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Inactivation of Aldophosphamide by Human Aldehyde Dehydrogenase Isozyme 3

Francesco Giorgianni, Peter K. Bridson,† Brian P. Sorrentino,‡ Jörg Pohl§ and Raymond L. Blakley*‖*

*Department of Molecular Pharmacology and ‡Departments of Biochemistry and Hematology/Oncology, St. Jude Children's Research Hospital, Memphis, TN 38105, U.S.A.; †Department of Chemistry, The University of Memphis, Memphis, TN 38152, U.S.A.; and §ASTA Medica AG, D-60314 Frankfurt am Main, Germany

ABSTRACT. Tumors resistant to chemotherapeutic oxazaphosphorines such as cyclophosphamide often overexpress aldehyde dehydrogenase (ALDH), some isozymes of which catalyze the oxidization of aldophosphamide, an intermediate of cyclophosphamide activation, with formation of inert carboxyphosphamide. Since resistance to oxazaphosphorines can be produced in mammalian cells by transfecting them with the gene for human ALDH isozyme 3 (hALDH3), it seems possible that patients receiving therapy for solid tumors with cyclophosphamide might be protected from myelosuppression by their prior transplantation with autologous bone marrow that has been transduced with a retroviral vector causing overexpression of hALDH3. We investigated whether retroviral introduction of hALDH3 into a human leukemia cell line confers resistance to oxazaphosphorines. This was examined in the polyclonal transduced population, that is, without selecting out high expression clones. hALDH3 activity was 0.016 IU/mg protein in the transduced cells (compared with 2×10^{-5} IU/mg in untransduced cells), but there was no detectable resistance to aldophosphamide-generating compounds (mafosfamide or 4-hydroperoxycyclophosphamide). The lack of protection was due, in part, to low catalytic activity of hALDH3 towards aldophosphamide, since, with NAD as cofactor, the catalytic efficiency of homogeneous, recombinant hALDH3 for aldophosphamide oxidation was shown to be about seven times lower than that of recombinant hALDH1. The two polymorphic forms of hALDH3 had identical kinetics with either benzaldehyde or aldophosphamide as substrate. Results of initial velocity measurements were consistent with an ordered sequential mechanism for ALDH1 but not for hALDH3; a kinetic mechanism for the latter is proposed, and the corresponding rate equation is presented. BIOCHEM PHARMACOL **60**;3:325–338, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. kinetic mechanism; mafosfamide; retroviral transduction; 4-hydroperoxycyclophosphamide; protection from oxazaphosphorines; aldophosphamide preparation

Cyclophosphamide and related oxazaphosphorines are clinically important antineoplastic agents. Cyclophosphamide is activated by hepatic cytochrome P450 to produce an equilibrium mixture of aldophosphamide and its tautomers, the *cis* and *trans* isomers of 4-hydroxycyclophosphamide [1]. Aldophosphamide undergoes a non-enzymic β-elimination reaction to give the active antineoplastic agent phosphoramide mustard. Some ALDHs¶ catalyze the NAD-dependent oxidation of aldophosphamide to carboxyphosphamide, a biologically inactive compound, thereby aborting the spontaneous generation of phosphoramide mustard [2]. From this evidence, and from the observed overexpression of ALDHs in drug-resistant tumor cell lines [3–7], it has been proposed that ALDH overexpression confers resistance to oxazaphosphorine drugs.

Of several isozymes of hALDH that have been identified, the predominant isozyme in the liver cytosol (hALDH1) appears to have the greatest activity for aldophosphamide oxidation [8]. However, when DNA expression vectors were constructed using hALDH1 cDNA and transfected or transduced into cells, variable results were obtained. Whereas *transfection* into hamster lung fibroblast cell lines resulted in 20-fold increased drug resistance, associated with a high copy number of the gene [9], retroviral *transduction* of the hALDH1 gene into human leukemia cell lines [10, 11], or into the L1210 murine leukemia cell line [10], resulted in lower levels of resistance. In unselected polyclonal populations of transduced cells, expression was very low or undetectable [12, 13]. Subsequently, it was shown that mRNA for hALDH1 transcribed from the proviral DNA template is unstable, resulting in very poor expression of hALDH1 in transduced cells [12]. Similarly,

‖ Corresponding author: Dr. Raymond L. Blakley, 5777 Elizabeth Ann Way, Fort Myers, FL 33912. Tel. (941) 466-8424; FAX (941) 466-9482; E-mail: RAYB1926@aol.com

¶ *Abbreviations*: ALDH, aldehyde dehydrogenase; hALDH1, human aldehyde dehydrogenase isozyme 1; hALDH3, human aldehyde dehydrogenase isozyme 3; 4-HAP-Sepharose, 4-hydroxyacetophenone Sepharose; PCR, polymerase chain reaction; MES, 2-(*N*-morpholino)ethanesulfonic acid; and MSCV, murine stem cell virus.

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expression of transduced hALDH1 in CCRF-CEM cells was found to be very poor.*

When a DNA expression vector was used to *transfect* hALDH3 cDNA into hamster lung fibroblast cell lines, resistance to mafosfamide increased 13-fold and that to 4-hydroperoxycyclophosphamide 2.7-fold [14], but *transduction* experiments with a retrovirus have not been reported. On the other hand, hALDH3 purified from gastric mucosa has been reported to have no activity for aldophosphamide oxidation [5], and that from tumors has little activity [5–7].

Although transfection with ALDH1 or ALDH3 expression vectors can lead to cellular resistance to oxazaphosphorines, it is not clear whether similar results can be obtained in primary bone marrow cells. The ability to modify bone marrow cells to overexpress ALDH isozymes could be useful in protecting hematopoiesis from the toxic effects of chemotherapy. To study the feasibility of this approach, we developed retroviral vectors containing the hALDH3 cDNA to determine whether protection could be obtained in a cell line of hematopoietic origin. A retroviral vector was chosen since bone marrow stem cells cannot be transfected directly, both because of their rarity and because of the low efficiency of integration with transfected constructs. In contrast, retroviral vectors efficiently integrate in primary hematopoietic cells, give stable expression of genes in transduced cells, and are the optimal system for bone marrow cell gene therapy [15]. On the other hand, copy numbers of introduced genes in retrovirus-transduced cells are low compared with those in transfected cells [16].

We found that although ALDH3 was expressed, transduced cells were not resistant, and the reasons for this were investigated. Kinetic studies revealed that the purified recombinant form of normal tissue hALDH3 did oxidize aldophosphamide, with either NAD or NADP as coenzyme. Furthermore, k_{cat} with aldophosphamide as substrate was similar to that for hALDH1. However, activity levels in extracts of transduced cells were low, and K_m was very high for aldophosphamide oxidation by hALDH3. The significance of these results as they impinge on the potential role of ALDH genes as drug resistance genes is discussed.

MATERIALS AND METHODS

Materials

L-Glutamine, penicillin, streptomycin, Dulbecco's modified Eagle's medium, and fetal bovine serum were obtained from BioWhittaker; leupeptin, pepstatin, phenylmethylsulfonyl fluoride, NAD, NADP, ampicillin, DNase I, lysozyme, HEPES, MES, Sepharose 4B, Q-Sepharose, carboxymethyl-Sepharose, and DEAE-Sepharose, from the Sigma Chemical Co.; benzaldehyde, propionaldehyde, dithiothreitol, and 4-hydroxyacetophenone, from the Aldrich Chemical Co.; and mafosfamide and 4-hydroperoxycyclophosphamide, from ASTA Medica. Isopropyl-β-d-thiogalactoside was obtained from Calbiochem; and restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs, Promega, and Gibco-BRL. Primers for PCR were synthesized in the Center for Biotechnology, St. Jude Children's Research Hospital. Competent cells of *Escherichia coli* strains were purchased from Stratagene. Other materials were obtained from the suppliers indicated in the text.

Cloning of hALDH1 cDNA

hALDH1 cDNA in the plasmid pT7–7 was provided by Dr. H. Weiner, Purdue University. The cDNA was excised and cloned into pDS5. The sequence of the resulting plasmid (pDS5-hALDH1) was as expected except for insertion of an additional base before the initiation codon of the cDNA. This did not adversely affect protein expression from the gene, as indicated by the fact that the expressed protein comprised 27% of the soluble protein from bacteria.

Cloning of hALDH3 cDNA

The hALDH3 cDNA (provided by Dr. Alan J. Townsend, Biochemistry Department, Bowman Gray School of Medicine, Wake Forest University Comprehensive Cancer Center) was released from the ΔpCEP-hALDH3 mammalian expression vector [14] by digestion with *Xho*I, which cuts at both ends of an ~1.5-kb fragment containing the cDNA for hALDH3. After gel purification of this fragment, it was ligated into the *Xho*I site of the MSCV-TK-Neo retroviral vector [17]. JM109 transformants were picked and analyzed for the presence and correct orientation of the cDNA. A plasmid prepared from one clone containing cDNA in the correct orientation was sequenced through the entire insert, including the hALDH3 coding region, to verify the sequence. For expression in *E. coli*, hALDH3 was cloned into pDS7, derived from pDS5 [18] by introduction of a polylinker region. When it was found subsequently that the yield of hALDH3 was poor from *E. coli* JM109 transformed with pDS7-hALDH3, the cDNA for hALDH3 was cloned into pET-26b(+) (Novagen).

Comparison of the sequence of the cDNA with the reported sequence [19] indicated one discrepancy. We, therefore, isolated hALDH3 cDNA by PCR amplification from a human stomach cDNA pool (QUICK-Clone™ cDNA; Clontech Laboratories) using primers with the following sequences: 5′-TGAGCAAGATCAGCGAGGCCGTGAA-3′ and 5′-CTGACCTTCAGGCCTTCATCATTC-3′. Sequencing of this PCR product confirmed the one-base difference from the published sequence.

DNA Sequencing

This was carried out by the St. Jude Children's Research Hospital Center for Biotechnology. DNA sequencing reactions were performed on a Beckman Biomek robotic system

* Giorgianni F, Sorrentino BP and Blakley RL, unpublished results.

using standard dye-terminator chemistry, *Taq* polymerase, 0.3 to 0.5 pmol of template DNA, 20 pmol of primer, and thermal cycling conditions described by the vendor (Perkin Elmer/Applied Biosystems Division). Reaction products were resolved on Perkin Elmer/Applied Biosystems Division model 373 and 377 automated DNA sequencers.

Cells and Cultures

The human 293 T cell line was grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The human lymphoid cell line CCRF-CEM was grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. Cells were grown at 37° in a humidified atmosphere with 5% CO_2.

Transduction of CCRF-CEM Cells with the MSCV-hALDH3 Retroviral Vector

293 T cells (1×10^7) were co-transfected by calcium phosphate precipitation with 30 μg of MSCV-hALDH3 retroviral expression vectors and 30 μg of pEQPAM3 packaging vector DNA [20], provided by Dr. Elio Vanin (Department of Experimental Hematology, St. Jude Children's Research Hospital). Twenty-four hours after transfection, the medium was changed, and 48 hr later the conditioned medium was harvested, passed through a 0.45-μm filter, and applied with 6 μg/mL of polybrene to CCRF-CEM cells (1×10^6).

Forty-eight hours after the cells had been transduced, the medium was replaced with RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1.0 mg/mL of active G418. After a selection period of 10–14 days the medium was replaced with RPMI 1640 containing no selection drug, and the cells were allowed to undergo 4–5 doublings before being used for growth inhibition studies.

Growth Inhibition Studies

CCRF-CEM cells at a density of 4×10^5/mL were centrifuged down and resuspended in serum-free RPMI 1640 medium at a density of 1.0×10^5/mL. Mafosfamide or reduced 4-hydroperoxycyclophosphamide was added at various concentrations to cell suspensions, and the resulting mixtures were incubated for 20–25 min at 37° in a tissue culture incubator. After exposure to drug, the cells were centrifuged, the supernatant solution was discarded, and the cells were washed in serum-free RPMI 1640 medium. Finally, the cells were resuspended in complete growth medium at a cell density of 1.0×10^5/mL. The cells were counted after 2–3 doublings (48 hr), and the results were used to calculate the IC_{50} values as described previously [21].

Activity of hALDH3 in Transduced Cells

Transduced CCRF-CEM cells (1×10^7) were collected by centrifugation, washed with PBS, and resuspended in 500 μL of ice-cold PBS containing 2 mM dithiothreitol, 2 mM EDTA, leupeptin (0.6 mg/L), pepstatin (0.6 mg/L), and phenylmethylsulfonyl fluoride (150 mg/L). Cells were sonicated three times for periods of 4–5 sec each time. Assay of the enzymes was carried out as described in the sections on purification of the recombinant enzymes except that they were conducted at room temperature (23°) using a microcell (1-cm light path). Cell extract containing 50 μg protein was used in each assay.

Western Analysis

The procedure was similar to that used previously [21] except that blocking was carried out overnight at 5° in 5% nonfat milk, and the blot was then washed and probed for 1 hr with a 1:2000 dilution of rabbit anti-rat ALDH1 antiserum [12] or a 1:10,000 dilution of rabbit anti-hALDH3 antiserum (HTI Bio-Products), followed by washing. The membrane was probed for 1 hr with horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:50,000; Amersham Life Science Inc.). To confirm equal sample loading in each lane, the blots were then washed extensively and reprobed for 1 hr with rabbit anti-human actin antiserum (1:1000; Sigma), washed, and developed.

Southern Analysis

Samples (15–20 μg) of genomic DNA isolated from cells by the use of the Puregene DNA Isolation Kit (Gentra Systems) were digested overnight with 30 U of *Asc*I at 37°. The digested DNA was electrophoresed overnight at 24 V on a 1% agarose gel (14 × 11 cm), and then was transferred to a Hybond-N^+ membrane (Amersham Life Science) using the downward transfer method [22]. Blots were hybridized with a ^{32}P-labeled neo^R probe. This was prepared by digesting 40 μg of Harvey-TK-Neo retroviral vector [23] with 40 U each of *Bsp*120I and *Mlu*I, and performing gel purification. The neo^R DNA (100 ng) was radiolabeled using the oligo-labeling kit from Pharmacia Biotech and [γ-^{32}P]dCTP (Amersham Life Science). Excess label was removed with a ProbeQuant G-50 Micro Column (Pharmacia Biotech). The final specific activity of the probe was 1×10^9 cpm/μg. The membrane was hybridized with the probe overnight at 50°, washed once in 1 × SSC (standard saline citrate), 0.3% SDS for 1 hr at 65°, then twice in 0.2 × SSC, 0.3% SDS at 65° for 1.5 hr each. Then the membrane was exposed to a phosphor screen. The image was developed after 2 days on a Molecular Dynamics PhosphorImager: 445 SI.

Purification of hALDH1

Transformation of JM109 *E. coli* with pDS5-hALDH1, growth in LB medium supplemented with ampicillin, induction of expression, and extraction of cells were done essentially as described previously [21]. The extract was treated with 0.2 vol. of 10% protamine sulfate (pH 6.0), which was added slowly with stirring. After removal of the precipitate by centrifugation at 30,000 *g* for 30 min, the solution was loaded on a CM-Sephadex column (2.5 × 45 cm) equilibrated with buffer A (30 mM sodium phosphate, 2 mM EDTA, 2 mM dithiothreitol, pH 6.0). The fractions containing the enzyme, which was not retained by the column, were combined and loaded onto a DEAE-Sepharose column (2.5 × 45 cm) equilibrated with buffer B (30 mM sodium phosphate, 2 mM EDTA, 2 mM dithiothreitol, pH 7.0), and the column was washed with the same buffer. The enzyme, recovered in the wash, was loaded onto a column of 4-HAP-Sepharose, synthesized according to Ghenbot and Weiner [24]. After loading, the column (2.5 × 30 cm), which had been equilibrated previously with buffer C (20 mM sodium phosphate, 10 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, pH 7.4), was washed with 2 L of buffer C, and the enzyme was eluted with buffer C containing 10 mM 4-hydroxyacetophenone. Fractions containing the enzyme were combined, the volume was reduced by ultrafiltration to about 4 mL, and the resulting enzyme solution was passed through a G75-Sephadex column (1.5 × 50 cm) equilibrated with buffer D (50 mM HEPES buffer, 2 mM EDTA, 2 mM dithiothreitol, pH 7.0) and washed with the same buffer. The enzyme was stored at −80° in buffer D containing 5% (v/v) glycerol. Enzyme activity was measured spectrophotometrically (340 nm) at 25° in 50 mM HEPES buffer, pH 7.5, supplemented with 2 mM EDTA, 1 mM dithiothreitol, 1 mM NAD, and 200 μM propionaldehyde.

Purification of hALDH3

BL21 (DE3) *E. coli* transformed with pET-26b(+)-hALDH3 was grown in 8 L of LB medium containing kanamycin (30 mg/L) at 30°. Expression was induced by 1 mM isopropyl-β-D-thiogalactoside, and harvested cells were resuspended in 0.5 L of buffer containing 30 mM Tris–HCl, 2 mM EDTA, 2 mM dithiothreitol, and 20% sucrose, pH 8.5. The resulting cell suspension was stirred at room temperature for 30 min and centrifuged (10,000 *g*) for 20 min, and the supernatant was removed and placed on ice. The pellet was resuspended in 0.5 L of 5 mM $MgSO_4$ solution, stirred at 0° for 30 min, and centrifuged for 30 min (10,000 *g*), and the supernatant was collected and combined with the previous extract. Purification was carried out by a modification of the method of Rose *et al.* [25]. The extract was loaded onto a Q-Sepharose column (1.5 × 45 cm) equilibrated with buffer D. After extensive washing with the same buffer (20 × the column volume), the enzyme was eluted with a KCl gradient generated with 250 mL of buffer D in the mixer and 250 mL of 0.5 M KCl in buffer D in the reservoir. The fractions containing the enzyme were combined, diluted by the addition of 1 vol. of cold, deionized water, and loaded onto a column (1.5 × 30 cm) of 5′-AMP-Sepharose 4B equilibrated with buffer E (25 mM potassium phosphate, pH 7.5, containing 2 mM EDTA, 2 mM dithiothreitol). After extensive washing with the same buffer (20 × the column volume), the enzyme was eluted with buffer E containing 0.5 mM NAD. Fractions containing the enzyme were combined, and the enzyme was stored in buffer E containing 5% glycerol. To remove NAD from a sample (3 mL, 1.2 mg/mL), it was passed through a G75-Sephadex column (1.5 × 50 cm) equilibrated with buffer D. This sample was stored at −80° in elution buffer containing 5% (v/v) glycerol and used for enzyme kinetics studies. Activity of samples during purification was measured spectrophotometrically (340 nm) at 25° in 66 mM sodium phosphate buffer, pH 8.5, containing 2 mM EDTA, 2 mM NADP, and 2 mM benzaldehyde.

N-Terminal Sequencing of hALDH3

A 5-μg sample of purified, denatured hALDH3 (100 pmol) was run on a 12% SDS–PAGE gel, and the protein was electroblotted onto an Immobilon P transfer membrane. The membrane was stained with Coomassie Blue and washed in 50% methanol, and the hALDH3 protein band was cut out of the membrane for amino-terminal protein sequencing by the Center for Biotechnology, St. Jude Children's Research Hospital. A Perkin-Elmer Applied Biosystems Procise 494 protein sequencer was used with standard cycles as supplied by the manufacturer.

Mass Spectrometry Analysis of Recombinant hALDH3

The molecular weight of recombinant hALDH3 was determined at the Charles B. Stout Mass Spectrometry Laboratory at the University of Tennessee, Memphis, using the Voyager DE-RP matrix-assisted laser desorption-ionization time-of-flight (MALDI–TOF) instrument (PerSeptive Biosystems Inc.). The measurement was done in the linear mode combined with delayed extraction, using sinapinic acid as matrix. The mass scale was calibrated with bovine serum albumin.

^{31}P NMR Analysis of Product Formation from Mafosfamide and 4-Hydroperoxycyclophosphamide

Aldophosphamide was generated by hydrolysis of mafosfamide or by reduction of 4-hydroperoxycyclophosphamide by modifications of previously published methods [26, 27]. The pH of the reaction mixtures did not change significantly during the reaction, except in the case of the reduction of 4-hydroperoxycyclophosphamide by thiosulfate, where it was necessary to adjust the pH at intervals. ^{31}P NMR spectra were recorded on a Varian VXR300 spectrometer operating at 121.4 MHz, and D_2O (final

concentration 10%) provided a deuterium lock. A 5000 Hz spectral width was recorded, with broad-band proton decoupling. Spectra were obtained by averaging 200 transients, using a 45-degree pulse and 2-sec acquisition time, with no further delay between pulses. Products were identified on the basis of their published chemical shifts [26], and concentrations were calculated from the integrated peak areas.

Preparation of Aldophosphamide for Enzyme Studies

Aldophosphamide can be generated *in vitro* by hydrolysis of mafosfamide, a reaction highly dependent on pH [28]. ^{31}P NMR indicated that at pH 5.0 in 50 mM MES buffer no hydrolysis took place within 24 hr at room temperature. At pH 7.4 in 70 mM sodium phosphate at 25°, mafosfamide reaction mixtures contained the interconverting tautomeric products, *cis-* and *trans*-4-hydroxycyclophosphamide and aldophosphamide mixed with unreacted *cis-* and *trans*-mafosfamide, and the product of aldophosphamide breakdown, phosphoramide mustard (Fig. 1A). The other undetected breakdown product, acrolein, would also be present. Aldophosphamide concentration slowly increased over 100 min, but the low concentration, uncertainty about its value at any given time (except by ^{31}P NMR analysis), and the presence of breakdown products made this a poor method to prepare aldophosphamide for kinetic studies.

Reduction of 4-hydroperoxycyclophosphamide with sodium thiosulfate also produces *cis-* and *trans*-4-hydroxycyclophosphamide and aldophosphamide [27]. Phosphoramide mustard and acrolein also form at some pH values. When 4-hydroperoxycyclophosphamide was reduced by a 5-fold molar excess of sodium thiosulfate at pH 5.0 in 50 mM MES buffer, the products formed in a 10-min incubation at 25° consisted of aldophosphamide (14.9%), *cis*-4-hydroxycyclophosphamide (51%), and *trans*-4-hydroxycyclophosphamide (34%). At pH 5 this mixture of tautomers remained unchanged in composition for a period of 24 hr at room temperature. However, at pH 7.4, the aldophosphamide concentration in the reaction mixture decreased with a half-life of 68 min, with formation of phosphoramide mustard (Fig. 1B). In addition, we found that aldophosphamide prepared by this method caused enzyme inactivation, and although inclusion of dithiothreitol in the assay mixture prevented this, sulfhydryl reagents react with aldophosphamide [26]. In addition, thiosulfate can react with the hydroperoxy group of the starting material to form a 4-thiosulfate derivative of cyclophosphamide, and with chloroethyl groups of oxazaphosphorines to form *S*-alkyl derivatives [27].

^{31}P NMR examination of the products when 4-hydroperoxycyclophosphamide was reduced by dimethyl sulfide, according to the procedure of Borch *et al.* [29], showed that, after a 20-min incubation at room temperature at pH 5.0 in MES buffer, the concentration of aldophosphamide was 14.9 ± 0.8% of the total phosphorus-containing compounds, the remainder being the tautomers with which it

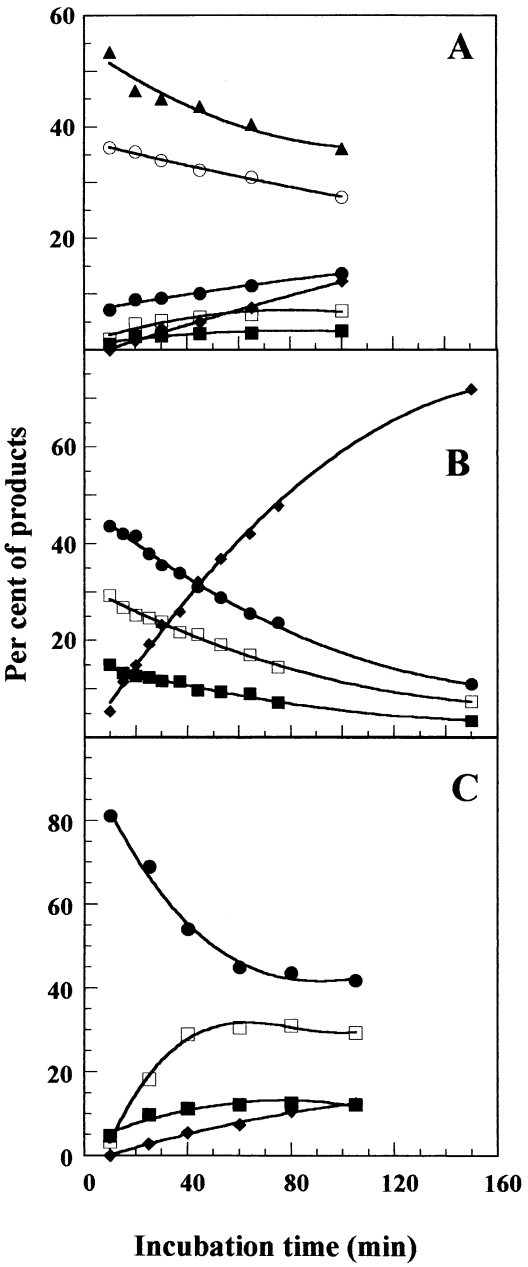


FIG. 1. Time course of formation of products from mafosfamide and 4-hydroperoxycyclophosphamide in 70 mM sodium phosphate buffer, pH 7.4, at 25°. Panel A: hydrolysis of 40 mM mafosfamide. Panel B: reduction of 25 mM 4-hydroperoxycyclophosphamide by 96 mM sodium thiosulfate. Panel C: reduction of 40 mM 4-hydroperoxycyclophosphamide by 136 mM dimethyl sulfide. Key: (▲) *cis*-mafosfamide; (○) *trans*-mafosfamide; (●) *cis*-4-hydroxycyclophosphamide; (□) *trans*-4-hydroxycyclophosphamide; (♦), phosphoramide mustard; and (■) aldophosphamide.

was in rapid equilibrium. There was no change in the concentration of aldophosphamide, or the formation of phosphoramide mustard or other by-products, over 24 hr at room temperature. Reduction at pH 7.4 produced a maximum concentration of aldophosphamide (12% yield) after 40 min with no significant further change up to 100 min

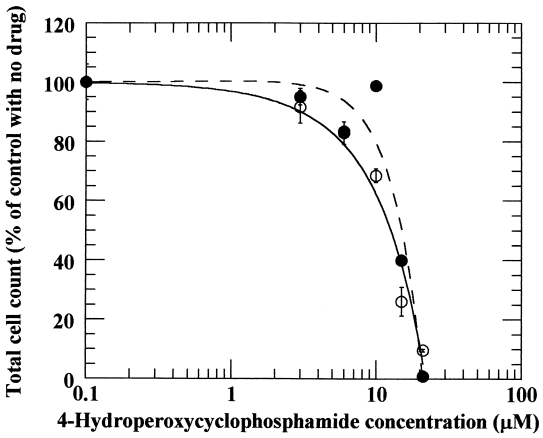


FIG. 2. Growth inhibition of CCRF-CEM cells by 4-hydroperoxycyclophosphamide. Experiments were performed as described in Materials and Methods. Data points are the means of values from three experiments, and standard errors are shown by the error bars. Where no error bar is visible, it is within the data symbol. Key: (○) untransduced cells; and (●) cells transduced with the MSCV-hALDH3 vector.

(Fig. 1C). This aldophosphamide preparation caused no enzyme inactivation under assay conditions. One reason for this was probably the significantly slower rate of formation of phosphoramide mustard and, by inference, acrolein, than with sodium thiosulfate reduction.

For routine preparations, 4-hydroperoxycyclophosphamide was dissolved in 0.5 mL of 50 mM MES buffer, pH 5.0, at a concentration of about 70 mM, and reduction was carried out by incubation with a 5-fold molar excess of dimethyl sulfide at room temperature for 20 min. The solution was usually kept on ice for use within the next 24 hr, but the product was stable when the solution was kept frozen at -80°. Since ^{31}P NMR showed that the yield of aldophosphamide was 14.9 ± 0.8%, the remainder being the tautomeric forms with which it was in equilibrium, this relationship was used to calculate the aldophosphamide concentration in stock solutions.

Determination of Steady-State Kinetic Parameters

The steady-state kinetic parameters for hALDH1 with propionaldehyde and NAD as substrates were determined at 25° and pH 7.4 in 50 mM HEPES buffer containing 2 mM EDTA, with substrate and cofactor concentrations ranging from 4 to 200 μM. The program LEONORA [30] was used to fit the recorded initial velocities to the rate equation for a sequential reaction mechanism.

The apparent K_m for aldophosphamide was determined using 70 mM sodium phosphate, containing 2 mM EDTA, at pH 7.4. The concentrations of aldophosphamide ranged from 40 to 800 μM with a 1000 μM fixed concentration of NAD. Rates were fitted to the Michaelis–Menten equation using the Kaleidagraph software package (Synergy Software).

The steady-state kinetic parameters of polymorphs of

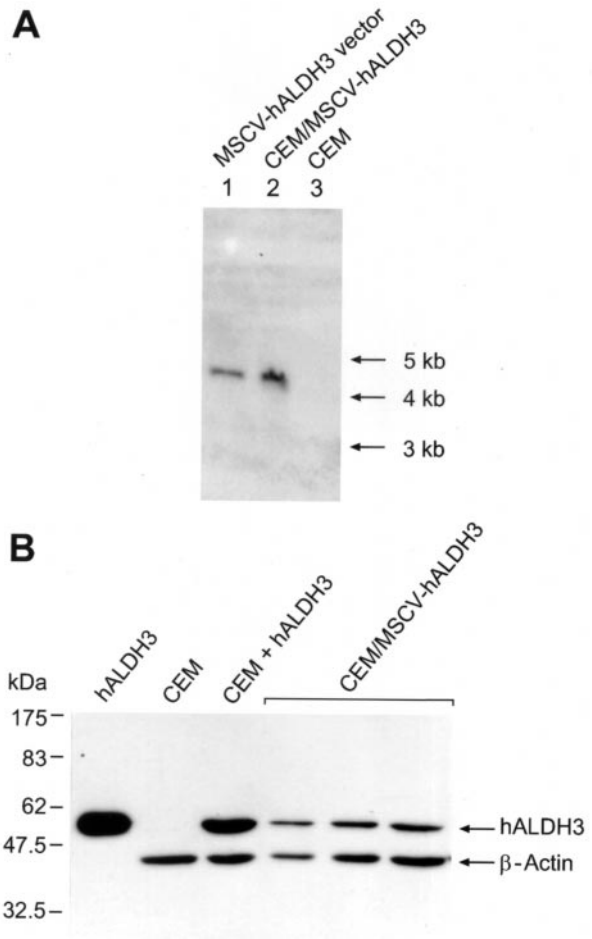


FIG. 3. (A) Southern analysis of extracts of transduced CCRF-CEM cells. Lane 1, *Asc*I-digested MSCV-hALDH3 vector (10 pg); Lane 2, *Asc*I-digested genomic DNA (30 μg) from cells transduced with MSCV-hALDH3; Lane 3, *Asc*I-digested genomic DNA (30 μg) from untransduced cells. The predicted size of the MSCV-hALDH3 fragment is 4.19 kb. (B) Western analysis of hALDH3 in extracts of CCRF-CEM cells. Lane 1, 25 ng of purified recombinant hALDH3; Lane 2, extract (40 μg protein) of untransduced cells; Lane 3, extract (40 μg protein) of untransduced cells and 25 ng of purified hALDH3; Lanes 4–6, extract of cells transduced with MSCV-hALDH3, containing 10, 20, and 40 μg protein, respectively.

hALDH3 for benzaldehyde with either NAD or NADP as cofactor were determined spectrophotometrically at 25°. Two buffers were used: either 66 mM sodium phosphate containing 2 mM EDTA, pH 8.5, or 100 mM sodium phosphate containing 2 mM EDTA, pH 7.4. The former corresponds to frequently used assay conditions, whereas the latter corresponds better to physiological conditions. The concentrations of benzaldehyde ranged from 50 to 2000 μM for the 328A, and 100 to 3600 μM for the 328P polymorph. NADP concentrations were 100-2000 μM and NAD concentrations 10–200 μM. The program LEONORA [30] was used to fit initial velocities to rate equations for sequential or ping-pong (substituted enzyme) mechanisms, with and without substrate inhibition. The best fit was obtained for the equation for the ping-pong mechanism with inhibition by benzaldehyde.

TABLE 1. Purification of recombinant hALDH1

Purification step	Volume (mL)	Protein (mg)	Activity (IU)*	Specific activity (IU/mg)	Yield (%)
Cell extract†	90	1078	235	0.298	
Protamine sulfate	103	1504	361	0.240	100
CM-Sephadex	128	551	211	0.382	90
DEAE-Sepharose	52	207	156	0.753	66
4-HAP-Sepharose	77	102	113	1.11	48
G75-Sepharose	30	87	97	1.11	41

*International units: the amount oxidizing 1 μmol substrate/min. Enzyme activity was assayed at 25° in 50 mM HEPES, pH 7.5, containing 2 mM EDTA, 1 mM dithiothreitol, 1 mM NAD, 200 μM propionaldehyde.

†Cell extract was obtained from 10 g of cell pellet.

The apparent K_m values for the oxidation of aldophosphamide by the hALDH3 polymorphs were calculated from rates measured at 25° using either buffer system in the presence of 500 μM NAD or 2000 μM NADP. The concentrations of aldophosphamide used ranged from 400 to 4000 μM. Rates were fitted to the Michaelis–Menten equation.

RESULTS

Sequence Analysis of hALDH3 cDNA

Sequencing of the cloned hALDH3 cDNA revealed a difference from the published sequence [19], which corresponded to the amino acid substitution P328→A for our determined sequence (numbering is for the protein as isolated, i.e. lacking the amino-terminal methionine). When we amplified hALDH3 cDNA from a cDNA pool from stomach tissue, the C→G change at nucleotide 985 in codon 328, resulting in a P→A substitution in the protein sequence, was again found. This mutation at nucleotide 985 occurs in about 25% of both Caucasians and Orientals [31], and therefore both of these polymorphic forms were investigated.

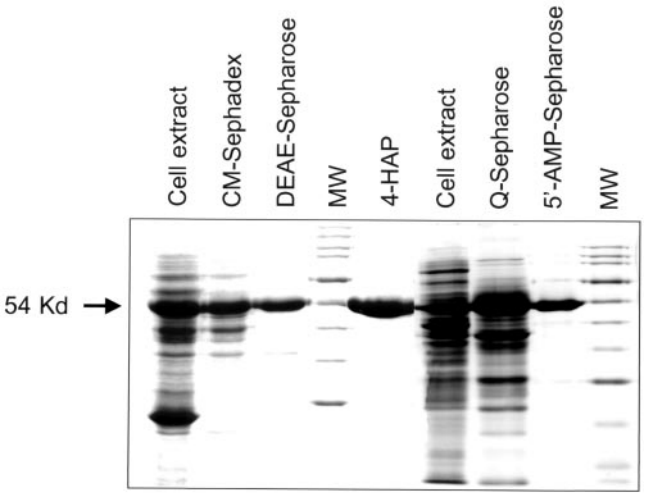


FIG. 4. SDS–PAGE analysis of recombinant hALDH1 and hALDH3. Lanes labeled MW were loaded with standards of known size. The first four sample lanes (left to right) were loaded with fractions from the purification of hALDH1, with protein loading of 30, 10, 12, and 6 μg, respectively. The next three sample lanes were from the purification of hALDH3 with 38, 28, and 5 μg protein, respectively. Bands were visualized by staining with Coomassie Blue.

Sensitivity of Transduced CCRF-CEM Cells to Aldophosphamide

Our objective was to determine whether it is possible to make cells of hematopoietic origin more resistant to oxazaphosphorines by causing overexpression of hALDH3 with a retroviral vector, and consequent oxidation of aldophosphamide to inactive carboxyphosphamide. A retroviral vector was chosen because bone marrow stem cells cannot be transfected directly, whereas retroviral vectors integrate in primary hematopoietic cells efficiently, and are the optimal system for bone marrow cell gene therapy [15]. We used the vector based on the MSCV, because of its efficiency in expressing other drug resistance genes in a variety of hematopoietic cells [32–34]. The CCRF-CEM line was chosen because it is a well-characterized cell line with a relationship, as a human lymphoid line, to human hematopoietic cells, and because it can be transduced by retroviral vectors [23, 35]; in addition, it has been useful previously for investigating retrovirus-mediated protection from antifolates [21, 23, 35]. Transduced cells were selected by growth in the presence of G418. For protection studies we used the polyclonal pool of transduced cells but without selecting highly expressing clones. Sensitivity to aldophosphamide was determined by measuring growth inhibition expressed as the IC_{50} for the parent compound, that is, the concentration of this compound that decreased growth to 50% of the growth observed in its absence. We have shown previously [21] that this method of measuring drug resistance of CCRF-CEM cells gives results equivalent to those obtained by the colony counting method. As may be seen in Fig. 2, there was no increase in resistance to 4-hydroperoxycyclophosphamide of the polyclonal culture of transduced cells compared with untransduced cells. Similar results were obtained when mafosfamide was used to generate aldophosphamide. In this case, the IC_{50} was 13.9 ± 1.8 μM for untransduced cells and 13.5 ± 0.8 μM for transduced cells.

TABLE 2. Purification of hALDH3

Purification step	Volume (mL)	Protein (mg)	Activity (IU)*	Specific activity (IU/mg)	Yield (%)
Crude† (periplasmic)	183	697	4923	7	100
Q-Sepharose	33		138	22	63
5′-AMP-Sepharose	45	55	3650	74	74

*International units: the amount oxidizing 1 μmol substrate/min. Enzyme activity was assayed at 25° in 66 mM sodium phosphate, pH 8.5, containing 2 mM EDTA, 2 mM benzaldehyde, and 2 mM NADP.

†Cell extract was obtained from 14 g of cell pellet.

Expression of hALDH3 in CCRF-CEM Cells

Southern analysis showed that CCRF-CEM cells transduced with the retroviral construct MSCV-hALDH3 contained integrated proviral sequences (Fig. 3A). Furthermore, extracts of transduced cells showed dehydrogenase activity with 16 ± 2.2 mIU/mg protein compared with a barely detectable level in untransduced cells (0.02 mIU/mg). Western analysis also confirmed the presence of hALDH3 protein in the transduced cells (Fig. 3B), and the level was similar to that obtained previously for hDHFR expressed in CCRF-CEM from the Harvey murine sarcoma vector [21]. As is usually the case with cells transduced with a retrovirus, expression of dehydrogenase was stable during many cell divisions.

Purification of hALDH1 and hALDH3

It is unclear why a high level of hALDH3 activity is required for protection of cells from oxazaphosphorines. Since low catalytic efficiency of hALDH3 with aldophosphamide as substrate might be one contributing factor, kinetic constants were determined for aldophosphamide oxidation by the recombinant enzyme. It was also of interest to determine whether one of the polymorphic forms was more efficient than the other. Determination of the corresponding kinetic constants for recombinant hALDH1 was also undertaken for comparison.

Isopropyl-β-D-thiogalactoside-induced expression of recombinant hALDH1 in the pDS5 vector in JM109 *E. coli* proved to be very efficient, the enzyme constituting about 27% of the soluble protein in cell extracts. The results for the purification of hALDH1 are summarized in Table 1. To purify the enzyme to homogeneity, three chromatographic steps were necessary. The purification protocol used was based on the method of Ghenbot and Weiner [24] with the following important modifications. First, a DEAE-Sepharose step was introduced and used to remove some protein contaminants. Under the conditions used, there was no retention of hALDH1 on the column. Second, in chromatography on 4-HAP-Sepharose, we found poor binding of hALDH1 in the presence of 50 mM NaCl as in the original procedure, and 10-fold better binding in the presence of 10 mM NaCl. The improved protocol allowed us to obtain 15–20 mg of pure hALDH1 (Fig. 4) per liter of *E. coli* culture.

Expression of hALDH3 from pDS7-hALDH3 in JM109

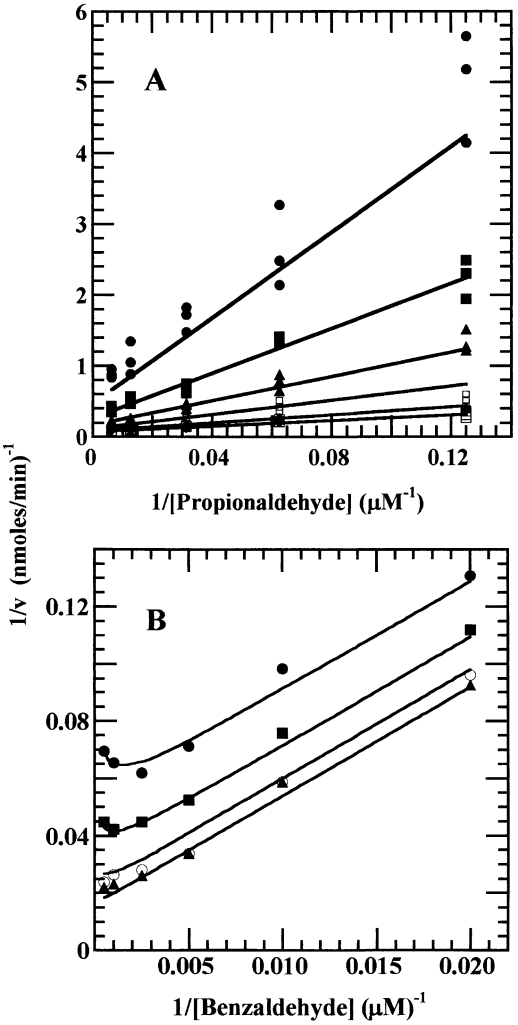


FIG. 5. Steady-state kinetic patterns for propionaldehyde oxidation by NAD in the presence of hALDH1 (A), and benzaldehyde oxidation by NADP in the presence of hALDH3 (328A polymorphic form) (B). The lines are derived from the fitting of all the data to the rate equation for a sequential reaction or ping-pong (substituted enzyme) mechanism with substrate inhibition (B). In panel A, the concentrations of NAD were 4 (●), 8 (■), 16 (▲), 32 (○), 80 (♦), and 200 (□) μM, and all data points are shown. The reaction volume was 1.0 mL, and 17.8 μg of hALDH1 was used in each reaction. In panel B, to simplify the presentation, means of triplicate determinations are shown. The concentrations for NADP were 50 (●), 100 (■), 250 (○), 500, 1150, and 2300 (▲) μM. For simplicity, the data sets for 500 and 1150 μM NADP are not shown. The reaction volume was 200 μL, and 0.1 μg of hALDH3 was used in each reaction.

TABLE 3. Limiting kinetic parameters for purified recombinant hALDH1 and hALDH3

Enzyme	Substrate and cofactor	Cofactor K_m (μM)	Substrate K_m (μM)	k^*_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} mM^{-1})
hALDH1	Propionaldehyde and NAD†	25.3 ± 3.5‡	21.5 ± 3.9	1.57 ± 0.05	73
hALDH3 (328A)	Benzaldehyde and NAD§	13.6 ± 1.2	168 ± 11	29.3 ± 1.4	174
	Benzaldehyde and NAD	20.6 ± 0.6	118.2 ± 4.1	24.1 ± 0.3	204
	Benzaldehyde and NADP§	135 ± 7	266 ± 9	76 ± 2	286
	Benzaldehyde and NADP	533 ± 35	214 ± 20	59.8 ± 3.4	279
hALDH3 (328P)	Benzaldehyde and NAD§	16.3 ± 1.1	163 ± 9.2	29.1 ± 1.0	179
	Benzaldehyde and NAD	19.8 ± 1.7	106 ± 11	19.9 ± 0.9	188
	Benzaldehyde and NADP§	198 ± 7	287 ± 11	78 ± 1	272
	Benzaldehyde and NADP	498 ± 36	255 ± 21	59.1 ± 3.6	263

*Calculated from M_r of 110 kDa for hALDH3.
†K_{ia} = 170 ± 0.13 mM.
‡Mean ± SEM for three or more determinations.
§Determined at pH 8.5. Other values for hALDH3 were determined at pH 7.4.

E. coli resulted in most of the hALDH3 forming insoluble inclusion bodies, with very little soluble enzyme. Attempts to solubilize the protein by treatment with a denaturing agent, such as guanidinium hydrochloride, followed by slow renaturation, yielded inactive enzyme. The use of a detergent (Triton X-100) increased the amount of soluble, active enzyme only 3-fold. A significant improvement in the expression of hALDH3 in *E. coli* was achieved by cloning the cDNA into the pET-26b(+) vector. The latter is designed to promote the export of the expressed protein into the periplasmic space by fusing the protein to a small leader peptide. Once the protein enters the periplasmic space, the leader peptide is cleaved by a localized protease. Expression of hALDH3 in BL21(DE3) *E. coli* using the pET-26b(+) construct yielded 20 times as much enzyme activity as with the pDS7 vector construct. An additional advantage of this method was that the hALDH3 recovered from the periplasmic space had a specific activity about 10-fold higher than that recovered from the cytoplasm. Growth of the host bacteria at 30° can in some cases increase the yield of properly folded, soluble protein, and use of this temperature further increased the yield of soluble hALDH3 3-fold.

In initial studies, hALDH3 was purified by the method used by Rose *et al.* [25] for purification of recombinant rat ALDH3. The method involves two chromatographic steps: cation exchange (carboxymethyl-Sepharose), followed by affinity chromatography on 5′-AMP-Sepharose. Although this procedure afforded a final product that was homogeneous by SDS–PAGE, the yield of active enzyme that we obtained from the cation exchange step was only about 10%. When Q-Sepharose anion exchange was used instead of carboxymethyl-Sepharose, enzyme recovery was ~70% (Table 2). The combination of a better expression vector with the optimized purification protocol yielded 8–10 mg of pure enzyme per liter of culture. The final product was homogeneous as shown by SDS–PAGE with Coomassie Blue staining (Fig. 4).

Since the vector used in the expression of hALDH3 attaches a leader sequence to the protein expressed, we carried out further analysis of the purified protein to ensure that the leader peptide had been removed completely. Protein sequence analysis indicated that the first 15 residues were as predicted from the cDNA except for the loss of the N-terminal methionine. Mass spectral analysis showed a major peak with m/z of 50,280 ± 150, which is in close agreement with an M_r of 50,247 predicted from the cDNA. These results indicated that both the leader peptide and the N-terminal methionine had been removed from the protein isolated.

Kinetic Constants with Propionaldehyde and Benzaldehyde as Substrates

Limiting values of k_{cat} and K_m for hALDH1 and hALDH3 were determined by measuring initial rates at various concentrations of both cofactor and substrate (propionaldehyde for hALDH1, and benzaldehyde for hALDH3) and fitting the data to the appropriate rate equation. As shown in Fig. 5A, the data obtained for hALDH1 gave a pattern of double-reciprocal plots consistent with a sequential mechanism. The limiting K_m and k_{cat} values (Table 3) obtained for recombinant hALDH1 oxidation of propionaldehyde were very close to the values reported for enzyme purified from autopsy liver by Yin *et al.* [36].

In contrast to hALDH1, hALDH3 gave a pattern characteristic of a ping-pong (substituted enzyme) kinetic mechanism (Fig. 5B). With NAD as coenzyme, the steady-state kinetics gave the same pattern as with NADP (data not shown). In addition, inhibition by high concentrations of benzaldehyde was evident, particularly at lower concentrations of cofactor. When we fitted the data to the rate equation that includes substrate inhibition, the sum of squares was 295.9 compared with 461.3 for fitting without substrate inhibition ($P < 0.0001$). In the case of hALDH3 (328A polymorph), the substrate inhibition constant obtained was 5.11 ± 1.08 mM.

The results for hALDH3 in Table 3 did not differ greatly from those reported for hALDH3 from normal tissue [37], which were determined at 37° and pH 8.1. Like hALDH3

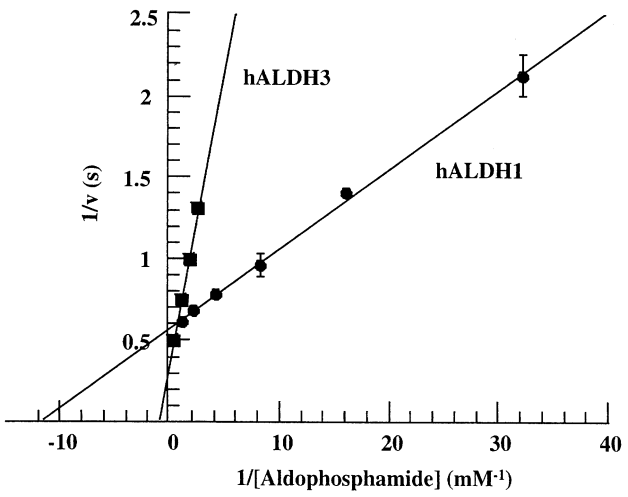


FIG. 6. Plots of reciprocal reaction rate versus reciprocal aldophosphamide concentration for hALDH1- and hALDH3-catalyzed reactions. Reactions were carried out at 37° in the presence of 0.5 mM NAD. For other details, see Materials and Methods.

from normal tissue, recombinant hALDH3 had greater catalytic efficiency with NAD as cofactor than with NADP, due primarily to a much higher K_m for NADP. Furthermore, K_m for NADP was much more dependent on pH than that for NAD. With either coenzyme the K_m for benzaldehyde was relatively high. There was no significant difference between the kinetic parameters of the two polymorphic forms of hALDH3.

Kinetics with Aldophosphamide as Substrate

For kinetic analysis, aldophosphamide was generated by the method we found to be optimal as described in Materials and Methods, reduction of 4-hydroperoxycyclophosphamide with dimethyl sulfide. Because of the limited amounts of 4-hydroperoxycyclophosphamide available for the generation of aldophosphamide, we were able to determine only apparent kinetic constants for this substrate, that is, constants obtained at a fixed concentration of coenzyme. However, since the concentrations of coenzyme were high (500 μM NAD or 2000 μM NADP) compared with the K_m values in Table 3, the apparent constants calculated were probably close to limiting values. In the case of both enzymes, the data for aldophosphamide oxidation fit well to the Michaelis–Menten equation as indicated by the linear double-reciprocal plots in Fig. 6.

The K_m that we obtained for aldophosphamide as substrate for recombinant hALDH1 (Table 4) was somewhat higher and the k_{cat} lower than the previously published values for the partially purified enzyme isolated from human liver [8]. hALDH3 purified from stomach mucosa was reported to have no detectable activity for aldophosphamide [5], but our homogeneous recombinant enzyme had considerable activity. There was little difference in the kinetics of the two polymorphic forms of hALDH3 with either coenzyme and either substrate. Although k_{cat} was higher with NADP as coenzyme than with NAD, K_m was also higher, so that the catalytic efficiency at low substrate concentrations (k_{cat}/K_m) was about the same for both coenzymes. hALDH3 was about 7 times less efficient than hALDH1 in catalyzing the oxidation of aldophosphamide to carboxyphosphamide.

DISCUSSION

Protection of Cells by hALDH3

Since the possibility of protecting hematopoietic cells from oxazaphosphorines by retroviral introduction of hALDH3 had not been explored previously, we used the MSCV-hALDH3 vector to transduce CCRF-CEM cells. In these studies we used polyclonal populations of transduced cells, selected for the presence of the neomycin resistance gene but not selected for resistance to oxazaphosphorines. It is very likely that clones with relatively high expression of hALDH3 were present in the polyclonal population. Thus, we have previously shown the presence of high expression clones when CCRF-CEM cells were transduced with vectors introducing genes for resistance to antifolates [21, 23, 35]. However, we chose not to select for such clones because *in vivo* selection for stem cells expressing high levels of vector-encoded ALDH is highly unlikely. Primitive hematopoietic cells already express high levels of the endogenous *ALDH1* gene [38]. This is the basis for bone marrow purging strategies with mafosfamide, where when bone marrow is cultured with the drug, although clonogenic

TABLE 4. Apparent kinetic parameters for oxidation of aldophosphamide by purified recombinant hALDH1 and hALDH3

Enzyme	Substrate and cofactor	Substrate K_m (μM)	k^*_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} mM^{-1})
hALDH1	Aldophosphamide and NAD	84 ± 7†	1.7 ± 0.1	20
hALDH3 (328A)	Aldophosphamide and NAD	820 ± 30	2.4 ± 0.04	2.9
	Aldophosphamide and NADP‡	1180 ± 190	2.3 ± 0.2	2.2
	Aldophosphamide and NADP	1040 ± 260	2.9 ± 0.4	2.8
hALDH3 (328P)	Aldophosphamide and NAD	1010 ± 130	3.1 ± 0.2	3.2
	Aldophosphamide and NADP	1870 ± 330	5.4 ± 0.7	2.9

*Calculated from M_r of 110 kDa for hALDH1 and 50 kDa for hALDH3.
†Mean ± SEM for three or more determinations.
‡Determined at pH 8.5. Other values for hALDH3 were determined at pH 7.4.

progenitors are killed, the engrafting stem cells are preserved. This high level of endogenous *ALDH1* expression would be predicted to obscure the selective advantage for stem cells expressing an ALDH3 vector.

We found no protection of the polyclonal population of transduced CEM cells from 4-hydroperoxycyclophosphamide or mafosfamide. Nevertheless, the cDNA for hALDH3 was expressed as shown by western blotting (Fig. 3B), and by ALDH3 activity in transduced cells (16 mIU/mg, compared with an endogenous level of 0.02 mIU/mg). However, this level of hALDH3 activity in the polyclonal transduced CCRF-CEM cells was quite low compared with that of selected clones of resistant V79 hamster cells transfected with hALDH3 (5.9 IU/mg for a line 12-fold resistant to mafosfamide) [14]. In those hamster cell clones, activity had to increase steeply to raise resistance to mafosfamide, a 250-fold increase in intracellular activity to 1 IU/mg being necessary for a 4.8-fold increase in resistance. It is not surprising, therefore, that the level in our transduced cells was too low to provide protection. When intracellular hALDH3 activity is low, increased non-enzymatic conversion of aldophosphamide to phosphoramide mustard and acrolein may result in inactivation of hALDH3 by acrolein, further decreasing cell resistance.

Kinetics of Recombinant Enzymes

It was unclear why such high levels of hALDH3 activity were needed to increase resistance to oxazaphosphorines. No data on the kinetics of aldophosphamide oxidation catalyzed by hALDH3 of normal tissue or its activity with this substrate relative to hALDH1 were available. Although some kinetic data have been reported for tumor ALDHs that appear to be of the class 3 type [5–7], no protein or nucleic acid sequences have been reported for these enzymes, so that their relationship to the enzyme in normal tissue is unknown. For a better understanding of the activity of both hALDH1and hALDH3 with aldophosphamide as substrate, we expressed the recombinant enzymes in *E. coli* using prokaryotic expression vectors that gave high yields of enzyme. The enzymes were purified by modifications of published procedures that gave homogeneous enzyme in good yield, and kinetic parameters were determined. It was found that the kinetic constants of the recombinant enzymes resemble those previously reported for the enzymes purified from normal tissue. In addition, it was found that there was no significant difference in kinetic behavior for the polymorphic forms of hALDH3 (Table 3), at least with the substrates used, so that it remains unclear why both polymorphic forms exist in the human population.

Reaction Mechanisms

hALDH1 was found to give the initial velocity pattern expected for an ordered sequential kinetic mechanism. This is consistent with a mechanism in which NAD binds to unmodified apoenzyme and NADH dissociates last from the product complex. Some evidence exists for this order of substrate addition and product release [39].

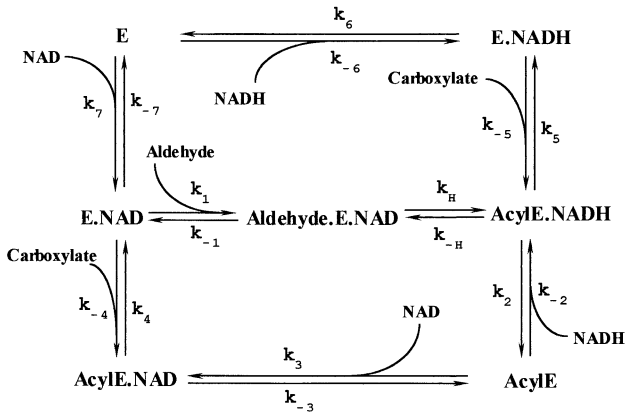
However, in contrast to this result, the initial velocity data for hALDH3 conform to the pattern predicted for a ping-pong or modified-enzyme kinetic mechanism. There is much evidence to indicate that, as proposed some time ago [40], a thiohemiacetal is formed between the aldehyde substrate and the active site cysteine residue (Cys 243 in hALDH3). Hydride transfer to bound NAD then oxidizes the thiohemiacetal to a thioester, and detailed schemes for the role of enzyme residues in catalyzing these steps have been proposed [41, 42]. These steps are represented on the center line of Scheme 1, where the aldehyde substrate binds to the enzyme–NAD complex to form a ternary substrate complex, which undergoes transformation to the NADH–enzyme thioester complex. This product complex could break down in two ways. If the upper loop in Scheme 1 is followed, with hydrolysis of the thioester and release of the carboxylic acid before release of NADH, the ordered sequential mechanism attributed to ALDH1 will occur. However, if the lower loop is followed, for example, if $k_2 \gg k_5$ and $k_3 \gg k_4$, then dissociation of NADH and binding of NAD occur before dissociation of the carboxylic acid. The full rate equation for the latter mechanism, derived by the King and Altman method [43], is as follows.

$$v = \frac{V_f[A][N] - (V_r K_A K_{iN})[C][NH]/(K_{NH}K_{iC})}{K_N[A] + K_A[N] + [A][N] + (K_A K_C K_{iN}[NH]/(K_{NH}K_{iC}) + K_A K_{iN}[C]/K_{iC} + (K_A K_{iN}[C][NH])/(K_{NH}K_{iC}) + K_N[A][NH]/K_{iNH} + K_A[C][N]/K_{iC}}$$

where the kinetic constants are defined according to the nomenclature of Cleland [43], and A is the aldehyde substrate, N is NAD, C is the carboxylate product, and NH is NADH. For initial velocities in the absence of products this equation becomes

$$v = \frac{V_f[A][N]}{K_N[A] + K_A[N] + [A][N]}$$

This equation has the same form as that for a true ping-pong mechanism, that is, where the first substrate dissociates before the second substrate binds. Although this is not the case for the mechanism described by the lower loop in Scheme 1, the equation has the same form, and therefore, predicts parallel double-reciprocal plots as the fixed substrate concentration is varied, like those shown in Fig. 5 for hALDH3. It should be noted that the form of the equation is not changed if hydrolysis of thioester occurs in the steps associated with k_H, k_2, or k_3, and it does not predict whether or not hydride transfer is rate-limiting, i.e. whether k_H is greater or smaller than the rate constants for subsequent steps in either loop. This mechanism is closely



SCHEME 1. Kinetic mechanisms proposed for ALDH. The following abbreviations are used: E, enzyme; AcylE, thioester product of the oxidation of the thiohemiacetal formed between the aldehyde and the active site Cys. In the *upper* loop the AcylE.NADH complex is converted to E.NAD via the intermediates E.NADH and E. In the *lower* loop the pathway is via AcylE and AcylE.NAD. When NADP is used as coenzyme instead of NAD, NADP replaces NAD in the scheme, and NADPH replaces NADH.

related to that for glyceraldehyde-3-phosphate dehydrogenase [44, 45], for which similar parallel initial velocity plots are obtained [44].

The active sites of ALDH1 and ALDH3 are structurally very similar [42], with conservation of residues believed to be involved in catalysis (Asn 114, Glu 209, Cys 243, Glu 333, and Phe 335 in ALDH3). Nevertheless, the structural differences between ALDH1 and ALDH3 appear to be sufficient to alter the relative magnitude of rate constants for binding and dissociation steps so that the upper loop of the kinetic mechanism predominates in one case and the lower loop in the other, despite the fact that the catalytic mechanism is the same in both cases.

The substrate inhibition observed for hALDH3 (Fig. 5) perhaps may be due to aldehyde binding to the enzyme–NADPH complex. Although the steady-state level of this complex must be small since the lower loop of the scheme predominates, binding of aldehyde to enzyme–NADPH could cause accumulation of enzyme–NADPH–aldehyde complex, which would be the case if dissociation of both ligands is slow, and this would result in a decreased overall reaction rate.

Kinetic Constants for Aldophosphamide Oxidation

Aldophosphamide of known concentration and free from other contaminants is required for steady-state kinetics. Although mafosfamide has been used to generate aldophosphamide for kinetic studies [46], ^{31}P NMR showed that mafosfamide is unsuitable as a precursor, since the aldophosphamide generated is low in concentration and accompanied by decomposition products and unreacted mafosfamide. Reduction of 4-hydroperoxycyclophosphamide with thiosulfate is also unsatisfactory, but dimethyl sulfide reduction at pH 5 gives a stable solution of aldophosphamide in which there are no compounds other than its tautomers, *cis*- and *trans*-4-hydroxycyclophosphamide, which always exist in equilibrium with it in aqueous solution.

The limited amount of 4-hydroperoxycyclophosphamide available made it impractical to determine limiting kinetic parameters with aldophosphamide as substrate. However, the apparent values obtained in the presence of a high concentration of NAD or NADP are expected to differ very little from limiting values. When NAD is used as cofactor, K_m for aldophosphamide is 0.8 and 1 mM for the polymorphic forms of hALDH3, as compared with 0.08 mM for hALDH1. Consequently the catalytic efficiency (k_{cat}/K_m) for aldophosphamide oxidation by hALDH3 is 7 times lower than for hALDH1, even though k_{cat} is slightly higher for hALDH3. At the concentrations likely to be present in cells, even with dose escalation, the activity of hALDH3 for aldophosphamide oxidation would be very low.

Prospects for Protection of Hematopoietic Cells from Oxazaphosphorines by Transduction with hALDH3

Our results indicate that for protection with hALDH3, considerably higher activity for intracellular aldophosphamide oxidation must be achieved, either by increasing expression in a polyclonal population, or by increasing the catalytic efficiency of hALDH3 with aldophosphamide as substrate by mutagenesis. K_m is probably high because of difficulty in accommodating the bulky, polar aldophosphamide in the narrow, hydrophobic funnel that constitutes the substrate binding site. Our modeling studies based on the published crystallographic data of Liu *et al.* support this concept [47]. In unpublished work, we have made some progress towards improving k_{cat}/K_m for aldophosphamide.*

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