Purification, Characterization, and Partial Sequence of the Glutathione-Dependent Formaldehyde Dehydrogenase from *Escherichia coli*: A Class III Alcohol Dehydrogenase†

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Received July 5, 1991; Revised Manuscript Received September 24, 1991

ABSTRACT: The glutathione-dependent formaldehyde dehydrogenase from *Escherichia coli* has been purified to homogeneity and characterized. It is a 83 000-kDa homodimer containing 4 g-atom of zinc per dimer with a specific activity of 60 units/mg toward *S*-(hydroxymethyl)glutathione and NAD^+ as substrates. Its isoelectric point, 4.4, is consistent with both its amino acid composition and chromatographic behavior on DEAE HPLC. The N-terminus is unblocked, and 47 residues from the N-terminus were sequenced. A computer search of the Swiss-Prot protein sequence data bank shows that the N-terminal sequence, MKSRAAVAFAPGKPLEIVEIDVAPXKKGEVLIKVTHTGVcetDAFGL, is homologous with the mammalian class III alcohol dehydrogenases with 27 identities when compared to the human enzyme. Like the human, rat, and rabbit enzymes, it has high formaldehyde dehydrogenase activity in the presence of glutathione and catalyzes the oxidation of normal alcohols (ethanol, octanol, 12-hydroxydodecanoate) in a reaction that is not GSH-dependent. In addition, hemithiolacetals other than those formed from GSH, including ω-thiol fatty acids, also are substrates. The wide distribution and high degree of similarity of this enzyme to the plant and animal alcohol dehydrogenases suggest that the *E. coli* enzyme is closely related to the ancestor of the plant and animal dimeric zinc alcohol dehydrogenases.

The alcohol dehydrogenases are an important family of enzymes with varying degrees of structural and functional homology (Jörnvall et al., 1987a; Kaiser et al., 1988; Eklund et al., 1990). All animal forms of this family contain enzymatically essential zinc, and the primary structure and kinetic properties of most of them have been determined. The crystallographic structure of one, the horse EE isozyme, is known (Eklund & Brändén, 1987). Among the mammalian ADHs[1] the class III form is unique, particularly in regard to its distribution and specificity (Kaiser et al., 1989). It is remarkably ineffective in oxidizing ethanol ($K_m > 3$ M), but it readily catalyzes the oxidation of long-chain primary alcohols such as *n*-octanol, 12-hydroxydodecanoic acid (Wagner et al., 1984), and 20-hydroxyleukotriene B_4 (W. P. Dafeldecker, personal communication).

The class III ADH and the glutathione-dependent formaldehyde dehydrogenase (FDH) isolated from rat liver are one and the same enzyme (Koivusalo et al., 1989), as has been confirmed for the enzyme from human liver (Holmquist & Vallee, 1991). Thus, in addition to its alcohol dehydrogenase activity, class III ADH catalyzes the oxidation of *S*-(hydroxymethyl)glutathione which is formed in a spontaneous reaction between GSH and formaldehyde and was once thought to be the only substrate for FDH. We have recently shown that in conjunction with formaldehyde other thiols such as 8-thiooctanoate and 6-thiohexanoate are nearly as active as GSH with the human (Holmquist & Vallee, 1991) and rabbit (L. Kunze, unpublished results) enzymes and that among the ADH classes this hemithiolacetal dehydrogenase activity is unique to class III ADH: neither class I nor class II ADHs are active toward them. The enzyme is also the one of this family of zinc metalloenzymes which is most conserved, and on the basis of its close homology to the plant ADHs, it has been suggested that it may be the evolutionary precursor of the class I and class II ADHs (Kaiser et al., 1988).

GSH-dependent FDH activity is widespread, being present in most, if not all, plants, animals, bacteria, and yeasts. Purified enzymes with this activity have been isolated from various yeasts, animal tissues, and bacteria and from pea seeds [for review see Uotila and Koivusalo (1989)]. All appear to be homodimers of molecular weight near 80 000. Beyond their activity toward HM-GSH, however, little is known about the substrate specificity, metal content, or structure of any of the non-mammalian enzymes that would allow comparison with the mammalian ADHs. The zinc metalloenzyme nature of the mammalian FDHs was not established until it was shown that they are identical with the class III (χχ-ADH) alcohol dehydrogenases.

The recognition that the mammalian FDHs are members of the ADH class III family raises several questions concerning the structural and evolutionary relationships among the dimeric zinc ADHs which we address here in part through the isolation, characterization, partial sequence determination, and specificity studies of the FDH from *Escherichia coli*. The

† This work was supported by a grant from the Samuel Bronfman Foundation, with funds provided by Joseph E. Seagram and Sons, Inc. W.G.G. was supported by a postdoctoral fellowship from the National Heart Lung and Blood Institute, No. 1F32HL08314-01.

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[1] Abbreviations: ADH, alcohol dehydrogenase; AMP, adenosine monophosphate; DEAE, (diethylamino)ethyl; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); FDH, formaldehyde dehydrogenase; GSH, reduced glutathione; HM-, *S*-hydroxymethyl; HM-GSH, *S*-(hydroxymethyl)glutathione; NADH, nicotinamide adenine dinucleotide (reduced form); NAD^+, nicotinamide adenine dinucleotide (oxidized form); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; 12-HDA, 12-hydroxydodecanoic acid; TFA, trifluoroacetic acid; GPC, gel permeation chromatography.

0006-2960/92/0431-475$03.00/0

FDH activity of *E. coli* has been observed previously and purified partially (Koivusalo et al., 1982), and the formation of HM-GSH from [^{13}C]formaldehyde and its oxidation to *S*-formylglutathione has previously been detected in *E. coli* by NMR studies (Mason & Sanders, 1986). We find that the enzyme is a homodimeric zinc metalloenzyme with activity toward long-chain alcohols, in addition to HM-GSH, and has a high degree of sequence homology to the human class III ADH.

Materials and Methods

Materials. NAD^+ (grade III and grade AA-1), NADH (grade III), glutathione, benzamidine, and DTT were purchased from Sigma Chemical Co., St. Louis, MO. Formaldehyde, as a methanol-free 20% aqueous solution, was purchased from Ladd Research Industries, Burlington, VT. Ethanol was obtained from U.S. Industrial Chemicals Co., New York, NY. PMSF and precast SDS–PAGE 10–20% gradient gels were from Enprotech, Hyde Park, MA. Sucrose was from BRL, Gaithersburg, MD. AMP–Sepharose was from Pharmacia/LKB, Piscataway, NJ. Other reagents were of reagent quality or better.

Purification. E. coli paste (strain B) was a generous gift from Albert Chu, E.-Y. Laboratories, San Mateo, CA, and was stored frozen at −80 °C until use. Typically, 250 g of frozen *E. coli* paste was suspended in 2 L of 20 mM Tris/10% sucrose/0.25 mM benzamidine/1 mM PMSF, pH 9.0. Cells were lysed with a Branson (Danbury, CT) Model 450 sonicator using a power setting of 7, 70% on, for four sets of 15 pulses, and stored on ice between sets of pulses. The sonicate was centrifuged at 10000*g* for 20 min. Cold saturated ammonium sulfate solution was slowly added to the recovered supernatant (approximately 2000 mL) with stirring to bring the solution to 50% saturation. The suspension was centrifuged for 20 min at 10000*g*, and solid (finely ground) ammonium sulfate was slowly added to the supernatant to 70% saturation (146 g/L) (Green & Hughes, 1955). The suspension was centrifuged at 10000*g* for 20 min, and the pellets were recovered. The pellets were dissolved in 200 mL of 10 mM potassium phosphate/0.1 mM DTT, pH 7.5, and loaded onto an AMP–Sepharose column (50-mL bed volume) equilibrated in the same buffer. The column was washed with 300 mL of buffer and then eluted with a 200-mL linear gradient of 0–150 mg/L of NADH in buffer collecting 4-mL fractions. Fractions were assayed for activity using the standard assay described below. Active fractions were combined and concentrated to about 10 mL on an Amicon PM-30 membrane. The concentrate, desalted by a 10-fold dilution with water and reconcentrated, was then chromatographed by DEAE HPLC on a Waters Protein-Pak DEAE 5PW column (Waters Chromatography Divison, Milford, MA) eluting with a 1.0 mL/min gradient of 0–300 mM NaCl in 10 mM Tris/0.1 mM DTT, pH 7.9. The protein eluted at around 150 mM NaCl. Active fractions were combined and concentrated to 100–200 μL and then applied to a 3 × 30 cm Waters X125 GPC column equilibrated and eluted with 0.1 M ammonium acetate at 0.5 mL/min. Active fractions were collected (0.5 mL), pooled, and stored at −80 °C. Separate fractions at the peak and near both half-heights were taken for amino acid analyses.

Activity Assays. Activity assays were performed by following the formation of NADH at 340 nm in 100 mM sodium phosphate, with 1.0 mM GSH, 1.0 mM formaldehyde, and 2.4 mM NAD^+ as substrates, at pH 8.0 in 1-mL cuvettes at 25 °C. One unit of activity is defined as the amount of protein necessary to catalyze the conversion of 1 μmol of NAD^+ to NADH in 1 min on the basis of a molar absorptivity of 6220 M^{-1} cm^{-1} for NADH. Assays of other hemithiolacetals were performed in 0.1 M sodium phosphate buffer at pH 8.0, and activity toward alcohols was monitored in 0.1 M glycine buffer at pH 10.0; both were at 2.4 mM NAD^+. Concentrations of thiols were established with DTNB (Anderson & Meister, 1989).

Electrophoresis Gels. Samples were prepared in 60 mM Tris/10% glycerol/1% SDS/0.05% bromphenol blue/40 mM DTT, pH 6.8 and placed in a boiling water bath for 10 min. Gradient SDS–PAGE gels were run with a running buffer of 25 mM Tris–base/200 mM glycine/0.1% SDS. Gels were fixed in 15% methanol/15% acetic acid and stained with Coomassie Blue.

Western Blots. Rabbit anti-human χχ-ADH antisera was from an earlier study (Montavon et al., 1989). Proteins from an SDS–PAGE gel were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by electroblotting in SDS–PAGE buffer containing 20% methanol. The membranes were blocked against nonspecific protein binding by treatment for 2 h in phosphate-buffered saline (PBS; 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, 2.2 g/L $Na_2HPO_4 \cdot 7H_2O$) containing 0.05% Tween 20 [Poly(oxyethylene)sorbitan monolaurate, Sigma] (PBS/Tween). The membrane was then incubated for 2 h in rabbit antiserum diluted 1:500 in PBS/Tween, washed in PBS/Tween 3 × 10 min, incubated in alkaline phosphatase labeled goat anti-rabbit (Pierce, Rockford, IL) for 1 h, and washed again in PBS/Tween. The membrane was then developed by incubating in 50 mL of a 0.1 M barbital buffer (Sigma) containing 2.5 mg of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidide and 5.0 mg of nitroblue tetrazolium. When developed sufficiently (15 min), the reaction was stopped by washing in water.

Isoelectric Focusing. A Pharmacia Phast gel system was used with samples loaded onto pH range 3.5–10.0 gels. Gels were stained with Coomassie Blue.

N-Terminal Sequence and Amino Acid Analyses. Quantitative amino acid analysis was performed using the Picotag system (Millipore, Milford, MA) based on the phenyl isothiocyanate precolumn derivatization method (Bidlingmeyer et al., 1984). N-Terminal sequencing was performed on a Beckman System 890 spinning cup sequencer as described previously (Bond & Strydom, 1989). Protein concentrations were also determined by the Bradford assay (Bradford, 1976) using the Bio-Rad reagent (Bio-Rad, Richmond, CA). Tryptophan was determined by hydrolyzing the sample with methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole prior to HCl hydrolysis. Cysteine was determined as cysteic acid following treatment of samples with performic acid prior to HCl hydrolysis.

Zinc Analyses. The zinc content of the HPLC-purified enzyme was determined using the following procedure. A Centricon 30 concentrator (Amicon, Beverly, MA) was rendered metal-free by rinsing with Milli-Q ultrafiltration purified water (Millipore), centrifugation with 10 mM HCl, and centrifugation with metal-free buffer (5 mM Hepes, 0.3 mM NaCl, pH 7.5, made metal-free by extraction with dithizone in CCl_4) (Holmquist, 1988). The sample was then diluted into 1 mL of metal-free buffer in the Centricon tube and centrifuged. The dilution/concentration step was repeated once more at which time the zinc content of the drop-through was equal to that of the buffer. The sample was then concentrated, diluted into 0.2% HNO_3 (Ultrex, J. T. Baker, Phillipsburg, NJ), and the zinc content was measured by graphite furnace atomic absorption spectroscopy on a Perkin-Elmer 5000 equipped with a HGA 500 furnace and AS 40 autosampler,

Table I: Summary of the Purification of *E. coli* FDH[a]

step	volume (mL)	concn (mg/mL)	total protein (mg)	act. (milli-units/mL)	total act. (units)	sp act. (units/mg)
sonication	2000	3.95	7900	13.4	26.8	0.0034
ammonium sulfate	1000	0.495	495	153	153	0.31
AMP–Sepharose	180	0.028	5.06	441	79.4	15.7
DEAE HPLC	12	0.115	1.38	4120	49.4	35.8
GPC	3.0	0.230	0.69	13000	39.0	56.5

[a] From 250 g of *E. coli* paste.

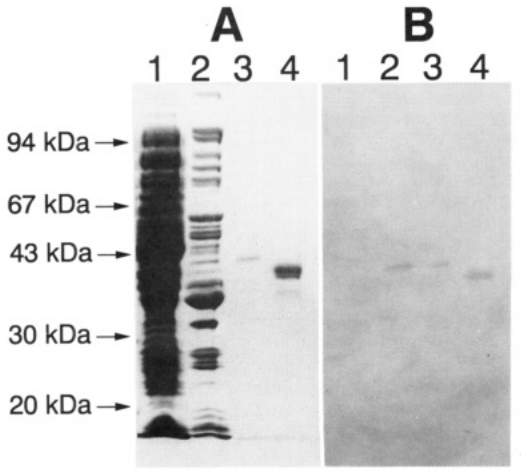

FIGURE 1: Electrophoresis gels illustrating anti-human χχ-ADH antisera cross-reactivity with the *E. coli* enzyme. Gels A and B were run on the two halves of an SDS–PAGE gel as described in the text. The gel was then divided. Half was stained with Coomassie Blue (A), and the other half was used for a Western blot with anti-human χχ-ADH antisera (B). Lane 1: 50–70% ammonium sulfate fraction. Lane 2: post-AMP–Sepharose column fraction. Lane 3: post gel permeation chromatography fraction (0.65 μg). Lane 4: human χχ-ADH (1 μg).

and the protein concentration was determined by amino acid analysis.

Sequence Analysis. The N-terminal sequence was used to search the Swiss-Prot protein sequence database. The programs PLSEARCH and PIMA.SH, version 2.1 (Smith & Smith, 1990), available at the Molecular Biology Computer Research Resource (MBCRR), Harvard Medical School, were used for the analysis of sequence homologies and alignment.

Results

Protein Purification. The method employed (Table I) provides essentially homogeneous material. The activity of the sonic extract is relatively low but is increased by ammonium sulfate fractionation, presumably due to the removal of inhibitors. This fractionation is necessary both to remove some impurities which are rather difficult to separate otherwise from the mixture and to lower the titer of AMP–Sepharose binding proteins sufficiently so as to preclude overloading the column. The AMP–Sepharose chromatography increases the specific activity 50-fold and reduces the total protein from 500 to 5 mg. The DEAE HPLC step significantly increases the purity; SDS–PAGE indicates a minor contaminating band of somewhat higher molecular weight than the subunit. The gel permeation chromatography step results in homogeneous enzyme with a specific activity of 60 units/mg.

Western Blot Analysis. Anti-human χχ-ADH antiserum cross-reacts with the *E. coli* enzyme (Figure 1). This antiserum does not cross-react with human class I or II ADHs (Montavon et al., 1989). In contrast, the antibodies of other classes of the human ADHs (class I and II) are known to cross-react to a significant extent (Montavon et al., 1989). The sensitivity for both the human and *E. coli* enzymes is approximately the same, suggesting similar structures. Even the crude *E. coli* fractions contain only a single band, suggesting that the reactivity observed is specific for this single protein and is not the result of a nonspecific antigenic response.

Table II: Comparison of the Amino Acid Composition of Three Fractions from the GPC Chromatography[a]

amino acid	fraction no. 28	29	30	mean ± SD	human (from sequence)
Cys	10.3			10.3	15
Asp	32.2	31.1	29.0	30.8 ± 1.36	23
Glu	31.6	31.4	31.1	31.35 ± 0.22	32
Ser	17.4	19.8	18.0	18.43 ± 1.07	21
Gly	50.2	51.7	52.5	51.49 ± 0.97	40
His	12.0	11.2	11.5	11.59 ± 0.32	7
Arg	17.5	17.7	18.0	17.75 ± 0.19	9
Thr	22.7	22.0	22.7	22.51 ± 0.30	23
Ala	31.9	32.3	33.4	32.56 ± 0.63	34
Pro	20.0	19.9	19.1	19.67 ± 0.41	16
Tyr	7.23	7.57	7.84	7.55 ± 0.25	7
Val	33.8	32.3	33.9	33.36 ± 0.76	39
Met	5.85	4.86	3.57	4.76 ± 0.95	7
Ile	20.0	20.0	20.2	20.08 ± 0.06	28
Leu	23.2	23.2	23.5	23.29 ± 0.13	22
Phe	14.1	14.1	13.4	13.88 ± 0.36	14
Lys	20.2	20.1	22.4	20.91 ± 1.07	32
Trp	2.98			2.98	4
sums	373	360	360	372.5	373

[a] Percent RMS deviation for amino acid analysis of fractions = 2.7%.

Isoelectric Focusing. Isoelectric focusing indicates a single sharp band at a p*I* of 4.4 for the *E. coli* enzyme, consistent with a previous preliminary report of 4.0 (Uotila & Koivusalo, 1989). This value is significantly lower than those for the human χχ-ADH (6.4; Wagner et al., 1984), rabbit χχ-ADH (6.78; L. Kunze, unpublished results), rat χχ-ADH (5.95–6.3; Juliá et al., 1987), and pea FDH (6.2; Uotila & Koivusalo, 1979).

Amino Acid Analysis. Amino acid analyses of three fractions covering the peak eluting from the GPC column (Table II) do not differ significantly, indicating the presence of homogeneous fractions. The amino acid composition differs sufficiently from human χ-ADH to account for the lower isoelectric point of the *E. coli* enzyme.

N-Terminal Sequence and Analysis. In contrast to the mammalian ADHs in which the N-terminus is acylated (Egestad et al., 1990), that of the *E. coli* enzyme is an unblocked Met, thereby allowing sequencing of 47 amino acids with one unknown and three uncertain assignments (Figure 2). Only a single polypeptide chain was observed during sequencing. The resultant sequence aligns surprisingly well with that of the human χ-ADH subunit and those of the large class of zinc-containing ADHs. The alignment scores assessing homology show that human χ-ADH is the closest match for the *E. coli* enzyme, followed in order by other mammalian χ-ADHs, the plant ADHs, and the class I and class II (human π) ADH sequences from vertebrates. The distantly related tetrameric yeast and fungus ADH sequences and the tetrameric sorbitol and threonine dehydrogenases are not included in this alignment but are homologous to a lesser extent (Jörnvall et al., 1987a). An evolutionary tree, constructed from the pairwise alignment of these N-terminal sequences (Figure 3), is consistent with the postulate that the *E. coli* enzyme is a close relative to the predecessor of the plant and animal ADHs.

Zinc Analysis. Triplicate analysis of a single preparation of the enzyme revealed an average of 2.1 g-atom of zinc/

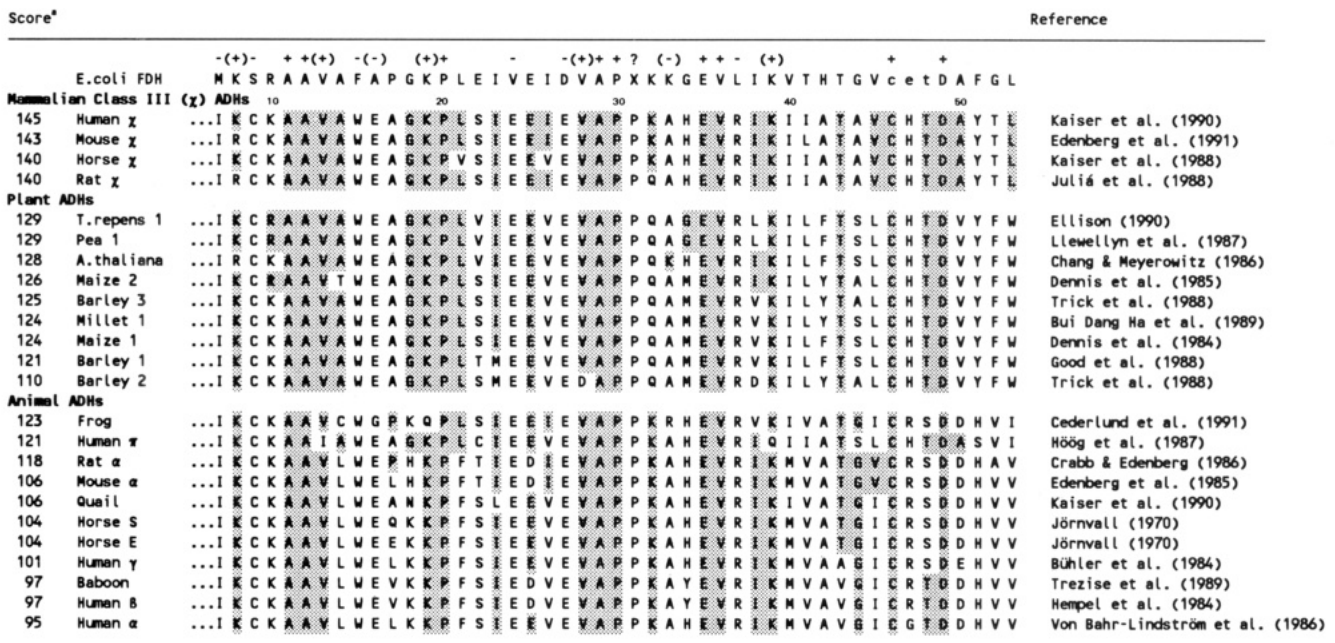

FIGURE 2: *E. coli* class III ADH N-terminal sequence and its alignment with the N-terminal sequences of other Zn ADHs. Shaded areas denote amino acids conserved between the compared sequence and that found in *E. coli* FDH. The plus signs indicate those residues which are conserved between *E. coli* FDH and all members of the class III ADHs, the plant ADHs, and the animal ADHs. The minus signs denote those amino acids which are strictly conserved in all members of the class III ADHs, the plant ADHs, and the animal ADHs but are different in the *E. coli* FDH sequence. Parentheses indicate where there are less than three sequences deviating from the marking criteria. The question mark denotes the undetermined residue, and lower-case letters denote uncertain assignments. The numbering of residues is based on that of the human class III ADH (Kaiser et al., 1988). The N-terminal sequence for the plant ADH millet 1 was from material submitted to a data bank (D. Bui Dang Ha, D. Buffard, C. Breda, and R. Esnault, submitted to EMBL/GenBank in September, 1989). Footnote *a*: alignment score with the *E. coli* sequence in roughly descending order.

Table III: Substrate Specificity of *E. coli* Class III ADH[a]

substrate	k_{cat}[b] (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
Hemithiolacetals (pH 8.0)			
HM-GSH	9350	94	99
HM-thioheptanoate[c]		>200	0.124[c]
HM-thiooctanoate	1680	280	5.9
Alcohols (pH 10.0)			
ethanol		>3000000	0.00084[d]
octanol[c]		>1000	0.00027[c]
12-HDA	400	640	0.63

[a] Reactions were as described under Materials and Methods. K_m values indicated with > indicate that no saturation could be observed and only minimal values are estimated. [b] Values were based on a specific activity of 60 units/mg toward 1 mM GSH/1 mM formaldehyde, pH 8.0/2.4 mM NAD^+/0.1 mM sodium phosphate and a molecular weight of 83 000. [c] Values were based on initial velocity measured at 1 mM substrate. [d] The value was based on initial velocity measured at 0.5 M substrate.

subunit, a value consistent with that established for the dimeric ADHs.

Characterization of Activity toward Various Substrates. The kinetic parameters of the *E. coli* enzyme toward hemithiolacetals and normal alcohols (Table III) were examined under conditions allowing comparison with those obtained for the human enzyme (see Discussion). When assayed in the presence of 1 mM formaldehyde, HM-GSH is the best substrate, but there is significant activity toward the hemithiolacetals of thiooctanoate and thioheptanoate. The ω-thiol fatty acids are nearly as good substrates for the human liver enzyme as HM-GSH. In comparison to the human enzyme, however, the K_m and k_{cat} values of the *E. coli* enzyme are considerably higher. With HM-GSH, the K_m for the human enzyme is 4 μM as compared to 94 μM for *E. coli*, while the k_{cat} values are 200 and 9350 min^{-1}, respectively. The specific activities of the enzymes, 3 units/mg for human and 60 units/mg for that from *E. coli*, reflect their respective k_{cat} values. Relative to HM-GSH, the activity toward normal alcohols is low but significant. Activity toward normal alcohols is highest with 12-HDA, with that of ethanol having a k_{cat}/K_m value at least 75-fold lower than 12-HDA.

Discussion

The physical and enzymatic characteristics, in particular the N-terminal sequence, of the *E. coli* glutathione-dependent FDH clearly identify it as a class III ADH. Prior to this study, beyond their common capacity to oxidize formaldehyde in a GSH-dependent reaction, it was not known what relationship there might be between the GSH-dependent FDH of lower species and that of mammals.

The presence of a GSH-dependent FDH in *E. coli* has been observed previously, and the enzyme responsible for the activity was partially purified and characterized (Uotila & Koivusalo, 1983). The purification procedure used here to obtain homogeneous enzyme is essentially that described for the isolation of the human class III ADH (Moulis et al., 1991) but with two additional steps. An ammonium sulfate fractionation was added as the first and GPC HPLC was added as the final step (Table I). The ammonium sulfate fractionation increases the total activity, presumably through the removal of inhibitors. The DEAE HPLC step provides nearly homogeneous enzyme with a slight contamination by a higher molecular weight contaminant that is removed in the GPC HPLC procedure. A 25% recovery of activity is achieved between the ammonium sulfate fractionation and the final product. From 8 g of soluble protein, 0.69 mg of purified enzyme is obtained. A single band on SDS–PAGE and isoelectric focusing gels, identical amino acid analysis of active fractions eluting from the GPC HPLC column (Table II), and the detection of a single N-terminal residue all indicate a high degree of purity.

Physical Characterization and Amino Acid Composition. On SDS–PAGE the protein exhibits an apparent molecular

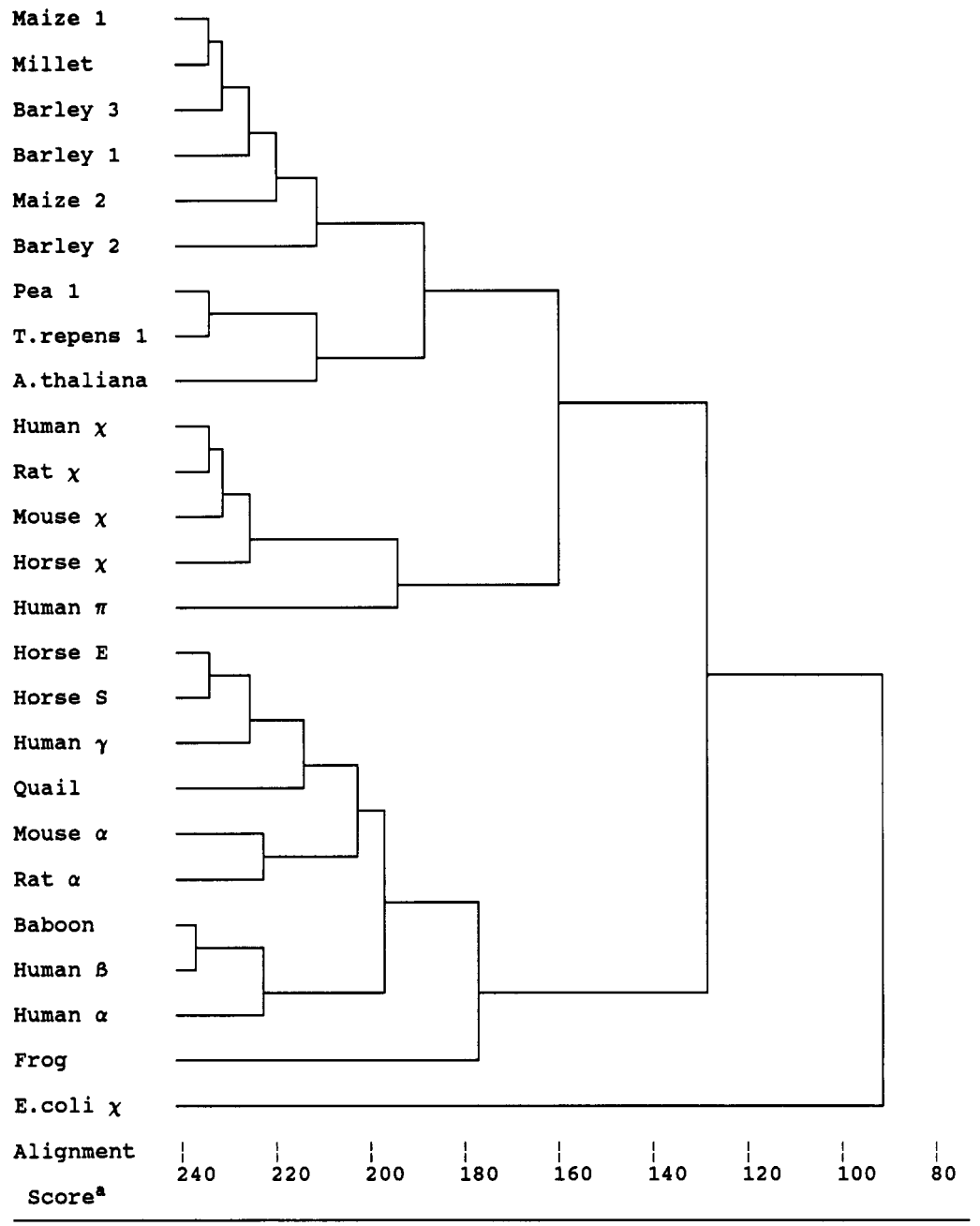

FIGURE 3: Evolutionary tree constructed by the MBCRR program, PIMA.SH, using the N-terminal sequence alignment shown in Figure 2 illustrating the major clustering characteristics of this class of proteins. This tree should be considered as only suggestive as it distorts some relationships found among the proteins when the complete sequences are used in the analysis.

weight of 41 500, somewhat higher than 40 000 for the human χ-ADH. Nondenaturing GPC HPLC demonstrates a molecular weight of about 80 000, indicating that the protein exists as a dimer, as does the human χχ-ADH (Wagner et al., 1984). The p*I* of 4.4 is close to that reported (Koivusalo et al., 1982). This value is considerably lower than the p*I* of 6.4 of the human enzyme (Koivusalo et al., 1982; Wagner et al., 1984) but consistent with the difference in amino acid composition and the salt concentration necessary for elution from the DEAE HPLC column (50 mM for the human vs 150 mM for the *E. coli* enzyme). The *E. coli* protein contains 2.1 g-atom of Zn/subunit, in agreement with a value of 2 g-atom/subunit found for all other class III ADHs and for all members of the dimeric Zn ADHs (Vallee & Auld, 1990).

Catalytic Characterization. The catalytic properties of the *E. coli* enzyme also clearly mark it as a class III ADH; it is active toward both hemithiolacetals and normal alcohols (Table III). In this regard it is quite similar to the class III ADHs from rat, human, and rabbit, all of which exhibit reactivity toward HM-GSH; those from rabbit and man have also been shown to oxidize hemithiolacetals generated from ω-thiol fatty acids (Holmquist & Vallee, 1991; L. Kunze, unpublished results). The comparison of its activity to that of the human enzyme (Table IV) clearly shows correspondence between the specificity constants (k_{cat}/K_m) of the two enzymes. Both exhibit low activity toward ethanol, a characteristic that distinguishes class III from the other animal ADHs. With the human enzyme, in spite of the low K_m for ethanol, the rate of ethanol oxidation can exceed that of HM-GSH when ethanol is 3 M or above, indicating that it is only the very high K_m, and not k_{cat}, that is the source of the low specificity constant. Both enzymes show highest activity toward HM-GSH. While this appears to indicate that the specificity between the two enzymes is very conserved, the individual kinetic parameters reveal distinct differences, the major one being the 47-fold higher k_{cat} of the *E. coli* enzyme toward

Table IV: Comparison of Human and *E. coli* Class III ADH Substrate Specificity

substrate	k_{cat}/K_m (M^{-1} min^{-1})	
	human	*E. coli*
ethanol	45[a]	84
octanol	185 000[b]	270
12-HDA	2 820 000[b]	630 000
HM-thioheptanoate	8 300 000[c]	124 000
HM-thiooctanoate	32 000 000[c]	5 900 000
HM-GSH	50 000 000[c]	99 000 000

[a] Wagner et al. (1984). [b] Moulis et al. (1991). [c] B. Holmquist, unpublished results.

HM-GSH, 9350 min^{-1}, as compared to that of the human enzyme, 200 min^{-1}. With few exceptions, the k_{cat} values for all substrates of the human enzyme are similar, near 200 min^{-1}, a value likely representing the rate-limiting dissociation of NADH after product release (Moulis et al., 1991). The much higher k_{cat} value of the *E. coli* enzyme for the hemithiolacetals, particularly HM-GSH, may be indicative of a mechanism differing from the ordered mechanism, proposed by Theorell-Chance, in which NADH dissociates first followed by rate-limiting dissociation of the GSH thiolester product.

Homology of E. coli FDH with Other Dimeric Zn-Containing ADHs. The Western blot analysis (Figure 1) demonstrates the close relationship between the human class III ADH and the *E. coli* enzyme. The anti-human χχ-ADH antibody is selective only for the *E. coli* class III enzyme and does not cross-react with other *E. coli* proteins. The N-terminal amino acid sequence, homologous to that of the mammalian class III ADHs (Figure 2), confirms this close relation. This is the first sequence information obtained for a nonmammalian GSH-dependent FDH. In comparison with the sequence of human class III ADH and its relation to that of the horse ADH EE isozyme structure (Kaiser et al., 1988), this segment contains residues involved in coenzyme binding (47, 48, and 51), substrate interactions (48), and metal binding (46). Three of the residues, Glu-35, Asp-49, and Cys-46, the latter a ligand to the catalytic zinc atom, are conserved in all zinc alcohol dehydrogenases (Jörnvall et al., 1987b) while eight residues are conserved in the plant and animal ADHs (Figure 2).

The high degree of homology with human χ-ADH [27 out of 47 residues (60%) strictly conserved] is remarkable considering the evolutionary distance. A lesser, but still highly significant, homology is seen for the plant ADHs, the sequences of which are generally obtained from c-DNA, few having been characterized at the protein level either structurally or catalytically. The GSH-dependent FDH from pea (Uotila & Koivusalo, 1979) has been studied most intensively. Though its purification scheme, dimeric nature, and molecular weight are consistent with it being a class III ADH, neither its metal content nor its activity toward normal alcohols has been determined. Due to the lack of such data or sequence information, whether the plant ADHs of Figure 2 are class III ADHs remains in question. Next in degree of homology are the animal class I and II ADHs. The frog enzyme is the one of this set which is most homologous while the human and primate class I ADHs are the least. Even the least conserved of these sequences, the human α, has 40% sequence homology with the *E. coli* N-terminal sequence.

The evolutionary tree (Figure 3) based on the ADH N-terminal sequences (Figure 2) suggests that the *E. coli* class III ADH may represent a close relative to the evolutionary precursor of the entire family of plant and animal dimeric zinc ADHs. Its sequence aligns with both the plant and animal branches. A complete amino acid sequence for the *E. coli* FDH will allow a more definitive assessment of the relationships between these proteins. However, several considerations can be proposed based upon the partial sequence available. The data suggests that FDH represents the ancestral ADH and that, even at the time at which plant and animal branches of evolution separated, the enzyme had achieved a high degree of optimization of its catalytic potential. In order for FDH to have remained so highly conserved, and given its widespread distribution in nature, it must serve a vital role in the physiology of the organisms of which it is a part, including man. On the basis of the sequence of human class III ADH and its comparison to other ADH primary sequences, it was suggested that it is the most plausible candidate to have been the precursor for the animal and plant zinc-containing ADHs (Kaiser et al., 1988). The N-terminal sequence of the *E. coli* FDH provides evidence for both of these views as well as a glimpse at what the common branch between the animal and plant ADHs may be.

Thus far, a precise role for this class III enzyme in animals or other species has not been demonstrated. Indeed, the identical enzyme (bearing different names) has now been isolated on the basis of activity toward three different substrates: ethanol, HM-GSH, and a 20-hydroxyleukotriene (Gotoh et al., 1989). The decrease in the rate of formaldehyde metabolism upon lowering the endogenous glutathione concentration by treatment with diethyl maleate suggests that the primary function of GSH-dependent FDH in *E. coli* is in the metabolism of formaldehyde formed endogenously (Uotila & Koivusalo, 1983). A possible role in normal alcohol metabolism and in detoxification of exogenous formaldehyde remains a possibility. Formaldehyde is formed from a wide variety of normal metabolic processes as well as from exogenous compounds (Uotila & Koivusalo, 1989) and constitutes between 0.05 and 0.5 μmol/g wet weight of rat tissues (Heck et al., 1982). Like mammalian tissues the GSH level is near 1 mM (Mason et al., 1986), and thus, the components required for reaction are present. However, in addition to the GSH-dependent FDH, several enzymes can metabolize formaldehyde. These include the nonspecific formaldehyde dehydrogenases, catalase, and several tetrahydrofolate-dependent enzymes.

ACKNOWLEDGMENTS

We thank Dr. Daniel Strydom for help in protein sequencing and Dr. Lisa Kunze for the zinc analyses.

Registry No. ADH, 9031-72-5; HM-GSH, 32260-87-0; 12-HDA, 505-95-3; HM-thioheptanoate, 137394-51-5; HM-thiooctanoate, 137394-52-6; zinc, 7440-66-6; ethanol, 64-17-5; octanol, 111-87-5.

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