Cloning and Characterization of Human Guanine Deaminase

PURIFICATION AND PARTIAL AMINO ACID SEQUENCE OF THE MOUSE PROTEIN*

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Mouse erythrocyte guanine deaminase has been purified to homogeneity. The native enzyme was dimeric, being comprised of two identical subunits of approximately 50,000 Da. The protein sequence was obtained from five cyanogen bromide cleavage products giving sequences ranging from 12 to 25 amino acids in length and corresponding to 99 residues. Basic Local Alignment Search Tool (BLAST) analysis of expressed sequence databases enabled the retrieval of a human expressed sequence tag cDNA clone highly homologous to one of the mouse peptide sequences. The presumed coding region of this clone was used to screen a human kidney cDNA library and secondarily to polymerase chain reaction-amplify the full-length coding sequence of the human brain cDNA corresponding to an open reading frame of 1365 nucleotides and encoding a protein of 51,040 Da. Comparison of the mouse peptide sequences with the inferred human protein sequence revealed 88 of 99 residues to be identical. The human coding sequence of the putative enzyme was subcloned into the bacterial expression vector pMAL-c2, expressed, purified, and characterized as having guanine deaminase activity with a $K_m$ for guanine of 9.5 ± 1.7 μM. The protein shares a 9-residue motif with other amino- hydrolases and amidohydrolases (PGX[V]I[D]X[H][TV][H]) that has been shown to be ligated with heavy metal ions, commonly zinc. The purified recombinant guanine deaminase was found to contain approximately 1 atom of zinc per 51-kDa monomer.

Guanine deaminase (guanine aminohydrolase, EC 3.5.4.3) catalyzes the hydrolytic deamination of guanine. By producing xanthine and ammonia, this reaction irreversibly eliminates the guanine base from further reutilization as a guanylate nucleotide in mammals. The product xanthine is a substrate for xanthine oxidase in the production of uric acid. Although it is an enzyme of purine catabolism, guanine deaminase is not ubiquitously expressed and exhibits a general absence in lymphoid tissues and variable expression elsewhere (1, 2). The highest levels of expression were found in the proximal section of the small intestine of the mouse (3). There are greater than 50-fold differences in guanine deaminase among different regions of the mouse brain; the cerebral cortex and amygdala have the highest activity (4), whereas there is essentially no activity in the cerebellum of the mouse or cat (4, 5). There are greater than 10-fold increases in the level of expression of guanine deaminase in the liver, kidney, and brain during the 40-day postnatal development of the rat (6), and alterations in embryonic expression have also been characterized (7). In the adult mouse, fractional increases in brain and liver enzyme activity occur in response to intraperitoneal administration of a bolus of guanine (8). The tissue-specific expression and the developmental and induced changes in expression suggest a potential role for guanine deaminase in the regulation of the guanine nucleotide pool. Cellular GTP has an important role not only in specific enzyme reactions and protein synthesis, but also in signal transduction pathways. The cloning and characterization of guanine deaminase will advance our understanding of this key enzyme in the catabolism of guanine metabolites.

Guanine deaminase has been purified from a number of mammalian sources (9–13). We describe here the purification to homogeneity of mouse erythrocytic guanine deaminase. Cyanogen bromide cleavage and peptide sequencing facilitated the identification and retrieval of human ESTs clones that were used to isolate a full-length guanine deaminase cDNA from human brain. Subsequent expression of the recombinant human protein and characterization of its catalytic activity and kinetics properties confirmed this cDNA to encode human guanine deaminase.

MATERIALS AND METHODS

Reagents—Xanthine oxidase, uricase, and peroxidase were purchased from ICN Pharmaceuticals. Guanine and 4-aminopyrine were from Sigma, and 2,4,6-tribromo-3-hydroxybenzoic acid was from Boehringer Mannheim. $[^{32}P]dCTP$ and $[^{32}P]dATP$ were from Amersham. Restriction enzymes, Taq DNA polymerase, and isoprropyl-β-thiogalactopyranoside were obtained from Life Technologies, Inc.

Purification of Mouse Erythrocyte Guanine Deaminase—Heparinized blood was collected from C57BL/6J adult mice as described previously (14). Blood pooled from 30–50 mice was centrifuged at 900 × g for 5 min, and the erythrocyte pellet was resuspended and washed once with 10 volumes of isotonic saline. The cell pellet was lysed by adding an equal volume of 2% Buffer A (20 mM Tris-HCl, 5 mM MgCl₂, and 1 mM DTT, pH 6.0) followed by three 30-s pulses at the maximum setting on the Polytron (Brinkmann Instruments). The lysate was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was used in subsequent steps.

The crude supernatant (approximately 20 ml) was applied to a 25-ml DEAE-Sepharose column previously equilibrated in Buffer A and washed with 10 column volumes of Buffer A. The enzyme was eluted with a gradient formed between equal volumes of Buffer A and Buffer B (buffer A containing 0.5 M KCl) at 1.5 ml/min. 3-ml fractions were collected, and guanine deaminase was typically eluted at approximately
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150 mM KCl. Fractions containing guanine deaminase were pooled and concentrated using an Amicon ultrafiltration YM10 membrane.

Subsequent steps were performed on a Pharmacia fast protein liquid chromatography system. 1× volume of 100 mM sodium phosphate, pH 6.8, 1 mM DTT, and 3.2 mM (NH₄)₂SO₄ was added to the concentrated fraction, and the sample was loaded on a 10-ml phenol-Sepharose high performance column (Pharmacia) equilibrated in Buffer C (50 mM sodium phosphate, pH 6.8, and 1 mM DTT) containing 1.6 mM (NH₄)₂SO₄. The column was washed at 1 ml/min with 8 volumes of loading buffer and then eluted by a stepwise gradient of 10 ml each in descending 0.2-x increments of (NH₄)₂SO₄ in Buffer C, collecting 1-ml fractions. Guanine deaminase eluted at approximately 0.6 M (NH₄)₂SO₄. Fractions were pooled and concentrated by Amicon ultrafiltration as described above.

Combined fractions were concentrated to 1 ml and adjusted to 3.2 mM (NH₄)₂SO₄ in Buffer C. The sample was applied to a 1-ml Source 15PHE column (Pharmacia) and eluted at 1 ml/min, collecting 1-ml fractions. The column was washed with Buffer D (10 ml of Buffer C containing 3.2 mM (NH₄)₂SO₄) and then eluted stepwise with decreasing amounts of (NH₄)₂SO₄, formed from mixtures of Buffers C and D as follows: 0–5 min, 100% to 75% Buffer D; 5–15 min, held at 75% Buffer D; 15–75 min, 75% to 45% Buffer D; and 75–100 min, 45% to 0% Buffer D. Guanine deaminase typically eluted at approximately 45 min, and fractions containing activity were pooled and concentrated, and the buffer was changed to 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 0.25 mM DTT. For storage at −20 °C, 20% glycerol was added to the final buffer.

The native molecular mass was determined by fractionation of purified erythrocyte guanine deaminase on a Superose 6 HR column (Pharmacia) (bed volume, 24 ml) eluted with 10 mM Tris-HCl, 150 mM NaCl, and 2 mM (NH₄)₂SO₄, and then eluted by a stepwise gradient of 10 ml each in descending amounts of (NH₄)₂SO₄. Guanine deaminase typically eluted at approximately 45 min, and fractions containing activity were pooled and concentrated, and the buffer was changed to 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 0.25 mM DTT. For storage at −20 °C, 20% glycerol was added to the final buffer.

**RESULTS**

**Purification of mouse erythrocyte guanine deaminase**

<table>
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<tr>
<th>Step</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Fold purification</th>
<th>Recovery</th>
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<tr>
<td>1. Cell supernatant</td>
<td>580</td>
<td>10</td>
<td>100</td>
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</tr>
<tr>
<td>2. DEAE-Sepharose</td>
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<td>11.2</td>
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<td>3. Phenol-Sepharose</td>
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<td>4. Source PH15</td>
<td>0.0096</td>
<td>9529</td>
<td>953</td>
<td>1.6</td>
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</table>

60 °C. A single positive phase was isolated from approximately 500,000 colonies. The 2.2-kb insert was PCR-amplified using lambda gt10 forward (AGCAGATTGCTGCTCTAAG) and reverse (TAATGAGATTTCCTCCAGGG) primers followed by direct sequencing (21).

From the kidney cDNA sequence, two primers were generated for amplification of the complete coding sequence from human brain Marathon™ Ready cDNA (CLONTECH). Primers derived from the human kidney cDNA and EST sequences were 5′ GDA (AAAGAATTCTGCTGCGGCTCAGATGC, bp −1 to 20) and 3′ dGDA. Both strands of the product were sequenced using the ABI prism TM Dye terminator cycle sequencing ready reaction kit (Perkin Elmer).

**Expression of Human Guanine Deaminase Protein**—Two oligoucleotides, 5′ GDA and 3′ dGDA, were used to amplify the full-length coding sequence. The resulting fragment was digested with EcoRI and HindIII, subcloned into the pMAL-c2 expression vector (New England Biolabs), and transformed into DH5α cells. The insert was sequenced to verify its structure. The expressed fusion protein was prepared essentially as described previously (14) and according to the supplier’s guidelines. The cleaved protein contained an extra four amino acids, LSEF, at the N terminus. The protein was analyzed by 12% SDS-polyacrylamide gel electrophoresis.

Mouse erythrocyte guanine deaminase was purified 90-fold to homogeneity (Table I). The purified mouse protein was analyzed by SDS-gel electrophoresis and gave a subunit molecular mass of 50 kDa (Fig. 1A). Gel exclusion chromatography on a Superose 6 HR column revealed the native protein to consist principally of a dimer of 100 kDa with approximately 5% of the protein present as a 200-kDa species, consistent with the presence of a minor tetrameric component (Fig. 1B). Purified mouse erythrocyte guanine deaminase gave a Kₘ of 22.7 ± 2.9 µM for guanine.

Attempts to sequence the intact native protein failed, indicating that the protein was N-terminal modified. The purified protein was subjected to cyanogen bromide cleavage followed by SDS-gel electrophoresis. Three principal bands were obtained, and these were electrophoresed onto a polyvinylidene difluoride membrane, cut out, and subjected to N-terminal gas phase sequencing. Two of these bands were comprised of two peptides each; however, their sequences could be distinguished by the differential signal intensity of the individual residues (Table II). Overall sequence was obtained for five peptides ranging from 12 to 25 residues and corresponding in total to 99 amino acids.

The strategy for the retrieval of guanine deaminase cDNA is outlined in Fig. 2. Basic Local Alignment Search Tool analysis of expressed sequence databases (20) using the mouse peptide sequence resulted in the identification of highly homologous human cDNA sequences; none were represented in the database for the mouse. Only peptide IIa corresponded to an expressed sequence clone. Three EST clones were retrieved, and a determination of their size and partial sequence indicated that the clones were essentially identical. The sequence of EST clone 48404 yielded coverage of approximately 80% of the predicted open reading frame, including some 3′ noncoding sequence. The entire coding region of the dbEST clone was used to screen a human kidney cDNA library and resulted in the retrieval of a single colony. Upon sequencing this clone, the
open reading frame appeared to be lacking the equivalent of approximately 40 residues at the N-terminal end; however, an out-of-frame ATG codon was apparent in approximately the right position near the 5' end of this sequence. Primers were designed corresponding to the putative 5' and 3' coding ends of the gene, and a PCR product was obtained from a human brain cDNA library (Marathon™, Ready cDNA). The sequence of this cDNA product gave an open reading frame of 1365 bp, which is in agreement with the subunit mass of the purified mouse protein (Fig. 1A). By comparison, there was a single base deletion at position 911 (Fig. 2, ΔC) of the EST clone that was not present in either the kidney or brain cDNA sequences. In addition, a 4-bp deletion at position 124–127 (ΔATAG) of the EST clone was not present in the brain cDNA sequence and accounted for the absence of a complete open reading frame in the kidney cDNA sequence.

The complete cDNA sequence is given in Fig. 3. The human translated sequence and the corresponding mouse peptide sequences are identified (Table II and Fig. 3). For each mouse peptide, the residue preceding the N terminus corresponded to a methionine in the human sequence, consistent with the cyanogen bromide cleavage strategy.

Conclusive verification of the identity for the cloned human sequence was obtained by subcloning the entire coding portion into the pMAL expression vector and the production of recombinant human protein (Fig. 4). The cleaved protein had an apparent molecular mass of 51 kDa and had the extra LSEF residues at the N terminus. The recombinant protein catalyzed the conversion of guanine to xanthine, with a Km for guanine of 9.5 ± 1.7 μM for guanine. The turnover number (kcat) was 17.4 s−1, and the catalytic efficiency (kcat/Km) was 11.8 × 105 s−1 M−1. The enzyme exhibited optimal activity at pH 7.0, with the Km remaining constant between pH 6.5 and pH 7.5, as described previously for the rabbit liver enzyme (12). Therefore, the pseudo first-order rate constant (Vmax/Km) also showed a sharp optimum at pH 7.0. The human enzyme showed no increase in activity or dependence upon zinc or magnesium ion; however, the addition of manganese consistently resulted in approximately 40-fold increased activity. Optimal activity was obtained at approximately 0.5 mM and remained constant to 10 mM manganese chloride. The divalent metal content of guanine deaminase was determined by atomic absorption. No detectable manganese was found (<0.02 atom Mn2+ per monomer), whereas 0.70–0.91 atom Zn2+ was associated per monomer of guanine deaminase.

**DISCUSSION**

Mouse erythrocytic guanine deaminase was purified to homogeneity (Fig. 1), and the amino acid sequence was obtained
from five cyanogen bromide cleavage products (Table I). The peptide sequences enabled the retrieval of human EST clones, and a portion of these was used to clone the full-length human cDNA guanine deaminase. Comparison of the mouse amino acid sequence from five cyanogen bromide peptides showed an identity of 88 of 99 residues with the inferred human protein sequence (Table II). Further verification of the function of the retrieved human cDNA was obtained by expression of the recombinant protein and its characterization as having guanine deaminase activity. The predicted molecular mass of the human cDNA, 51,040 Da, is in good agreement with the subunit composition for the mouse protein estimated at 50,000 Da (Fig. 1A). These findings are in general agreement with previous studies of purified mammalian guanine deaminases that gave $M_r$ subunit estimates of $M_r$ 50,000 for pig brain (9), $M_r$ 52,000 for rabbit liver (10) and rat brain (11), $M_r$ 55,000 for rabbit liver (12), and $M_r$ 59,000 for human liver (13). The native enzyme has generally been found to be dimeric (10, 11, 13, 22). The purified native mouse erythrocytic protein was principally dimeric in composition with a minor tetrameric component (Fig. 1B).

The $K_m$ values for the purified mouse erythrocytic protein and recombinant human guanine deaminase were similar (22.7 and 9.5 $\mu$M, respectively). Michaelis constants for guanine deaminase with guanine from other sources were also in this range: 4.2 $\mu$M for bovine liver (22), 11 $\mu$M for pig brain (9), 12.5 $\mu$M for rabbit liver (12), and 15.3 $\mu$M for human liver (13). The purified enzymes have exhibited $pK_a$ values consistent with the involvement of essential cysteine and histidine residues (9, 12, 13), and inactivation with $p$-hydroxymercuribenzoate (9, 13).

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Fig. 3. cDNA and predicted amino acid sequence of human guanine deaminase. Amino acid residues corresponding to mouse peptide sequences are shown in bold.

Fig. 4. Expression and purification of recombinant human guanine deaminase as monitored by SDS-polyacrylamide gel electrophoresis. Lane 1, molecular weight standards; lane 2, amylose column purified maltose-binding protein-guanine deaminase fusion product (2 $\mu$g); lane 3, factor Xa cleaved fusion product (2 $\mu$g); lane 4, purified recombinant guanine deaminase (0.5 $\mu$g).
and rose bengal or diethylpyrocarbonate (23), respectively, has substantiated an essential role for these residues. Comparative sequence analysis now provides further evidence for the importance of histidine residues in guanine deaminase.

Guanine deaminase shares a 9-residue N-terminal sequence previously recognized in other aminohydrolases and amidohydrolases (24, 25). Inspection of the SWISS-PROTEIN and TrEMBL databases using the Scan Prosite tool (26) revealed that this motif is shared by 28 enzymes from 20 different families. Metal analysis of the recombinant human guanine deaminase confirmed the association of bound zinc with histidine residues in dihydroorotase results in both loss of enzymatic activity (30). Guanine deaminase has His-Ile-His at residues 82–84, with a third histidine at residue 112. This sequence is also known to be associated with zinc with an additional His at 214, but it does not share the preceding sequence of the 9-residue motif (30). Guanine deaminase has His-ile-His at residues 82–84, with a third histidine at residue 112. This sequence is also likely to be responsible for the apparent 2-fold activation seen by mapping guanine deaminase and looking for human disease indicators of liver disease (1) as a consequence of its near absence in normal human serum, erythrocytes, and lymphoid cells (2). There is a single report of a full-term infant who presented with acute anoxia at birth and died from respiratory distress at day 2 who was found to be deficient in brain guanine deaminase activity (37). Further studies may now be initiated to determine whether guanine will be reutilized or catabolized and eliminated.

Guanine deaminase has been immunohistochemically localized to the cytoplasm of human liver (36), consistent with its isolation from the supernatant of cell and tissue lysates. The serum enzyme activity in man is among the most sensitive indicators of liver disease (1) as a consequence of its near absence in normal human serum, erythrocytes, and lymphoid cells (2). There is a single report of a full-term infant who presented with acute anoxia at birth and died from respiratory distress at day 2 who was found to be deficient in brain guanine deaminase activity (37). Further studies may now be initiated by mapping guanine deaminase and looking for human disease correlates using model systems such as the mouse to explore the relationship between guanine deaminase deficiency and clinical and histological phenotypes.

Acknowledgment—We thank Florence Yang for assistance with manuscript preparation.

REFERENCES


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Aminohydrolase and amidohydrolase enzymes sharing a 9-residue motif

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