Biochem. J. (1993) 290, 457–462 (Printed in Great Britain)

Demonstration of adenosine deaminase activity in human fibroblast lysosomes

Elizabeth R. LINDLEY and Ronald L. PISONI*

Department of Pediatrics and Communicable Diseases, Division of Biochemical Genetics and Metabolism, University of Michigan, Ann Arbor, MI 48109–2029, U.S.A.

Human fibroblast lysosomes, purified on Percoll density gradients, contain an adenosine deaminase (ADA) activity that accounts for ~ 10% of the total ADA activity in GM0010A human fibroblasts. In assays of lysosomal ADA, the conversion of [^{3}H]adenosine into [^{3}H]inosine was proportional to incubation time and the amount of lysosomal material added to reaction mixtures. Maximal activity was observed between pH 7 and 8, and lysosomal ADA displayed a K_m of 37 μM for adenosine at 25 °C and pH 5.5. Lysosomal ADA was completely inhibited by 2.5 mM Cu^{2+} or Hg^{2+} salts, but not by other bivalent cations (Ba^{2+}, Cd^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}). Coformycin (2.5 mM), deoxycoformycin (0.02 mM), 2′-deoxyadenosine (2.5 mM), 6-methylaminopurine riboside (2.5 mM), 2′-3′-isopropylidene-adenosine (2.5 mM) and *erythro*-9-(2-hydroxy-3-nonyl)adenine (0.2 mM) inhibited lysosomal ADA by > 97%. In contrast, 2.5 mM *S*-adenosyl-L-homocysteine and cytosine were poor inhibitors. Nearly all lysosomal ADA activity is eluted as a high-molecular-mass protein (> 200 kDa) just after the void volume on a Sephacryl S-200 column, and is very heat-stable, retaining 70% of its activity after incubation at 65 °C for 80 min. We speculate that compartmentalization of ADA within lysosomes would allow deamination of adenosine to occur without competition by adenosine kinase, which could assist in maintaining cellular energy requirements under conditions of nutritional deprivation.

INTRODUCTION

Lysosomes are a major intracellular site for the degradation of a wide variety of macromolecules, including proteins, complex carbohydrates, glycolipids and nucleic acids. Many of the metabolites generated as a result of lysosomal macromolecule breakdown are transported back into the cytosol, where they can be reutilized for the synthesis of new macromolecules or participate in various metabolic pathways. The role of lysosomes in supporting cellular activities is especially important under conditions of nutritional deprivation, in which the lysosome can be viewed as a major recycling centre for providing the basic building blocks for synthesis of essential proteins, nucleic acids, carbohydrates and lipids necessary for cell survival.

Lysosomes contain a repertoire of enzymes capable of degrading nucleic acids completely to their constituent nucleosides [1–5]. Under conditions of nutritional deprivation, ~ 65% of total rat liver cytoplasmic RNA is degraded per day, with 70–85% of this degradation occurring within the lysosomal compartment [6,7]. The nucleosides that result from nucleic acid breakdown within lysosomes are returned to the cytosol by the lysosomal nucleoside-transport system [8]. In the case of adenosine, however, substantial deamination of this nucleoside to inosine occurs within human fibroblast lysosomes [8]. In the present paper, we describe the characteristics of the adenosine deaminase (ADA) activity present in human fibroblast lysosomes and discuss how lysosomal compartmentalization of adenosine deaminase may be important for maintaining cellular energy requirements under conditions when cytosolic adenosine levels are low.

EXPERIMENTAL

Cell culture and preparation of Percoll-purified lysosomes

Normal human fetal skin fibroblasts (GM0010A) and the ADA-deficient human fibroblast cell line, GM469, were obtained from the Human Genetic and Mutant Cell Repository. Fibroblasts were grown and maintained in an atmosphere of air/CO_2 (19:1) in 100 mm tissue-culture dishes or 850 cm^2 roller bottles in Coon's modification of Ham's F-12 medium (Sigma). The medium was supplemented with 10% horse serum (Sigma) rather than fetal-bovine serum, because fetal-bovine serum has been found to contain a significant amount of ADA activity, whereas horse serum contains very little [9,10]. When nearly confluent, human fibroblast monolayers were routinely split 1:4 by using a mixture of collagenase, trypsin and chicken serum (CTC) as described previously [11], and were not used beyond passage 18. Lysosomes were prepared from six to seven roller bottles of nearly confluent human fibroblasts by centrifugation on Percoll density gradients as described previously [12,13], and stored at −20 °C in ~ 0.22 ml of 0.25 M sucrose. Three lysosomal preparations were pooled, freeze/thawed three times to lyse the lysosomes completely, divided into 75 μl batches and stored at −20 °C. This pooled lysate was used throughout the enzyme-characterization studies.

Lysosomal ADA assay

For most assays, lysosomal ADA activity was assayed at 25 °C by mixing a portion of the lysosomal lysate described above with 0.02 mM [^{3}H]adenosine (0.29 mCi/ml) in 35.7 mM Mes/NaOH buffer, pH 5.5, to yield a reaction mixture with a final adenosine concentration of 0.015 mM. Assays were terminated at desired time intervals by removing 12 μl samples which were then added to tubes containing 8 μl of 25 mM adenosine and 50 mM inosine in 0.1 M HCl (termination solution). The samples were immediately frozen in a solid-CO_2/ethanol bath and stored at −20 °C until high-voltage electrophoresis could be performed. Lysosomal ADA displays no activity at the acidic pH of the

Abbreviations used: ADA, adenosine deaminase; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine.

* To whom correspondence should be addressed.

termination solution, and inosine and adenosine are included to serve as internal standards for high-voltage electrophoresis.

The product of the ADA reaction, inosine, was separated from the substrate, adenosine, by high-voltage electrophoresis. This separation was achieved by wetting electrophoresis paper (CAMAG S+S 2043B paper, 20 cm × 40 cm) with 6% formic acid, blotting away any excess liquid and spotting the entire volume (20 μl) of the samples described above. The samples were then electrophoresed in a CAMAG high-voltage electrophoresis apparatus for 13 min at 3500 V. Electrophoretograms were dried in an oven at 85 °C, and nucleoside spots were observed under a short-wave u.v. lamp. The inosine spots were cut out and allowed to equilibrate in 16 ml of scintillation fluid for at least 5 h before counting of radioactivity in a Beckman LS 100C scintillation counter. Inosine migrated with an average R_F of 0.13 with respect to adenosine.

Blanks were run in triplicate for each experiment. Each blank consisted of 9 μl of the [^{3}H]adenosine solution described above, 8 μl of the termination solution, and 3 μl of 0.25 M sucrose in place of the lysosomal suspension. The blanks were frozen, electrophoresed, and their inosine spots were cut out and counted for radioactivity as described above. The amount of radioactivity migrating with each of the inosine spots of the blanks was averaged and subtracted from the amount of radioactivity migrating with the inosine spots of the experimental samples. One unit of ADA activity as measured by this assay is defined as 1 pmol of inosine formed/min at 25 °C and pH 5.5. Assays were designed so that substrate was always in large excess at the time when sampling points were taken so as to reflect initial-rate kinetics.

For determination of the K_m and $V_{max.}$ of lysosomal ADA activity, 2.26 μCi of [^{3}H]adenosine (30 Ci/mmol, 0.18 mCi/ml in 41 mM Mes/NaOH buffer, pH 5.5) was added to each incubation mixture along with unlabelled adenosine solutions prepared in 50 mM Mes/NaOH buffer, pH 5.5, to give the final adenosine concentrations indicated in a final volume of 0.03 ml.

N-Acetyl-β-hexosaminidase assay

The amount of lysosomal material used in each lysosomal ADA assay was quantified per unit of *N*-acetyl-β-hexosaminidase activity, measured in the presence of 0.1% Triton as described previously [8]. One unit of *N*-acetyl-β-hexosaminidase activity is defined as the amount of enzyme producing 1 nmol of *p*-nitrophenol/min at 37 °C.

Analysis of kinetic data

The kinetic constants K_m and $V_{max.}$ were calculated by applying the Gauss–Newton non-linear least-squares method to the kinetic data by using Cleland's HYPER program [14].

Miscellaneous

Cytoscint ES was obtained from ICN (cat. no. 882453); coformycin (Pentostatin) and deoxycoformycin were generously provided by Warner Lambert Co., Ann Arbor, MI, U.S.A.; *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) was a gift from Burroughs–Wellcome Co., Research Triangle Park, NC, U.S.A.; [^{3}H]adenosine (30 Ci/mmol) was purchased from Moravek Biochemicals; adenosine, inosine and other chemicals were obtained from Sigma.

RESULTS

In a previous investigation of nucleoside transport by human fibroblast lysosomes, we had observed that a substantial portion of the radiolabelled adenosine taken up by fibroblast lysosomes was converted into inosine [8]. After a 2.5 min uptake of 1 mM [^{3}H]adenosine, approx. 15% of the radioactivity recovered from human fibroblast lysosomes was found to be inosine, and after [^{3}H]adenosine uptakes of 15–20 min inosine accounted for the great majority of the total radioactivity recovered from lysosomes. The goal of the present investigation was to examine the characteristics of this adenosine-deaminating activity present in human fibroblast lysosomes and to determine if this enzyme differs from other ADA activities which have been characterized previously.

In previous subcellular fractionations of the human fibroblast, only lysosomes have been found to sediment in the bottom third of 30–40% Percoll density gradients, as judged by the appearance of this fraction on electron micrographs and by the distribution of the marker enzymes β-hexosaminidase, succinate dehydrogenase, galactosyltransferase and 5′-nucleotidase [13,15–17]. The purity of this dense Percoll fraction as being that of lysosomes is further substantiated by the characterization of a large number of lysosomal transport systems from this fraction, which display properties that significantly differ from their analogous counterparts on other cellular membranes [18]. To demonstrate the presence of ADA activity within human fibroblast lysosomes, fibroblasts were lysed, nuclei were removed by centrifugation, and a granular fraction was obtained which contained 13% of the total human fibroblast ADA activity. When the granular fraction was centrifuged on a 31%-Percoll density gradient, much of the ADA activity co-migrated with the lysosomal marker enzyme β-hexosaminidase, with most of both of these enzymes being found in the bottom third of the Percoll density gradient (Figure 1). Some ADA activity is also observed at the top of the Percoll gradient, which may be non-vesicularized enzyme or enzyme associated with a buoyant organelle such as endosomes or plasma-membrane vesicles. ADA activity has been found to be associated with the plasma membrane of various cells [19–24]. The ADA characterized in the present investigation was taken only from the bottom third of the Percoll density gradients, in which only lysosomes have been found.

Human fibroblasts have been shown to contain a large- and a small-molecular-mass form of ADA [9,10,25,26]. With confluent

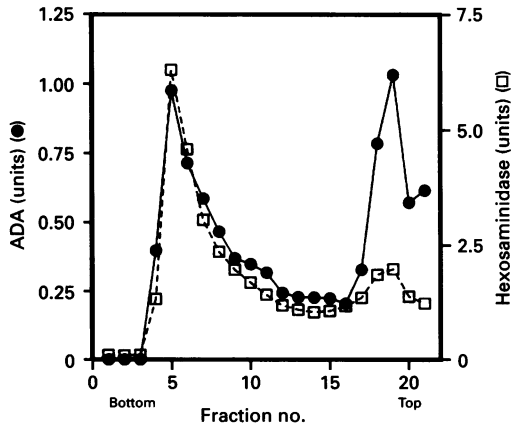

Figure 1 Distribution throughout a 31%-Percoll density gradient of *N*-acetyl-β-hexosaminidase and ADA activity contained in the human fibroblast granular fraction

The granular fraction prepared from human fibroblasts was layered on top of 10 ml of a 31% Percoll solution and centrifuged for 38 min at 17800 rev./min in a SS-34 rotor at 4 °C as described in the Experimental section. Fractions (0.5 ml) were collected from the bottom of the gradient and assayed for *N*-acetyl-β-hexosaminidase (□) and ADA activity (●).

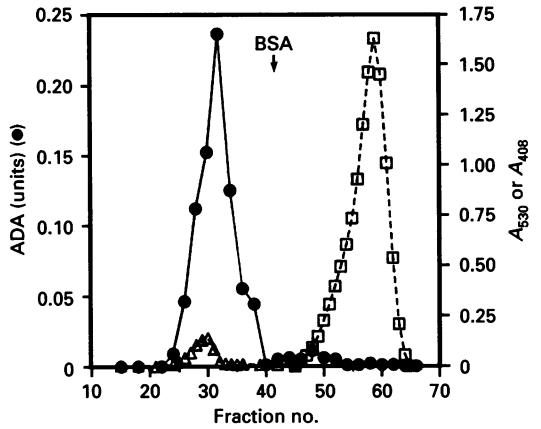

Figure 2 Gel filtration of lysosomal ADA on a Sephacryl S-200 column

Human fibroblast lysosome lysate (100 μl) was mixed with 50 μl of a column marker solution containing Blue Dextran, cytochrome *c* and sucrose, prepared in distilled water. The entire sample volume was loaded on a Sephacryl S-200 column and eluted with 0.5 M NH_4HCO_3. Fractions (1.15 ml) were collected at a flow rate of 18 ml/h. Selected fractions were assayed for 30 min for ADA activity (●) as described in the Experimental section. The distribution of Blue Dextran (△) and cytochrome *c* (□), was determined by measuring the absorbance of indicated fractions at 530 nm or 408 nm respectively. The distribution of BSA was determined in a separate run by measuring the absorbance at 280 nm.

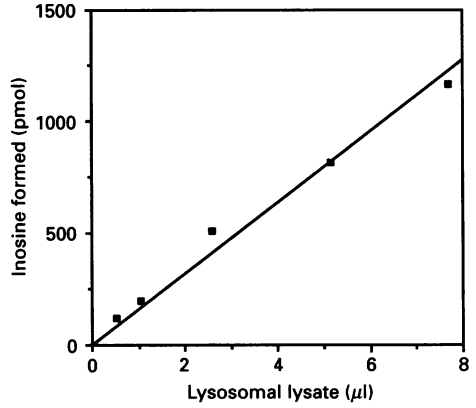

Figure 3 Dependence of lysosomal ADA activity on the amount of lysosomal material added to incubation mixtures

Samples (30 μl) of 250 μM [^{3}H]adenosine (0.29 μCi/μl) in 35.5 mM Mes/NaOH buffer, pH 5.5, were mixed with the indicated amount of human fibroblast lysosome lysate, which was added to reaction mixtures with 0.25 M sucrose to give a final reaction volume of 40 μl. Reactions were run at 25 °C for 4 min, and the amount of [^{3}H]inosine formed was measured as described in the Experimental section. Lysosomal lysate contained 3.2 units of β-hexosaminidase activity/μl.

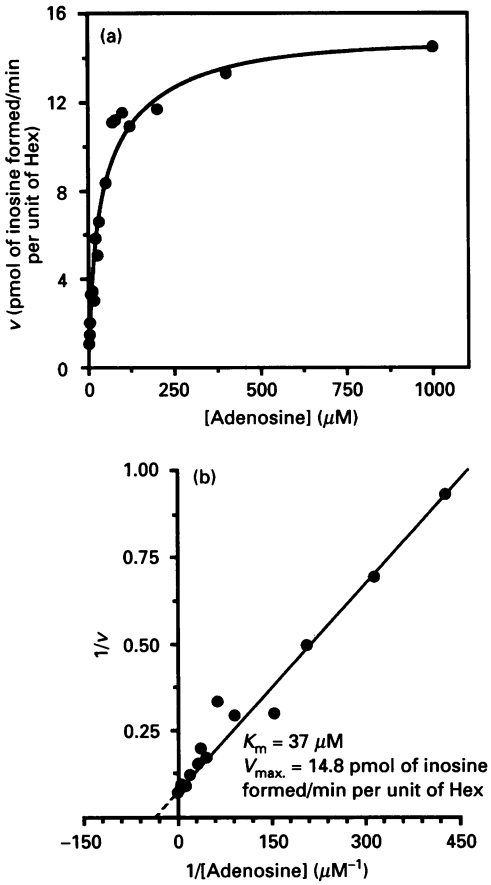

Figure 4 Kinetics of the initial rate of converting [^{3}H]adenosine into [^{3}H]inosine by lysosomal ADA as a function of adenosine concentration

Human fibroblast lysosome lysate was incubated at 25 °C in [^{3}H]adenosine solutions prepared in 50 mM Mes/NaOH, pH 5.5, at the concentrations indicated. Samples were removed after 8 min (after 3 min for the 3.33 and 2.50 μM concentrations), and the amount of [^{3}H]inosine formed was measured as described in the Experimental section. Lower adenosine concentrations (72.5 μM, 52.5 μM, 32.5 μM, 22.5 μM, 12.5 μM and 5.83 μM) were performed in duplicate. (**a**) Michaelis–Menten plot of the initial rate of conversion of [^{3}H]adenosine into [^{3}H]inosine by lysosomal ADA as a function of adenosine concentration. (**b**) Lineweaver–Burk (double-reciprocal) plot of the same data. Abbreviation: Hex, *N*-acetyl-β-hexosaminidase.

fibroblast monolayers, the large-molecular-mass form of ADA (~ 298 kDa) predominates, which is composed of two molecules of the small-molecular-mass form of ADA (~ 38 kDa) bound to a specific ADA-binding protein (~ 213 kDa) [10,27,28]. To assess the approximate molecular size of human fibroblast lysosomal ADA, lysosomal material was applied to a Sephacryl S-200 column, from which most (> 85%) of the ADA activity was eluted just after the void volume (shown by Blue Dextran elution) and displayed a molecular mass > 200 kDa (Figure 2). In addition, a very low level of ADA activity was observed in some of the fractions eluted after the high-molecular-mass ADA peak. These smaller ADA activities may represent ADA species generated from the lysosomal degradation of the high-molecular-mass ADA form.

Daddona et al. [10] have shown previously that the ADA-deficient human fibroblast cell line, GM469, contains ~ 2% of the normal level of ADA activity. We quantified the total amount of ADA activity in lysosomes from GM469 fibroblasts, and found that lysosomal ADA activity is also greatly decreased in this cell line to ~ 5% of that found in the GM0010A normal human fibroblast cell line (results not shown). These results suggest that the catalytic portion of lysosomal ADA is derived from the same gene as that which is defective in ADA deficiency (i.e. that coding for the 38 kDa catalytic subunit).

Since a focus of this study was to define the characteristics of ADA functioning within the environment of the lysosomal compartment, all assays were performed at pH 5.5, which is near the intralysosomal pH previously reported for human fibroblast lysosomes [29]. Lysosomal ADA activity was found to be directly dependent on the amount of lysosomal material added to incubation mixtures (Figure 3), and assays were linear with time until a plateau was reached when all adenosine had been converted into inosine (results not shown).

A Michaelis–Menten plot of the initial rate of lysosomal adenosine deaminase activity as a function of the concentration

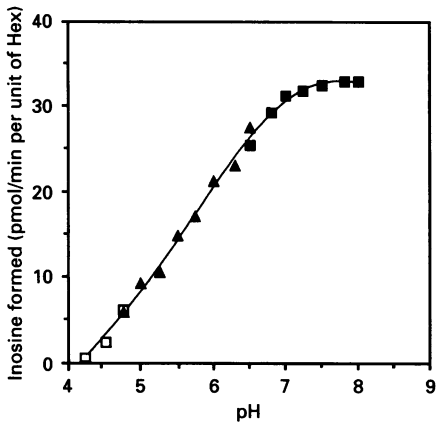

Figure 5 Effect of pH on the $V_{max.}$ of lysosomal ADA

Human fibroblast lysosome lysate [8 μl in 0.25 M sucrose, containing 1.19 units of *N*-acetyl-β-hexosaminidase (Hex)/μl] was incubated at 25 °C with 8 μl of 7.5 mM [^{3}H]adenosine (0.625 μCi/μl in dH_2O) and 8 μl of buffer titrated to the indicated pH. Samples (12 μl) were removed from each reaction mixture after 105 min, and the amount of [^{3}H]inosine formed was measured as described in the Experimental section. Three different buffers were used over the tested pH range: pH 4.25–4.75, 50 mM acetic acid/NaOH (□); pH 4.75–6.5, 50 mM Mes/NaOH (▲); pH 6.5–8.0, 50 mM Mops/NaOH (■).

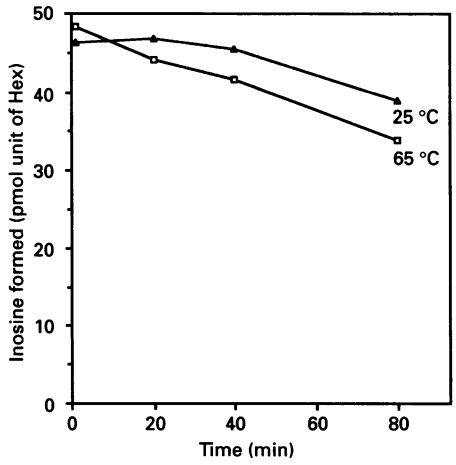

Figure 6 Effect of incubating lysosomal ADA at pH 5.5 and 65 °C on the stability of enzymic activity

Human fibroblast lysosomal lysate (80 μl), containing 128 units of *N*-acetyl-β-hexosaminidase (Hex) in 0.25 M sucrose, was incubated in a water bath at 25 °C (▲) or 65 °C (□). At the indicated times, ten samples were removed and ADA activity was immediately assayed in an 8 min reaction at pH 5.5 and 25 °C with 14.8 μM [^{3}H]adenosine.

of adenosine demonstrates saturability of the enzyme (Figure 4a). The Lineweaver–Burk plot is linear, suggesting that lysosomal adenosine deamination is catalysed by only one enzyme, with a K_m of 37 ± 4 μM at 25 °C and pH 5.5 (Figure 4b). This K_m is somewhat lower than the K_m of 60–65 μM reported previously for other human fibroblast ADA activities measured at pH 7.5 and 37 °C [9]. A $V_{max.}$ of 14.8 pmol of inosine formed/min per unit of *N*-acetyl-β-hexosaminidase was found for lysosomal ADA at pH 5.5 and 25 °C. The $V_{max.}$ of lysosomal ADA varies with pH in a manner similar to that reported for other ADA activities [30–34], being greatest in the neutral pH range pH 7–8, and declining steadily over the range pH 7–4, with little or no enzymic activity observed at pH values less than 4 (Figure 5). This pH profile coincides well with recent X-ray-crystallographic data suggesting that the side-chain carboxyl group of an aspartate residue located in the active site of ADA is important for the catalytic mechanism of this enzyme [35]. Since this carboxyl group is located in a highly hydrophobic environment [35], its pK_a is expected to be higher than for an aspartate side-chain carboxyl group free in aqueous solution.

Hirschhorn et al. [9] have shown that a 60% inactivation of ADA activity occurs when human fibroblast ADA is incubated at 56 °C for 80 min. In contrast, the residual ADA activity present in several different ADA-deficient fibroblast cell lines exhibits a much greater heat-stability, losing only 10–20% of activity when incubated at 56 °C for 80 min [9]. Lysosomal ADA from the normal human fibroblast used in the present study also was found to be very heat-stable, losing 30% of its initial activity when incubated at 65 °C for 80 min (Figure 6).

In addition to the existence of ADA within human cells, an aminohydrolase capable of deaminating adenosine has been shown to be present in human spleen tissue and B-lymphoblast cells [31,36]. This aminohydrolase displays a K_m of 2–3 mM for adenosine which is 40–50 times higher than that of ADA. The aminohydrolase also exhibits a molecular-mass of 92–110 kDa, appears to be extremely heat-stabile at 68 °C, and is not inhibited by 0.2 mM EHNA, which has been shown to inhibit ADA strongly [31]. To assess the specificity of the human fibroblast lysosomal ADA activity, we analysed the ability of various nucleobase, nucleoside and nucleotide analogues to inhibit lysosomal adenosine-deaminating activity (Table 1). Deoxycoformycin (0.02 mM), coformycin (2.5 mM), adenosine (2.5 mM), 2′-deoxyadenosine (2.5 mM), 6-methylaminopurine riboside (2.5 mM) and 2′,3′-isopropylidene-adenosine (2.5 mM) strongly inhibited lysosomal ADA activity by more than 97%. EHNA inhibited the lysosomal enzyme by $> 98\%$, thus further indicating that lysosomal adenosine deamination is not due to the action of a non-specific aminohydrolase. The pyrimidine cytosine (2.5 mM) and *S*-adenosyl-L-homocysteine (2.5 mM) poorly inhibited lysosomal ADA, by $< 10\%$. The other analogues varied from strong to moderate in their inhibitory effect of lysosomal ADA.

Daddona et al. [31] have demonstrated that the bivalent cations Hg^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} at pH 7.4 and at 2.5 mM concentration inhibit the small-molecular-mass form of ADA from human B-lymphoblast cells by $\geq 96\%$. In addition, Fe^{2+} and Ni^{2+} inhibited this form of ADA by 71–76%. We tested a variety of bivalent cations at pH 5.5 and at 2.5 mM concentration to determine their effect on lysosomal ADA (Table 2). Hg^{2+} and Cu^{2+} were found to inhibit lysosomal ADA completely, whereas the other bivalent cations had no effect on lysosomal ADA. The strong inhibitory action of Hg^{2+} and Cu^{2+} on both lymphoblast ADA and lysosomal ADA may reflect the binding of these bivalent cations to a thiol group that has been previously implicated as being important for ADA activity [33,34,37] or may inhibit the catalytic function of the essential active-site Zn^{2+} ion of ADA [35]. It is conceivable that the other bivalent cations inhibit the small form of ADA at pH 7.4 by binding in part to side-chain carboxyl groups which are ionized at pH 7.4 but not at pH 5.5, or bind to sites on the enzyme which are accessible in the small-molecular-mass form of lymphoblast ADA but not in the large-molecular-mass form of lysosomal ADA.

Lucarini et al. [38] have reported that human erythrocyte acid phosphatase activity is stimulated as much as 160% by 4.2 mM inosine. In view of these findings, we tested whether 4.2 mM inosine could modulate human fibroblast lysosome acid phosphatase activity, thereby implicating a role for ADA in up-regulating lysosomal phosphatase activity. In contrast with the results of Lucarini et al., we did not observe any significant effect

Table 1 Inhibition of lysosomal ADA by various nucleoside and nucleobase analogues

Lysosomal material was incubated with 14.5 μM [^{3}H]adenosine (0.18 μCi/μl) in the presence or absence of the given analogue each at a final concentration of 2.5 mM, unless otherwise indicated. Inhibitor solutions were prepared in 50 mM Mes/NaOH buffer, pH 5.5, and duplicate reactions were performed in a final volume of 22.5 μl at pH 5.5 and 25 °C for 10 min. Data are expressed as the percentage of ADA activity in the uninhibited control, in which 50 mM Mes/NaOH buffer, pH 5.5, was substituted for inhibitor solution. ADA activity in the uninhibited control corresponded to 6.33 ± 0.22 pmol of inosine formed/min per unit of hexosaminidase.

Inhibitor	Activity (% of uninhibited rate)
Deoxycoformycin (20 μM)	0.4 ± 0.1
6-Methylaminopurine riboside	0.8 ± 0.1
Coformycin	1.2 ± 0
2′-Deoxyadenosine	1.3 ± 0.1
Adenosine	1.5 ± 0.6
EHNA (200 μM)	1.6 ± 0.1
2′,3′-Isopropylidene-adenosine	2.6 ± 0.6
2,6-Diaminopurine	10 ± 1
6-Mercaptopurine riboside	11 ± 0.4
6-Methylmercaptopurine riboside	19 ± 1
Guanosine (1.42 mM)	30 ± 1
4-Amino-5-imidazolecarboxamide hydrochloride	35 ± 2
6-Chloropurine riboside	36 ± 3
Adenine (1.25 mM)	37 ± 2
Adenosine 5′-monophosphate	56 ± 0.2
Adenosine 3′-monophosphate	58 ± 1
Formycin A	71 ± 3
Adenosine 5′-triphosphate	74 ± 0
S-Adenosyl-L-homocysteine	93 ± 4
Cytosine	96 ± 0

Table 2 Effect of various bivalent cations on lysosomal ADA activity

Lysosomal material was incubated with 14.5 μM [^{3}H]adenosine (0.18 μCi/μl) in the presence of the indicated 2.5 mM bivalent cation solution, prepared in 50 mM Mes/NaOH buffer, pH 5.5. Duplicate reactions were performed in a final volume of 45 μl at pH 5.5 and 25 °C for 10 min. Data are expressed as the percentage of ADA activity in the uninhibited control, in which 50 mM Mes/NaOH buffer, pH 5.5, was substituted for bivalent-cation solution. ADA activity in the uninhibited control corresponded to 4.44 ± 0.32 pmol of inosine formed/min per unit of hexosaminidase.

Salt	Activity (% of control rate)
$CuCl_2$	0
Mercuric acetate	0
$ZnSO_4$	94 ± 3
Cadmium acetate	103 ± 2
$CaCl_2$	104 ± 6
$MgCl_2$	104 ± 4
$FeSO_4$	106 ± 2
$BaCl_2$	110 ± 1
$MnSO_4$	111 ± 3

of these nucleosides on lysosomal acid phosphatase activity (results not shown).

DISCUSSION

In this report, we present the first characterization of ADA activity within the lysosomal compartment of human fibroblasts. The first indication that lysosomes may contain an adenosine-deaminating activity was revealed during a previous investigation of the lysosomal uptake of adenosine, in which we observed deamination of [^{3}H]adenosine after its uptake by human fibroblast lysosomes [8]. The presence of ADA in lysosomes may account for a previous observation by van der Weyden and Kelley [28], who found that ~ 2% of leucocyte ADA activity was associated with a particulate fraction which was not identified. Many of the properties of lysosomal ADA are very similar to those reported for other ADA activities, including: (1) a K_m of 37 μM for adenosine, (2) maximum activity in the neutral pH range, which declines sharply at acidic pH values, and (3) a strong inhibition by deoxycoformycin, coformycin, deoxyadenosine, adenosine, EHNA, Hg^{2+} and Cu^{2+}. Furthermore, lysosomal ADA activity is greatly diminished in the ADA-deficient human fibroblast cell line GM469, which has been previously shown to exhibit only 2% of normal ADA activity. These results suggest that at least the catalytic portion of lysosomal ADA activity is derived from the same gene locus as that coding for the form of ADA which is deficient in the most common childhood form of severe combined immunodeficiency.

The presence of ADA activity within human fibroblast lysosomes raises questions as to how this activity is delivered to lysosomes, what is the stability of ADA within the lysosomal compartment, and what role does lysosomal deamination of adenosine play in overall cellular metabolism. A number of different isoenzymes and glycosylated forms of ADA are known [39,40], some of which could be preferentially targeted to lysosomes. The similar properties of lysosomal ADA and the large-molecular-mass form of ADA which predominates within human fibroblasts suggests that the large-molecular-mass form of ADA may be delivered to the lysosomal compartment and remain active in that location. The ADA-complexing protein could be especially important for maintaining the stability of ADA activity delivered to the lysosomal compartment by binding to the small-molecular-mass (38 kDa) form of ADA, resulting in a complex with greatly enhanced resistance to lysosomal degradation. The ability of lysosomal ADA to maintain 70% of its original activity after incubation at 65 °C for 80 min indicates that the active structural form of lysosomal ADA is held together strongly.

Previous studies have indicated that endocytosis of exogenous serum ADA does not significantly contribute to the total ADA activity of human fibroblasts [9,10,19,25]. We examined whether adding exogenous ADA to the culture medium of an ADA-deficient cell line, GM469, would result in an increase in the amount of lysosomal ADA subsequently recovered from these cells (results not shown). GM469 fibroblasts exhibit ~ 2% of the normal level of lysosomal ADA activity when cultured in media supplemented with 10% horse serum or 10% fetal-calf serum. Whereas horse serum contains only a small amount of ADA activity, fetal-calf serum contains substantial amounts of ADA activity [9,10]. On incubating GM469 fibroblasts for 1–24 h in media supplemented with ADA purified from normal human fibroblasts, we observed only marginal (0–2 fold) elevations in the amount of lysosomal ADA activity recovered from GM469 fibroblasts. These results support the previous indications [9,10,19,25] that receptor-mediated endocytosis of exogenous ADA does not significantly contribute to the total ADA content of human fibroblasts. It should be noted, however, that in four separate experiments when GM469 fibroblasts were cultured for 13 days or more in media containing 20% fetal-calf serum with several changes of media during this time, the amount of lysosomal ADA activity recovered from these cells was greatly increased, and instead of being 2% of the normal range, the lysosomal ADA activity was now at the same level as that found in normal human fibroblasts. If GM469 fibroblasts were cultured

for 13 days in media containing 20% fetal-calf serum and then placed in media containing 10% horse serum for 2 days, the amount of lysosomal ADA recovered from the cells returned to its low level of approx. 2% of the normal range. The mechanism by which culturing GM469 fibroblasts in 20% fetal-calf serum leads to a large increase in the lysosomal ADA level within this cell type is not known at present, and whether that mechanism is physiologically relevant to how ADA is delivered to lysosomes in normal human fibroblasts is not apparent. As one possibility, the enhancement of lysosomal ADA observed in cells grown in 20% fetal-calf serum, but not in 10% fetal-calf serum, may reflect the difference in the amount of ADA delivered to lysosomes via pinocytosis or plasma-membrane internalization versus the rate of degradation of ADA within the lysosomal compartment.

Lardeux and Mortimore [6] have shown that, in perfused rat liver under conditions of nutritional deprivation, approx. 65% of the total rat liver cytoplasmic RNA is degraded per day, with 70–85% of this turnover occurring within the lysosomal compartment. In this context, lysosomes clearly play a central role in generating nucleosides that can be re-utilized for the synthesis of nucleic acids necessary for cell survival. Moreover, inosine can serve as a fuel for the production of ATP to drive energy-requiring reactions within a cell. Inosine at concentrations as low as 40 nM can support the entire energy requirement of pig erythrocytes [41–43], and has been shown to support partially the cellular energy demands in a variety of other tissues [44–48]. In this context, inosine can be acted on by nucleoside phosphorylase to yield hypoxanthine and ribose l-phosphate [39], with this latter product entering glycolysis via the pentose phosphate pathway.

Compartmentalization of ADA may provide a pathway of metabolism that is advantageous under certain conditions. At low adenosine concentrations, adenosine is primarily phosphorylated in the cytosol by adenosine kinase, which has a much lower K_m for adenosine than does ADA [39,49]. In contrast, at high adenosine concentrations, adenosine inhibits adenosine kinase activity, allowing deamination by ADA to be the major route of adenosine metabolism [39,49]. By compartmentalizing ADA activity within lysosomes, however, adenosine deamination could occur without competition by the kinase reaction. The inosine thus formed within lysosomes can be transported to the cytosol and converted by nucleoside phosphorylase into hypoxanthine and ribose l-phosphate, which can be used to support cellular energy requirements, which may be particularly important under conditions of nutritional deprivation. Although lysosomal ADA constitutes only 10% of the total ADA activity of human fibroblasts, this compartmentalization, allowing ADA to function in the absence of adenosine kinase competition, may permit lysosomal ADA to play a significant role in overall adenosine deamination within the cell under normal conditions. Hence the concept of lysosomes as supplying critical nutrients within the cell may be further expanded by the finding of ADA activity within this compartment.

We especially thank Dr. Jess Thoene for his helpful comments in reviewing this manuscript. This work was supported by Grant DK40323 from the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1 Barrett, A. J. (1972) in Lysosomes, A Laboratory Handbook (Dingle, J. T., ed.), pp. 63–68, North-Holland, Amsterdam
2 Arsenis, C., Gordon, J. S. and Touster, O. (1970) J. Biol. Chem. **245**, 205–211
3 Arsenis, C. and Touster, O. (1968) J. Biol. Chem. **243**, 5702–5708
4 Bernardi, G. (1968) Adv. Enzymol. **31**, 1–49
5 Vaes, F. G. (1973) in Lysosomes and Storage Diseases (Hers, H. G. and Van Hoof, F., eds.), pp. 70–72, Academic Press, Orlando, FL
6 Lardeux, B. R. and Mortimore, G. E. (1987) J. Biol. Chem. **262**, 14514–14519
7 Heydrick, S. J., Lardeux, B. R. and Mortimore, G. E. (1991) J. Biol. Chem. **266**, 8790–8796
8 Pisoni, R. L. and Thoene, J. G. (1989) J. Biol. Chem. **264**, 4850–4856
9 Hirschhorn, R., Beratis, N. and Rosen, F. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 213–217
10 Daddona, P. E., Frohman, M. A. and Kelley, W. N. (1980) J. Biol. Chem. **255**, 5681–5687
11 Pisoni, R. L. (1991) J. Biol. Chem. **266**, 979–985
12 Pisoni, R. L., Thoene, J. G., Lemons, R. M. and Christensen, H. N. (1987) J. Biol. Chem. **262**, 15011–15018
13 Pisoni, R. L., Flickinger, K. S., Thoene, J. G. and Christensen, H. N. (1987) J. Biol. Chem. **262**, 6010–6017
14 Cleland, W. W. (1979) Methods Enzymol. **63**, 103–138
15 Rome, L. H., Garvin, A. J., Allietta, M. M. and Neufeld, E. F. (1979) Cell **17**, 143–153
16 Gieselmann, V., Pohlmann, R., Hasilik, A. and von Figura, K. (1983) J. Cell Biol. **97**, 1–5
17 Pertoft, H., Warmegard, B. and Hook, M. (1978) Biochem. J. **174**, 309–317
18 Pisoni, R. L. and Thoene, J. G. (1991) Biochim. Biophys. Acta **1071**, 351–373
19 Andy, R. J. and Kornfeld, R. (1982) J. Biol. Chem. **257**, 7922–7925
20 Pora, N., Gill, D. and Parola, A. H. (1988) J. Biol. Chem. **263**, 14608–14611
21 Bielat, K. and Tritsch, G. L. (1989) Mol. Cell. Biochem. **86**, 135–142
22 Franco, R., Aran, J. M., Colomer, D., Matutes, E. and Vives-Corrons, J. L. (1990) J. Histochem. Cytochem. **38**, 653–658
23 Aran, J. M., Colomer, D., Matutes, E., Vives-Corrons, J. L. and Franco, R. (1991) J. Histochem. Cytochem. **39**, 1001–1008
24 Franco, R. and Centelles, J. J. (1990) Biochem. Soc. Trans. **18**, 641–642
25 Hirschhorn, R., Beratis, N. G. and Martiniuk, F. (1978) Exp. Cell Res. **117**, 103–109
26 Inwood, M., Povey, S. and Delhanty, J. D. A. (1980) Cell Biol. Int. Rep. **4**, 327–335
27 Daddona, P. E. and Kelley, W. N. (1979) Biochim. Biophys. Acta **580**, 302–311
28 van der Weyden, M. B. and Kelley, W. N. (1976) J. Biol. Chem. **251**, 5448–5456
29 Smith, M. L., Greene, A. A., Potashnik, R., Mendoza, S. A. and Schneider, J. A. (1987) J. Biol. Chem. **262**, 1244–1253
30 Akedo, H., Nishihara, H., Shinkai, K., Komatsu, K. and Ishikawa, S. (1972) Biochim. Biophys. Acta **276**, 257–271
31 Daddona, P. E. and Kelley, W. N. (1981) Biochim. Biophys. Acta **658**, 280–290
32 Daddona, P. E. and Kelley, W. N. (1977) J. Biol. Chem. **252**, 110–115
33 Pfrogner, N. (1967) Arch. Biochem. Biophys. **119**, 147–154
34 Orsi, B. A., McFerran, N., Hill, A. and Bingham, A. (1972) Biochemistry **11**, 3386–3392
35 Wilson, D. K., Rudolph, F. B. and Quiocho, F. A. (1991) Science **252**, 1278–1284
36 Schrader, W. P., Pollara, B. and Meuwissen, H. J. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 446–450
37 Ronca, G., Bauer, C. and Rossi, C. A. (1967) Eur. J. Biochem. **1**, 434–438
38 Lucarini, N., Borgiani, P., Ballarini, P. and Bottini, E. (1989) Hum. Genet. **81**, 185–187
39 Kredich, N. M. and Hershfield, M. S. (1989) in The Metabolic Basis of Inherited Disease, 6th edn. (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 1045–1075, McGraw-Hill, New York
40 Swallow, D. M., Evans, L. and Hopkinson, D. A. (1977) Nature (London) **269**, 261–262
41 Jarvis, S. M., Young, J. D., Ansay, M., Archibald, A. L., Harkness, R. A. and Simmonds, R. J. (1980) Biochim. Biophys. Acta **597**, 183–188
42 Young, J. D., Paterson, A. R. and Henderson, J. F. (1985) Biochim. Biophys. Acta **842**, 214–224
43 Young, J. D., Jarvis, S. M., Clanachan, A. S., Henderson, J. F. and Paterson, A. R. (1986) Am. J. Physiol. **251**, C90–C94
44 Yoshiyama, M., Sakai, H., Teragaki, M., Takeuchi, K., Takeda, T., Ikata, M., Ishikawa, M. and Miura, I. (1988) Biochem. Biophys. Res. Commun. **151**, 1408–1415
45 Kovacevic, Z., Popovic, J., Brkljac, O. and Lelas, S. (1987) Biochem. J. **247**, 47–51
46 Zeidler, R. B., Metzler, M. H., Moran, J. B. and Kim, H. D. (1985) Biochim. Biophys. Acta **838**, 321–328
47 Zaporowska-Siwiak, E., Michalik, M., Kajstura, J. and Korohoda, W. (1985) J. Cell Sci. **77**, 75–85
48 Kipshidze, N. N., Karsanov, N. V., Chichinadze, N. A., Machitadze, T. N. and Azrumelashvili, M. I. (1983) Methods Find. Exp. Clin. Pharmacol. **5**, 163–170
49 Fox, I. H. and Kelley, W. N. (1978) Annu. Rev. Biochem. **47**, 655–686

Received 30 March 1992/10 September 1992; accepted 8 October 1992