the maximum concentration reached for octanol was 1 K_m, for 10-hydroxydecanoic acid 1.3 K_m, and for 12-hydroxydodecanoic acid 5 K_m. Results were expressed as the mean of at least three determinations, the variability of which was below 15%. Kinetic results were analyzed by a nonlinear regression data analysis program [19].

2.4. Protein structure analysis and comparisons

Peptides for amino acid sequence analysis were generated by digestions of the ^{14}C-carboxymethylated [2] protein with *Achromobacter* Lys-specific and *Astacus* Gly-specific proteases, followed by purifications by reverse phase HPLC on a C4 column using a linear gradient (0–70%) acetonitrile in 0.1% aqueous trifluoroacetic acid. Sequence analyses were performed by degradations in gas-phase or solid-phase sequencers, Applied Biosystems 470 with separate HPLC analysis, 477A with on-line 120A analyzer, or Milligen 6600 instruments. Total compositions were determined with an LKB Pharmacia Alpha Plus analyzer after hydrolysis for 24 h at 110°C in 6 M HCl, 0.5% phenol.

Nucleotide and amino acid sequences were compared against the EMBL, GenBank and SwissProt data banks using the FASTA program [20].

3. Results and discussion

3.1. Isolation of Saccharomyces cerevisiae class III alcohol dehydrogenase

The method employed to purify class III alcohol dehydrogenase from Baker's yeast provided homogeneous material. The activity of the crude extract was relatively high (Table 1), presumably due to the presence initially of not only class III alcohol dehydrogenase but also other activities, such as glutathione-independent formaldehyde dehydrogenase and glutathione reductase. The DEAE-Sepharose chromatography removed those interfering activities, while the hydroxylapatite chromatography step was necessary for the elimination of material which precluded the binding of the enzyme to Blue-Sepharose. Finally, Blue-Sepharose chromatography separated two peaks of activity. The corresponding forms are similar in specific activity (just below 50 U/mg) and identical in molecular weight, both giving a homogeneous band at approximately 45 kDa upon SDS-polyacrylamide gel electrophoresis. Isoelectric focusing of the material from both peaks revealed that the major fraction (fraction 2) is the one most basic, and fraction 1 the one more acidic. Both fractions, however, contained a further sub-band separated by charge. This charge multiplicity, with two major bands, is typical of class III alcohol dehydrogenases isolated also in other species [1,6,10,21] and has been concluded to reflect adduct formation and other chemical modifications. This seems also to be the case for the yeast enzyme, since Southern analysis revealed a single gene [22] and isoelectric focusing showed both bands in haploid yeast. In conclusion, it is clear that yeast has a class III protein recovered in expected yield, activity and minor multiplicity.

Table 1
Purification of yeast class III alcohol dehydrogenase

Purification (step)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	458	8020	0.057	1	100
DEAE-Sepharose	325	218	1.49	26	71
Hydroxylapatite	292	15.8	18.5	320	64
Blue Sepharose					
fraction 1	60	1.37	44	770	13
fraction 2	137	2.82	49	860	30

Values apply to the amount from 300 g bakers' yeast. Activity was measured with 1 mM formaldehyde/1 mM glutathione, 4 mM NAD^+ in 0.1 M sodium phosphate, pH 8.0.

Table 2
Terminal structures of *S. cerevisiae* class III alcohol dehydrogenase (top line) correlated with those of some other yeasts (below), mammals (middle) and prokaryotes (bottom) characterized

Origin	Class III region		
	N-terminal		C-terminal
S. cerevisiae	SAATVGKPI	KCIAAVAYDA.....	HNGDCLRTVL KSDEIK
C. maltosa	SESTVGKPI	TCKAAVAWEA.....	HAGDCIRAVV DLS
Human	ANEVI	KCKAAVAWEA.....	HSGKSIRTVV KI
Horse	SAEVI	KCKAAVAWEA.....	HAGKSIRTVV KL
Rat	ANQVI	RCKAAVAWEA.....	HSGNSIRTVL KL
Mouse	ANQVI	RCKAAVAWEA.....	HSGDSIRTVL KM
E. coli	M	KSRAAVAFAP.....	

3.2. Protein structure and DNA clone

Screening under high stringency of a genomic library from *Saccharomyces cerevisiae* strain X2180 with an approximately 600 bp fragment from X2180 as probe (cf. section 2), yielded a single λgt11 clone showing a strong hybridization signal. This clone contained a 4.3 kb insert that was sub-cloned into pBluescript SK(+) and sequenced, yielding a 1158 bp open reading frame encoding a 386-residue polypeptide chain characteristic of class III ADH. At the time of analysis, this structure was unknown, but soon after it was given in another report [14]. Our structure is identical to that report, except for a silent mutation at position 726, where we find C (instead of A in 14).

We also analyzed the protein structure of the purified class III alcohol dehydrogenase from Baker's yeast after reduction and carboxymethylation. Attempts at direct sequence analysis of the whole polypeptide revealed that the N-terminal residue was blocked as in a wide range of other alcohol dehydrogenases [23]. However, deblocking by treatment with trifluoroacetic acid in methanol (1:1, by vol.) for 16 h at room temperature was successful (cf. [2]) and subsequent sequence analysis revealed the deacylated start sequence to be Ser-Ala-Ala-Thr-. Together with the DNA sequence, this identifies the N-terminal structure as shown in Table II, and proves the acylated (pre-

Table 3
Enzymatic properties of yeast class III alcohol dehydrogenase

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)
Ethanol	NS	–	0.21
Octanol	2.4	1100	460
10-HDA	7.2	2700	370
12-HDDA	1.8	1800	960
HM-GSH	0.04	5300	134000
NAD^+	0.14	4100	29000

Alcohol activities were measured at pH 10.0, glutathione-dependent formaldehyde dehydrogenase activity at pH 8.0. NS, nonsaturable; 10-HDA, 10-hydroxydecanoic acid; 12-HDDA, 12-hydroxydodecanoic acid, HM-GSH, *S*-hydroxymethylglutathione.

sumably acetylated) nature of the native N-terminus. The C-terminal structure was determined by carboxypeptidase Y digestion, establishing the four last residues.

In addition, the carboxymethylated protein was cleaved by proteinases K and G, and peptides obtained were separated by reverse phase HPLC for sequence analysis. In this manner, 63% of the entire protein was checked, giving at all positions controlled, identical results with those deduced from the DNA analysis, except in one case: amino acid residue 307 was Gly by peptide analysis, but Glu from DNA analysis now and previously [14]. Thus, a strain difference within *Saccharomyces cerevisiae*, explaining this single difference between protein and nucleotide data is likely. Notably, strain differences were also found in the traditional ethanol-active yeast alcohol dehydrogenase [24]. The protein sequence of class III ADH from *Saccharomyces cerevisiae* has 71% residue identity with that from *Candida maltosa* [15] and 63% identity with that of the human class III enzyme [17].

In conclusion, protein and nucleotide data agree in defining class III yeast alcohol dehydrogenase, revealing one silent and one translated exchange between strains, apart from establishing protein start and end points, which differ in three groups (Table 2). The yeast enzymes are the ones longest at both ends, the mammalian enzymes intermediate, and the prokaryotic forms shortest, all most clearly visible at the N-terminal end (Table 2). Apart from this regular pattern, many residues close to the termini differ widely.

3.3. Enzymatic characterization

The fraction 1 and 2 preparations (Table 1) showed identical enzymatic properties against all substrates tested (Table 3).

Table 4
Comparison of enzymatic properties of class III alcohol dehydrogenases with *S*-hydroxymethylglutathione from different species

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)
Human	4	200	50,000
Rat	0.92	216	235,000
Octopus	1.5	300	200,000
D. melanogaster	6	960	160,000
S. cerevisiae	40	5300	134,000
E. coli	94	9350	100,000

Activities were measured as in Table 3. *S. cerevisiae* data are from this study, those from *E. coli*, fruit fly, octopus, rat, and the human enzymes from [1,4,8,25,26].

With ethanol, the enzyme was active only at very high substrate concentrations and did not reach saturation; with formaldehyde in the presence of glutathione the enzyme was highly active, properties typical of class III enzymes. Hydroxymethylglutathione is the best substrate. Relative to that activity, the one toward alcohols is low due to the very high K_m values for alcohols (Table 3).

3.4. Comparisons of enzymatic data and correlation with functional needs

In comparison to the human enzyme, the K_m and k_{cat} values of the yeast enzyme are significantly higher. The K_m for the human enzyme is 4 μM [25] with hydroxymethyl-glutathione, compared to 40 μM for the yeast enzyme. However, the k_{cat} values are 200 and 5300 min^{-1}, respectively (Table 4). Consequently, the specific activity is also much lower for the human (3 U/mg) than for the yeast enzyme (49 U/mg). All K_m values are higher for the yeast enzymes (0.04–0.26 mM) ([13], this work) than those for the human, rat, octopus and *Drosophila* enzymes (4–9 μM) [1,4,25,26], but the simultaneous increase in k_{cat} makes the k_{cat}/K_m ratio largely conserved for *S*-hydroxymethylglutathione (Table 4). Regarding coenzyme, the yeast enzyme is characterized by a low affinity (K_m 0.14 mM) compared to that of the human enzyme (K_m 0.009 mM) [27].

Notably, the formaldehyde concentration to which the cells of the human body can be exposed is low. Hence, it appears significant that the human and mammalian enzymes have very low K_m values, adapted to these conditions. In contrast, yeast is exposed to hazardous environments, with potentially higher content of formaldehyde. At those conditions, an enzyme with both higher K_m and higher k_{cat} values will be catalytically more efficient [28]. Interestingly, the methylotrophic yeasts are exposed to even higher formaldehyde concentrations, and consequently exhibit higher K_m values than the enzyme for *Saccharomyces*. Thus, K_m values for class III from *Candida boidinii*, *Kloeckera* and *Pichia pastoris* are from 3- to 6-fold that for *Saccharomyces* [13]. A class III form from another micro-organism, *E. coli*, also exhibits a high K_m and k_{cat} [8], resulting in a k_{cat}/K_m ratio similar to those for the yeast and animal enzymes (Table 4). Overall these data support the hypothesis of a close correlation between the class III catalytic values and the need for an efficient formaldehyde elimination in the corresponding organism, maintaining an optimal k_{cat}/K_m ratio, which appears to lead to a value of about 200,000 $mM^{-1} \cdot min^{-1}$ (Table 4). Depending on the formaldehyde concentration in their respective environments, the K_m and k_{cat} values would change in an evolutionary adaptation to acquire the highest catalytic rate with the maximal K_m value [28]. With low formaldehyde exposure, K_m values are low (and overall activity low). With less sensitive, unicellular organisms, both K_m and k_{cat} values are increased. This correspondence indicates a strong evolutionary restriction on the catalytic constants, and emphasizes the central role of class III alcohol dehydrogenase as an enzyme in formaldehyde detoxication.

Acknowledgements: Fellowships from Universitat Autònoma de Barcelona, and Subdirección General de Cooperación Científico-Técnica (Ministerio de Asuntos Exteriores, Spain) to MRF are acknowledged. This study was supported by grants from the Spanish Dirección General de Investigación Científica y Técnica (PB92-0624), the Swedish Medical Research Council (project 13X-3532), and the Comission of the European Union (BMH1-CT93:1601).

References

[1] Danielsson, O., Atrian, S., Luque, T., Hjelmqvist, L., Gonzàlez-Duarte, R. and Jörnvall, H. (1994) Proc. Natl. Acad. Sci. USA 91, 4980–4984.

[2] Parés, X., Cederlund, E., Moreno, A., Hjelmqvist, L., Farrés, J. and Jörnvall, H. (1994) Proc. Natl. Acad. Sci. USA 91, 1893–1897.

[3] Jörnvall, H. and Höög, J.-O. (1995) Alcohol and Alcoholism 30, 153–161.

[4] Koivusalo, M., Baumann, M. and Uotila, L. (1989) FEBS Lett. 257, 105–109.

[5] Burnett, K.G. and Felder, M.R. (1978) Biochem. Genet. 16, 443–454.

[6] Danielsson, O., Shafqat, J., Estonius, M. and Jörnvall, H. (1994) Eur. J. Biochem. 225, 1081–1088.

[7] Kaiser, R., Fernández, M.R., Parés, X. and Jörnvall, H. (1993) Proc. Natl. Acad. Sci. USA 90, 11222–11226.

[8] Gutheil, W.G., Holmquist, B. and Vallee, B.L. (1992) Biochemistry 31, 475–481.

[9] Ras, J., van Ophem, P.W., Reijnders, W.N.M., van Spanning, R.J.M., Duine, J.A., Stouthamer, A.H. and Harms, N. (1995) J. Bacteriol. 177, 247–251.

[10] Parés, X. and Vallee, B.L. (1981) Biochem. Biophys. Res. Commun. 98, 122–130.

[11] Estonius, M., Danielsson, O., Karlsson, C., Persson, H., Jörnvall, H. and Höög, J.-O. (1993) Eur. J. Biochem. 215, 497–503.

[12] Schütte, H., Flossdorf, J., Sahm, H. and Kula, M.R. (1976) Eur. J. Biochem. 62, 151–160.

[13] Allais, J.J., Louktibi, A. and Baratti, J. (1983) Agr. Biol. Chem. 47, 1509–1516.

[14] Wehner, E.P., Rao, E. and Brendel, M. (1993) Mol. Gen. Genet. 237, 351–358.

[15] Sasnaukas, K., Jomantiené, R., Januska, A., Lebediené, E., Lebedys, J. and Janulaitis, A. (1992) Gene 122, 207–211.

[16] Hoffman, C.S. and Winston, F. (1987) Gene, 57, 267–272.

[17] Sharma, C.P., Fox, E.A., Holmquist, B., Jörnvall, H. and Vallee, B.L. (1989) Biochem. Biophys. Res. Commun. 164, 631–637.

[18] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.

[19] Leatherbarrow, J.R. (1987) Enzfitter: A Nonlinear Regression Data Analysis Program for the IBM-PC, Elsevier Biosoft, Cambridge, UK.

[20] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.

[21] Julià, P., Farrés, J. and Parés, X. (1987) Eur. J. Biochem. 162, 179–189.

[22] Gömpel-Klein, P., Mack, M. and Brendel, M. (1981) Curr. Genet. 16, 65–74.

[23] Hjelmqvist, L., Hackett, M., Shafqat, J., Danielsson, O., Iida, J., Hendrickson, R.C., Michel, H., Shabanowitz, J., Hunt, D.F., and Jörnvall, H. (1995) FEBS Lett. 367, 237–240.

[24] Jörnvall, H. (1977) Eur. J. Biochem. 72, 425–442.

[25] Holmquist, B. and Vallee, B.L. (1991) Biochem. Biophys. Res. Commun. 178, 1371–1377.

[26] Fernández, M.R., Jörnvall, H., Moreno, A., Kaiser, R. and Parés, X. (1993) FEBS Lett. 328, 235–238.

[27] Uotila, L. and Koivusalo, M. (1989) in: Coenzymes and Cofactors. Glutathione. Chemical, Biochemical and Medical Aspects, Vol. III, Part A, (D. Dolphin et al., Eds.) Wiley, New York, p. 534.

[28] Fersht, A. (1985) Enzyme Structure and Mechanism, 2nd Edn., pp. 324–327, Freeman, New York.