Characterization of the Activity and Kinetics of Guanine Deaminase

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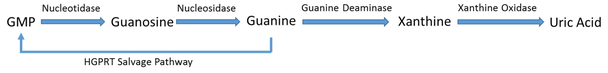
Introduction

Guanine deaminase, also known as "nedasin " or "cypin", catalyzes the purine catabolic commitment step from guanine (G), through xanthine (X), to the elimination product, uric acid. In rabbit, mouse and human, the enzyme appears to exist predominantly cytoplasmic as a homodimer, with catalytic domains for the Zn^{+2}-dependent hydrolytic deamination of guanine to xanthine plus ammonia. Genomic details for the GDA gene are mapped for several species, and expression profiling in certain tissues and organisms has been initiated, although the complement of various transcript variants is incomplete.

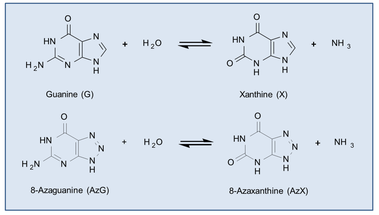
- Structure: c. 50 kDa subunits with sequences that vary at internal and terminal sites, due to exon selection; at least 4 significant forms predicting proteins of various lengths are known.
- Interactions: tubulin, snapin, and the post-synaptic domain protein 95 (PSD-95).
- PSD-95 binding is through the PDZ binding motifs present at the C-terminus of the dimeric structure.
- Sequence variants occur mostly at the protein binding domains, although minor variants lack the deaminase catalytic site.
- In mammalian brain, high enzymatic levels are in telencephalic brain regions; very low levels in white matter and cerebellum; moderate levels in liver and certain other organs. low levels in plasma/serum, notably altered by liver dysfunction. The S- transcript is predominant, coding for a 51 kDa monomer.
- Actual role of guanine deaminase in specialized organ metabolism and synaptic physiology is uncertain, and relatively little is known about the enzyme characteristics outside of rabbit, with significant detail available detail on gene expression patterns, but not at the protein level. Available protein expression surveys have suggested extensive post-translational modifications, that have not yet been detailed with certainty.
- Few useful inhibitors have been described for the enzyme, and the biochemical pharmacology is confused, in part due to poorly characterized enzyme properties.



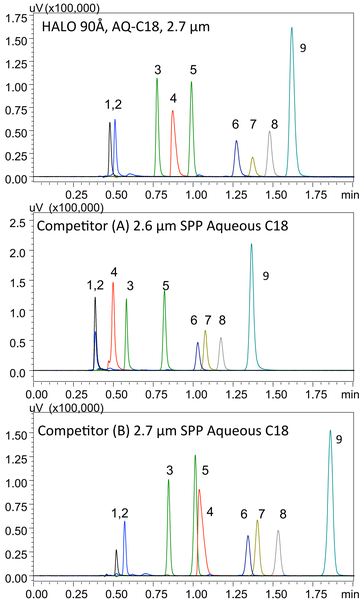
Purpose

- Previous enzyme assays are problematic, using either spectrophotometry (spectral shift of G to X, or ammonium capture), or coupled fluorometric enzyme assay of X (via Xanthine Oxidase produced H_2O_2), with attendant problems in kinetic parameter estimation.
- Available assays have poorly characterized analytical specificity, reproducibility and sensitivity, generating broad reported ranges for basic kinetic parameters (Km values and specific activities vary widely), and inhibitor characteristics.

To measure tissue enzyme levels, follow purification processes, define enzyme kinetics and effects of inhibitors, highly specific and sensitive methods would be useful. Direct generation of product xanthine uses fast separation of highly polar purine metabolites by a new reversed-phase material, HALO® AQ-C18. High sensitivity and selective detection uses selected reaction monitoring (SRM) LC-MS/MS. Analytical benefit was assessed across a range of substrate concentrations, in the presence of known and potential competitive inhibitors. The features of the assay are explored for the only two known substrates for guanine deaminase, Guanine and 8-Azaguanine. We have also initiated comparison of native GDA from bovine brain and liver tissue, to recombinant bacterial expressed human GDA.



Efficient Separations of Highly Polar Purine Metabolites



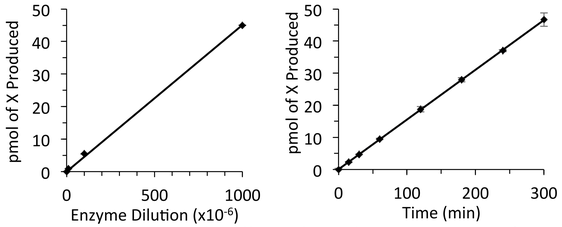
A selection of C18 phases stable in 100% Aqueous conditions were screened. The HALO 90Å, AQ-C18, 2.7 µm column was chosen for its ability to resolve the highly polar purine metabolites and inhibitors of interest. Under the current use conditions, the AQ-C18 column is stable for many thousands of samples, including tissue homogenates.

LC Conditions
Columns: 2.1 x 100 mm
Mobile Phase: 0.1% Formic Acid
Flow Rate: 0.5 mL/min
Temperature: 35 °C
Detection: UV 254 nm
Injection: 1 µL
Instrument: Shimadzu Nexera

Peak Identities
1: 5-Amino-imidazole-4-carboxamide (AICA)
2: Azepinomycin
3: Guanine - Substrate
4: 2,6-Diaminopurine (2,6-DAP)
5: Uric Acid
6: Xanthine - Product
7: 8-Azaxanthine
8: 8-Azaguanine
9: Allopurinol

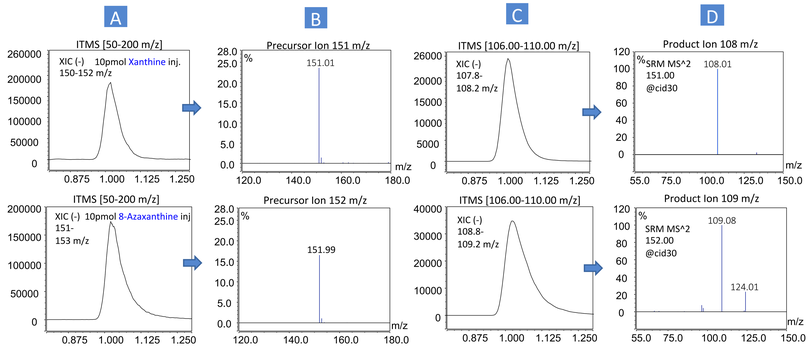
Standard Microplate Enzyme Assay and LC/MS Conditions

Shimadzu Nexera
HALO® AQ-C18 2.1x75mm 2.7µm 90Å, or HTP assay using 2.1 x 30 mm.
A=0.1%Formic Acid B=Acetonitrile
0.5ml/min, 35°C, 265nm, MTP, autosampler 25°C
Gradient: 0%B 0-1.5min to 70%B @1.7-2.2min to 0%B @2.3-4.2min
Thermo Orbitrap Velos Pro ETD
MS Run Time (min): 1.7; divert first 0.8 min from MS source
ITMS (-) 0.7 to 1.7min
HESI Source Type; Capillary 350°C; Heater 325°C; Sheath Gas 40; Aux Gas Flow 10
ITMS Full AGC 50K; ITMS SIM AGC 100K; ITMS MSn AGC 50K
Source Voltage (-kV) 2.70; Ion Trap Full Max Ion Time (ms) 200;
Segment (SEG) Information/Scan Event Details:
ITMS - c low injrf=70.0 ·(151.00000)->oS(98.00-118.00)
MS/MS: AT CID CE 30.0% Q 0.350 Time 10.000 IsoW 1.5, CV = 0.0V
Assay Conditions
50 mM Bicine pH 7.8, 5 µg/mL BSA, varying Guanine or 8-azaGuanine.
Reaction at 25°C for >15 minutes, typical volume of 100 µL
Stop Reaction by addition of 0.1 volume of 10% Formic Acid.
All purine reagent stocks are maintained in 15 mM NaOH/0.15 mM $MgCl_2$ until dilution.
Enzyme incubations are conducted in standard 96 well reaction plates, which are directly loaded in the Nexera Autosampler, for LC/MS analysis of reaction products.



Enzyme activity is linear over 4 orders of dilution, in this example, an enzyme concentration of 1 in 12,000 (50 mU/rx), X production is linear with incubation time to 300 minutes, consuming less than 10% of substrate at 100 uM G. Observed for 10 uL injection; LOQ for X < 1 fmol and linearity to > 10 pmol injected.

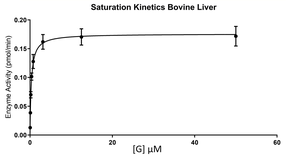
Selective Detection of Enzyme Reaction Products Using SRM in the Ion Trap

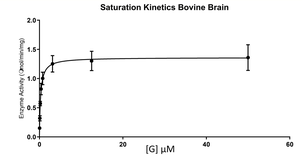


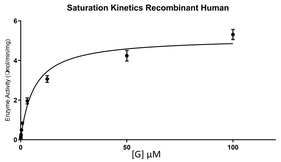
Panels A-D represent steps taken for selective detection of the enzyme reaction product, xanthine and 8-azaxanthine. Panel A shows a chromatogram of a 10 pmol injection of X or AzX using the extracted ions from 150 to 152 m/z. Panel B shows the spectra with xanthine and azaxanthine precursor ions $[M-H]^-$ at 151 m/z (X) or 152 (AzX) chosen for fragmentation. Panel C shows the extracted ion chromatograms centered at dominant fragment ions, and Panel D shows the product ions 108 m/z monitored for X and 109 m/z for AzX.

Xanthine Theoretical Mass [m/z]
152.033 Da monoisotopic, [151.025 m/z (-)]
8-azaxanthine Theoretical Mass [m/z]
153.029 Da monoisotopic, [152.021 m/z (-)]

Comparison of Kinetics Between Brain and Liver and Recombinant GDA

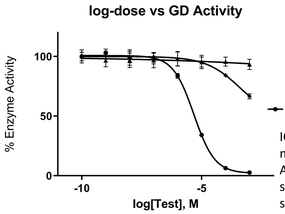


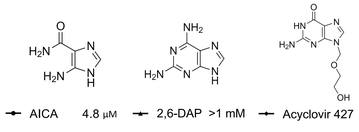




Km values for bovine brain and liver are similar, 0.286 ± 0.035(SEM) µM and 0.31 ± 0.03(SEM) µM respectively, and are much lower than literature values. This emphasizes the importance of highly specific and sensitive LC/MS enzyme assays. The Km values for bovine tissues are much lower than the assayed recombinant human enzyme, Km of 6.2 ± 0.2(SEM) µM. Vmax of bovine brain compares favorably with literature reports at 1.36 ± 0.04(SEM) µmole/min/mg but differs significantly from the recombinatnt, 5.2 ± 0.2(SEM) µmole/min/mg. AzG is a much poorer substrate.

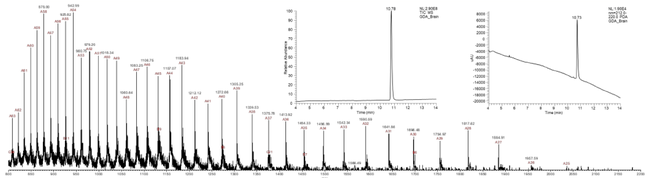
IC50 Determination for GDA





IC50 values were determined with G at 0.5 µM under normal microplate assay conditions (30 min reaction).
AICA showed modest inhibition, 2,6-diaminopurine (2,6-DAP) showed no inhibitory activity, while the antiviral Acyclovir showed very mild inhibitory activity.

LC/MS of GDA



Deconvolution resulted in a subunit MW of 50866. This is 193 Da from the predicted mass using genomic data, 51059, for the S isoform of the enzyme. Chromatograms and SDS PAGE show high enzyme purity.
Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm QExactive HF and Nexera X2
A: 0.1% DFA B: 0.1% DFA 1:1 nPropanol:ACN 0.25 mL/min 30%B 0-20min

Conclusions

A sensitive and specific LC/MS approach to study enzyme kinetics is demonstrated. Results differ from literature reports that use spectrophotometric methods highlighting the importance of the assay's specificity. The HALO® AQ-C18 phase provides robust separation in 100% aqueous conditions for these highly polar purines. High purity enzyme from tissue exhibited much lower Km than bacterial expressed recombinat enzyme and a subunit mass that diverges from DNA derived value.

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