Human Adenosine Deaminase

PURIFICATION AND SUBUNIT STRUCTURE*

PETER E. DADDONA AND WILLIAM N. KELLEY

From the Departments of Internal Medicine and Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the irreversible hydrolytic deamination of adenosine to inosine and ammonia (1). Reduced or absent adenosine deaminase activity is associated with severe combined immunodeficiency disease. At least 19 patients with this disorder have been described since the original report of two patients by Gibbitt et al. (2) in 1972. Affected patients present a defect of both cellular and humoral immunity characterized clinically by severe recurrent infections which ultimately prove fatal. The relationship of adenosine deaminase deficiency to severe immune dysfunction is still undefined, but may reflect (a) a generalized aberration in the cell which alters both adenosine deaminase activity and immune function, (b) a large genetic deletion which includes loci critical to both the immune response and adenosine deaminase activity, or (c) a direct causal relationship.

Multiple forms of adenosine deaminase have been reported in amphibian (3, 4), avian (5), and mammalian sources (6-16). Some human tissues display as many as four molecular species with estimated molecular weights of >20,000,000, 298,000, 114,000, and 36,000 (designated “particulate,” large, intermediate, and small form, respectively) (15). Interconvertibility of the soluble forms of the enzyme has been demonstrated in partially purified tissue extracts (15). Furthermore, the small form of the enzyme regardless of tissue source exhibits at least three electrophoretic variants by starch gel electrophoresis (10), isoelectric focusing (15), and DEAE-Sephadex chromatography (14).

The post-translational modification of normal adenosine deaminase and its regulation may be important to normal cell function and possibly to the complex interactions involved in the immune system. In this context, the purification and elucidation of the structure of the small form of the enzyme would provide not only a basis for future studies of the genetic defect leading to reduced or absent adenosine deaminase activity, but would also allow a more detailed examination of the mechanism(s) responsible for the molecular interconversions of the normal enzyme.

This study describes the first purification of normal human erythrocyte adenosine deaminase to apparent homogeneity and defines the subunit structure.

EXPERIMENTAL PROCEDURES

Materials

[8-14C]Adenosine (50.4 mCi/mmol) was purchased from New England Nuclear Corp. Sephadex G-100 (superfine), cyanogen bromide-activated Sepharose 4B, CM-Sephadex C-50, and blue dextran 2000 were obtained from Pharmacia. Sigma supplied bovine serum albumin, chymotrypsigen A, cytochrome c, adenosine deaminase from calf intestinal mucosa, basic fuchsin, 4-phenylbutylamine, ethanolamine, and catalase. Ultrapure urea, ultrapure sucrose, SDS bromophenol blue, and Coomassie brilliant blue were obtained from Schwarz/Mann; dansylhydrazine from Pierce Chemical Co.; ovalbumin from Nutritional Biochemicals; and DE52, microgranular, from Whatman, Reeves Angel. Moe, Heps, adenosine, and inosine came from Calbiochem; iminobismalamin and 1-cyclohexyl-3-(2-morpholinomethyl)carbodiimide metho-p-toluenesulfonate from Aldrich.

The abbreviations used are: SDS, sodium dodecyl sulfate; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Mes, 2-(N-morpholinoethanesulfonic acid; Heps, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; KP, potassium phosphate; NaCUP, 50 mM potassium phosphate and 0.15 mM sodium chloride; NaF, sodium phosphate.
Human Adenosine Deaminase

Preparation of Antibody Affinity Sepharose 4B

Dry CNBr-activated Sepharose 4B (0.829 g) was swollen and washed in 1 mM HCl. Immediately after equilibration of the gel in 0.15 M sodium citrate buffer, pH 6.5 (coupling buffer). 37.5 mg of purified anti-calf adenosine deaminase γ-globulin was added. The mixture was immediately rotated end over end for 12 h at 4°C, filtered on a sintered glass filter and washed with 10 volumes of coupling buffer. Protein analysis of the filtrate revealed that >99% of the antibody protein was bound to the Sepharose gel. The gel was then resuspended in 200 mM ethanalamine/HCl, pH 8.5, rotated end over end for 2 h, and washed on a sintered glass funnel successively with 10 volumes of NaCl/Pi buffer, pH 7.2, 50 mM KP, containing 1 mM NaCl, pH 7.2, 50 mM imidazole/HCl, pH 5.8, and finally 40 mM imidazole-HCl, pH 6.0, containing 8 M urea. The resulting affinity gel was equilibrated in NaCl/Pi buffer with 0.025% sodium azide could be stored at 4°C over 6 months without detectable loss of activity.

Enzyme Purification

Step 1: Preparation of Erythrocyte Hemolysate – Thirty units of outdated (older than 21 days) packed erythrocytes (4900 ml) were obtained from the American Red Cross. Unless otherwise stated, all subsequent steps were performed at 4°C. Residual plasma anduffy coat were removed by centrifugation at 3000 × g for 15 min. The erythrocytes were washed twice with equal volumes of 0.15 M NaCl and the packed cells were lysed by dilution with 2 volumes of distilled water. Stroma was precipitated and removed by adjusting the pH of the hemolysate to 8.8 with 0.1 M HCl, followed by centrifugation at 6000 × g for 20 min.

Step 2: CM-Sepharose Negative Adsorption Step – Dry CM-Sepharose C-50 (66 g/1000 ml) of original packed erythrocytes) was added slowly with stirring to the stroma-free hemolysate. The mixture was centrifuged at 15,000 × g for 30 min and stored at 4°C for 2 h. The mixture was again filtered as before. The combined filtrates were then treated by the same procedure using the Sepharose CM-50 column at 4°C. The second batch treatment resulted in an essentially hemoglobin-free filtrate.

Step 3: Ammonium Sulfate Precipitation – The filtrate was brought to 60% saturation with crystalline ammonium sulfate and stirred for 4 h. The mixture was centrifuged at 16,000 × g for 1 h. The precipitate was then dissolved in a minimum volume of NaCl/Pi buffer, pH 7.2, and dialyzed against three changes of 100 volumes of NaCl/Pi buffer for 2 h. The precipitate was then resuspended at 4°C for 2 h and washed on a sintered glass funnel. The resin containing most of the hemoglobin was then resuspended in enough distilled water to make a thin slurry and stirred for 10 min. The mixture was again filtered after centrifugation.

Step 4: Antibody Affinity Chromatography – The ammonium sulfate-purified fraction was applied to the antibody affinity column (0.7 x 6.5 cm) which was prepared as described previously and equilibrated with NaCl/Pi buffer, pH 7.2. Fractions (5 ml) were collected at a flow rate of 12 ml/h. After sample application, the column was washed in sequence with 10 ml of each of the following buffers: NaCl/Pi, pH 7.2; 0.5 M NaCl in 50 mM KP, pH 7.2; 1.0 M NaCl in 50 mM KP; pH 7.2; NaCl/Pi; and 50 mM imidazole/HCl, pH 6.0. Enzyme activity was eluted by a 1-ml step gradient of 5 to 8 M urea in 50 mM imidazole/HCl, pH 5.8, and 1-ml fractions were collected. Fractions containing peak enzyme activity were pooled and concentrated by ultrafiltration using a PM-10 membrane as described. The enzyme solution was then dialyzed against changes of 100 volumes of 20% glycerol in 40 mM imidazole/HCl buffer, pH 6.0, for 24 h. The enzyme could be stored in this buffer at 4°C for over 1 month without significant loss of enzyme activity.

Polyacrylamide Gel Electrophoresis

A Buchler electrophoresis apparatus equipped with a Beckman Duostat power source was used for all electrophoretic procedures. Disc gel electrophoresis was performed using a discontinuous Tris/glycine buffer system, pH 8.5, according to the method of Davis (23). Bromphenol blue was used as the tracking dye. Gels were either assayed for adenosine deaminase activity or stained for protein. Gels to be assayed were sliced into 1-mm sections and placed into 10 mM Tris/HCl buffer, pH 7.4, containing bovine serum albumin (1 mg/ml) to elute and stabilize the enzyme. After standing overnight at 4°C an aliquot of the supernatant was assayed for enzyme activity. Gels were stained for protein overnight with 0.3% Coomassie brilliant blue prepared according to the method of Weber and Osborn (24) and...
were determined at 37°C using a Radiometer
Tris/HCl (50 mM) for the pH range 7.4 to 9.5. The pH of the reaction
for the pH range 5.5 to 6.5, Hepes (50 mM) for pH range 7.0 to 8.0, and
Mes (50 mM) was employed
Amino Acid Analysis
Sucrose Gradient Ultracentrifugation
Sucrose density ultracentrifugation was performed with a Beck-
man SW 41 rotor in a Beckman model L5-50 ultracentrifuge. Isoki-
etic gradients of 10 to 28.2% w/w were prepared as described by
McCarty et al. (27), using 10 mM Tris/HC1, pH 7.4. After centrifuga-
tion at 40,000 rpm for 30 h at 4°C, fractions of 25 ml were collected.
Sedimentation coefficient ($s_{20, w}$) values were calculated on the basis
of the linear relationship of the $s_{20, w}$ to the distance migrated in the
isokinetic gradient (27). Bovine serum albumin, chymotrypsinogen A,
and catalase were used as standards for these calculations.

Gel Filtration
Gel filtration was performed on a Sephadex C-100 (superfine) column (1.7 x 87.5 cm) with 100 mM KCl, pH 7.4, as eluting buffer.
The void volume was determined with dextran blue and the column was calibrated with ovalbumin, chymotrypsinogen A, and cyto-
chrome c. The molecular weight standard was determined by the method of
Laurent and Killander (28) and the Stokes radius of erythrocyte adenosine deaminase was calculated according to the method of
Ackers (29). The Stokes radii or $s_{20, w}$ values for calibration standards
were obtained from the literature (27, 30).

Amino Acid Analysis
Amino acid analysis was performed on a Beckman 120C instru-
ment equipped with an electronic range expander and an Infotronics
integrator. The sample was hydrolyzed in vacuo in 5.7 n HCl for 22 h at
110°C (constant boiling). Composition was estimated by assuming a
molecular weight in the range of 35,000 to 38,000 and then fitting the
data to minimize the number of fractional residues. Values for
threonine and serine were increased by 5% and 10%, respectively,
to correct for destruction during hydrolysis. Valine and isoleucine were
rounded off to the next higher integral number of residues. Trypto-
phan and cysteine were not determined by this method nor was any
attempt made to quantitate the yield of ammonia from the acid
amides. The partial specific volume of erythrocyte adenosine deami-
nase was calculated from the amino acid composition (31).

Molecular Weight Estimation
Molecular weight and frictional ratio of adenosine deaminase were calculated from the Stokes radius, $s_{20, w}$, and partial specific
volume values by the following formulas (28):

\[ M_r = 6\pi \eta N / (1 - \epsilon \rho) \]

\[ f/f_0 = a (50M)^{1/3} \]

where $M_r$ is molecular weight, $\eta$ is viscosity of the medium, $N$ is Avogadro’s number, $\alpha$ is Stokes radius, $s$ is sedimentation coefficient, $\epsilon$ is partial specific volume, $\rho$ is density of the medium, and $f/f_0$ is frictional ratio.

pH Optimum
The pH optimum for affinity-purified adenosine deaminase was evaluated over the pH range 5.5 to 9.5. Mes (50 mM) was employed
for the pH range 5.5 to 6.5. Heps (50 mM) for pH range 7.0 to 8.6, and
Tris/HC1 (60 mM) for the pH range 7.4 to 9.5. The pH of the reaction
mixtures without enzyme was determined at 37°C using a Radiometer
Copenhagen pH meter equipped with a combination glass electrode.

**Enzyme Stability**

The stability of highly purified adenosine deaminase was evaluated
under a variety of conditions. The enzyme at a final concentra-
tion of 20 mg/ml was added to 10 mM Tris/HC1, pH 7.4, containing 5% or
20% glycerol, 1 mM or 10 mM EDTA, 5% or 10% dimethyl sulfoxide or
with no additive and stored at -70°C and 4°C for varying periods.
Stability of the enzyme in 100 mM KP, 100 mM NaF, and 50 mM KP,
containing either 0.05 M, 0.1 M, or 0.2 M ammonium sulfate was also
tested using the same storage conditions as described above.

**Enzyme Specificity**

The enzyme specificity of highly purified adenosine deaminase (Step 4) and a more crude preparation (Step 3) of the enzyme was
determined. All reaction mixtures contained [8-14C]adenosine, 100 
$\mu$m (2 $\mu$Ci/\muM), selected compounds, 2.5 mM, and adenosine deami-
nase (35.7 nmol/h). The effectiveness of the selected compounds to
act either as substrates or inhibitors for adenosine deaminase was
evaluated by their ability to inhibit the enzymatic conversion of [8-

**RESULTS**

Our initial attempts to purify the enzyme by conventional
protein purification techniques, including pH adjustment, am-
nionum sulfate precipitation, heat treatment, ion exchange
chromatography, gel filtration, and isoelectric focusing were
only partially successful. In addition, putative affinity col-
 umns using adenosine or inosine coupled to Sepharose 4B via
the ribose ring (position 2,3) with an iminobispropylamine
 spacer group (33, 34) or adenosine coupled via position C-8
with a hexane spacer group (P-1, Biochemicums, Inc.) failed to
bind adenosine deaminase significantly over control columns.
A series of homologous C-2 to C-10 n-alkane Sepharose col-
 umns (Miles-Yeda Ltd.) as well as synthetically prepared
phenyl and 4-phenylbutyl Sepharose columns (33, 35) were
found to bind adenosine deaminase to varying degrees, but
elution of enzyme activity by a linear salt gradient resulted in
no increase in purification. Attempts to selectively remove the
affinity-bound adenosine deaminase by saturating the elution
buffer (100 mM NaP, buffer, pH 7.0) with adenosine or by the
addition of a strong competitive inhibitor erythro-9-(2-hy-
droxy-3-nonyl) adenine (EHNA) at a final concentration of
1 mM failed to release the enzyme.

The failure of chemical affinity chromatography to provide a
substantial improvement in purification led us to develop an
antibody affinity chromatography system. Antisera produced
against purified calf intestinal adenosine deaminase cross-
reacted with human erythrocyte adenosine deaminase (Fig.
1). Furthermore, the antisera could be highly purified by
ammonium sulfate precipitation, DE52 chromatography, and/or
specific immunoglobulin precipitation with an apparently
homogeneous preparation of calf adenosine deaminase. Recov-
eries of active anti-calf adenosine deaminase antibodies from
these purification steps were approximately 85%, 9%, and
50%, respectively. All preparations of the original antisera
coupled to activated Sepharose were able to strongly
bind human erythrocyte adenosine deaminase. However, the
most highly purified immunoglobulin fraction resulting from
specific immunoprecipitation with calf adenosine deaminase
was bound to Sepharose for enzyme purification to minimize
non-specific protein binding.

Using the antibody affinity column an overall purification from
crude hemolysate of 500,000-fold to a specific activity of
538 $\mu$mol/min/mg at 37°C was achieved with a 35% recovery of
original adenosine deaminase activity (Table I). Routinely, all
of the adenosine deaminase activity present after Step 3
(equivalent to 30 units of hemolysate) could be bound to the
2.5-ml antibody affinity gel column prepared as described.
under "Experimental Procedures." Enzyme activity was eluted by a urea step gradient in order to remove a small amount of non-adenosine deaminase protein which was weakly bound. The antibody affinity gel has been reused at least 10 times under the conditions described without loss of binding capacity.

Polyacrylamide gel electrophoresis showed three bands by Coomassie stain, each of which had adenosine deaminase activity as determined by assay of an identical companion gel (Fig. 2A). SDS-polyacrylamide electrophoresis revealed one major band of protein by Coomassie stain with an apparent molecular weight of 41,700 ± 686 (Figs. 2B and 3) demonstrating the human erythrocyte adenosine deaminase to be a single polypeptide chain. Staining a companion SDS gel with periodic acid-Schiff reagent using either the chromophore basic fuchsin or fluorescent dansylhydrazine indicated the presence of carbohydrate on the protein.

A Stokes radius of 24 Å, an $s_{20,w}$ of $3.8 \times 10^{-13}$ s and a partial specific volume of 0.729 cm$^3$/g were calculated for the human erythrocyte adenosine deaminase purified by antibody affinity chromatography. From these data, a molecular weight of 38,200 and a frictional ratio of 1.077 was calculated.

The highly purified enzyme exhibited a broad pH optimum from pH 7.0 to 8.0. Furthermore, a Michaelis constant of 52 μM was determined from an Eadie-Hofstee plot. From a fixed concentration of inosine and variable concentrations of adenosine, the $K_i$ for inosine was calculated to be 700 μM.

The affinity-purified adenosine deaminase was evaluated for stability under a variety of conditions. The enzyme generally retained activity for up to 3 weeks at 4°C. However, at -70°C, glycerol or dimethylsulfoxide appeared to be necessary for preservation of activity during the freezing, or thawing process, or both.

![Fig. 1](image1.png)

**Fig. 1.** Precipitation of adenosine deaminase activity with anti-calf adenosine deaminase antiserum. A constant amount of adenosine deaminase was incubated with varying amounts of anti-calf adenosine deaminase antiserum. The enzyme was normally followed by a second incubation with goat anti-rabbit y-globulin antiserum (added at equivalence for rabbit y-globulin) as described under "Experimental Procedures." After centrifugation, the supernatant and precipitate were separated and the latter washed once with 200 μl of NaCl/PI and resuspended in 50 μl of 50 mM Tris/HCl, pH 7.4. The per cent of initial adenosine deaminase activity remaining in the supernatant and that appearing in the precipitate was determined. Δ—Δ, calf adenosine deaminase remaining in supernatant; ●—●, human erythrocyte adenosine deaminase remaining in supernatant; ○—○, human erythrocyte adenosine deaminase inhibition in the absence of goat anti-rabbit antiserum; ■—■, human erythrocyte adenosine deaminase activity appearing in the precipitate; Δ—Δ, adenine phosphoribosyltransferase activity in the presence of goat anti-rabbit antiserum.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (nmol/min × 10^6)</th>
<th>Total protein (mg)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude hemolysate diluted 1:3</td>
<td>12,672</td>
<td>10.2</td>
<td>1.52 × 10^6</td>
<td>0.673</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>pH adjust 5.8</td>
<td>13,526</td>
<td>10.2</td>
<td>1.16 × 10^6</td>
<td>0.881</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>CM-Sephadex batch treatment</td>
<td>12,626</td>
<td>8.78</td>
<td>3.53 × 10^4</td>
<td>24.9</td>
<td>37</td>
<td>86</td>
</tr>
<tr>
<td>0-60% [NH₄]₂SO₄</td>
<td>362</td>
<td>8.22</td>
<td>1.21 × 10^4</td>
<td>67.5</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>0.5</td>
<td>3.63</td>
<td>0.675</td>
<td>538,400</td>
<td>800,000</td>
<td>35</td>
</tr>
</tbody>
</table>
Substrate specificity of the affinity-purified enzyme (Step 4) was compared to that of a less pure preparation of the enzyme (Step 3). The selected compounds, AMP, dAMP, cAMP, dADP, ATP, cytosine, cytidine, CMP, dCMP, CDP, dCDP, and CTP gave 0% inhibition, whereas 4-amino-5-imidazole carboximide ribonucleoside, 4-amino-5-imidazole carboximide HCl, 2,6-diaminopurine sulfate, 6-chloropurine, 6-chloropurine ribonucleoside, iodopurine, 6-methylmercaptopurine ribonucleoside, and adenosine gave 15 to 60% inhibition, and finally deoxycytosine and 6-methylaminopurine ribonucleoside gave greater than 90% inhibition. In each case both enzyme preparations were inhibited to the same degree.

**DISCUSSION**

Previous attempts to purify adenosine deaminase from a human source have resulted in specific activities of 0.53 (14), 0.71 (36), and 12.9 (37) μmol/min/mg from erythrocytes and 47.4 μmol/min/mg (13) from stomach. In this communication, we describe the purification of human erythrocyte adenosine deaminase to a specific activity of 538.4 pmol/min/mg and an apparent homogeneity. The enzyme is shown to be a single polypeptide chain with an estimated molecular weight of 38,200 and a frictional ratio of 1.077.

**Table II**

<table>
<thead>
<tr>
<th>Amino acid composition of human erythrocyte adenosine deaminase</th>
<th>Residues/35,700</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>20</td>
</tr>
<tr>
<td>Histidine</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>10</td>
</tr>
<tr>
<td>Aspartic acid and asparagine</td>
<td>37</td>
</tr>
<tr>
<td>Threonine</td>
<td>17</td>
</tr>
<tr>
<td>Serine</td>
<td>21</td>
</tr>
<tr>
<td>Glutamic acid and glutamine</td>
<td>38</td>
</tr>
<tr>
<td>Proline</td>
<td>14</td>
</tr>
<tr>
<td>Glycine</td>
<td>30</td>
</tr>
<tr>
<td>Alanine</td>
<td>35</td>
</tr>
<tr>
<td>Valine</td>
<td>18</td>
</tr>
<tr>
<td>Methionine</td>
<td>8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>14</td>
</tr>
<tr>
<td>Leucine</td>
<td>28</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>7</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>313</strong></td>
</tr>
</tbody>
</table>

*Values for threonine and serine were increased by 5% and 10%, respectively, to correct for destruction during hydrolysis. Cysteine and cystine were not determined by this method nor was the yield of ammonia quantitated from acid amides.*

**REFERENCES**

Human adenosine deaminase. Purification and subunit structure.
P E Daddona and W N Kelley


Access the most updated version of this article at http://www.jbc.org/content/252/1/110

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/1/110.full.html#ref-list-1