Rescue of the Orphan Enzyme Isoguanine Deaminase

Daniel S. Hitchcock[§], **Alexander A. Fedorov**[‡], **Elena V. Fedorov**[‡], **Lawrence J. Dangott**[§], **Steven C. Almo**[‡], and **Frank M. Raushel**[§,Ψ,*]

[§]Department of Biochemistry & Biophysics, Texas A&M University, College Station, Texas 77843

[Ψ]Department of Chemistry, Texas A&M University, College Station, Texas 77843

[‡]Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Abstract

Cytosine deaminase (CDA) from *Escherichia coli* was shown to catalyze the deamination of isoguanine (2-oxoadenine) to xanthine. Isoguanine is an oxidation product of adenine in DNA that is mutagenic to the cell. The isoguanine deaminase activity in *E. coli* was partially purified by ammonium sulfate fractionation, gel filtration and anion exchange chromatography. The active protein was identified by peptide mass fingerprint analysis as cytosine deaminase. The kinetic constants for the deamination of isoguanine at pH 7.7 are $k_{cat} = 49$ s^{-1}, $K_m = 72$ μM, and $k_{cat}/K_m = 6.7 \times 10^5$ M^{-1} s^{-1}. The kinetic constant for the deamination of cytosine are $k_{cat} = 45$ s^{-1}, $K_m = 302$ μM, and $k_{cat}/K_m = 1.5 \times 10^5$ M^{-1} s^{-1}. Under these reaction conditions isoguanine is the better substrate for cytosine deaminase. The three dimensional structure of CDA was determined with isoguanine in the active site.

Keywords

orphan enzymes; isoguanine deaminase

A major challenge for all aerobic organisms is the prevention and management of oxidative damage to DNA (1). In bacteria such as *Escherichia coli* there are specific repair enzymes for the removal of modified bases from damaged DNA (2-5). This class of repair enzymes includes MutM for excising 8-oxoguanine (8-oxoG), 8-oxoadenine (8-oxoA) and formamidopyrimidines (FAPY) from DNA (5-7). MutT catalyzes the hydrolysis of 2'-deoxy-8-oxoguanosine triphosphate to the monophosphate (8). The removal of mismatched A and isoguanine (2-oxoadenine) from DNA is catalyzed by MutY (9).

Isoguanine is mutagenic to *E. coli* (10, 11). This base promotes A to C, G, and T transversions in addition to base substitutions and deletions (12). The formation of isoguanine in DNA occurs when 2'-deoxyATP is oxidized to 2'-deoxy-2-oxoadenosine triphosphate and then this modified base is incorporated into DNA by DNA polymerase III opposite guanine (14). To a lesser extent, adenine moieties in DNA can be oxidized directly to isoguanine (13).

A bacterial enzyme has recently been discovered that catalyzes the deamination of 8-oxoG to urate (14). This discovery suggests that other oxidized nucleotides may be metabolized in a similar manner. However, to the best of our knowledge, an enzyme that is able to

catabolize isoguanine has not been identified and characterized. The recently discovered 8-oxoguanine deaminase (8-OGD) was found in cog0402 within the amidohydrolase superfamily (AHS). This superfamily also contains enzymes known to deaminate guanine, cytosine, *S*-adenosyl homocysteine (SAH) and adenosine (14-20).

A sequence similarity network for cog0402 is presented in Figure 1 at an E-value cutoff of 10^{-70} (21). We postulated that there may be a subset of enzymes within cog0402 that is able to deaminate isoguanine to xanthine as shown in Scheme 1. The most likely candidate for this activity was predicted to be in a group of enzymes related to *S*-adenosylhomocysteine deaminase in Group **1** of Figure 1. Group **1** is the largest and most diverse group of enzymes within cog0402. SAH deaminase utilizes a conserved histidine residue (His-137) to hydrogen bond with N3 of the adenine moiety of SAH for substrate recognition (17). This particular histidine residue is fully conserved across all Group **1** enzymes of cog0402 except for three small subgroups, one of which contains a glutamine at this position. It was initially hypothesized that the carboxamide moiety of the glutamine side chain could be positioned in the active site of these enzymes to hydrogen bond with the C2/N3 carbamoyl group of isoguanine. From this small subgroup of enzymes, an uncharacterized protein from *Picrophilus torridus* was selected for purification and characterization. Since the DNA from this extremophilic archaeon was not commercially available, we purchased the codon-optimized gene (gi|48477797) from GenScript and then attempted to express the protein in an *E. coli* host.

The target protein was largely insoluble when expressed from either a pET-28 or GenScript PGS-21a vector in *E. coli* BL21. However, an isoguanine deaminase activity was detected in cell extracts after centrifugation. The enzymatic activity was not thermostable and we were unable to isolate the protein using standard nickel affinity or GST columns, as would be expected for the recombinant protein with GST and polyhistidine tags. These results suggested that *E. coli* contained a native isoguanine deaminase. In *E. coli* there are two uncharacterized putative deaminases from cog0402 within the AHS. These proteins are YahJ (gi|16128309; Group **9**) and SsnA (gi|33347706; Group **5**). Both of these proteins were purified to homogeneity, but no isoguanine deaminase activity could be detected with either enzyme. We therefore attempted to identify the specific enzyme responsible for the isoguanine deaminase activity in *E. coli* through classical purification methods.

E. coli BL21 (DE3) cells were grown in an LB medium to stationary phase and harvested. The isoguanine deaminase activity was determined at each step of the purification scheme by monitoring the decrease in absorbance at 300 nm using a $\Delta\varepsilon = -5.0 \times 10^3$ M^{-1}cm^{-1} for the conversion to xanthine. The cells were lysed by sonication and the DNA was removed through precipitation with protamine sulfate. Ammonium sulfate (40% - 50% of saturation) was used to fractionally precipitate the protein mixture. The pellet was redissolved in 50 mM HEPES, pH 7.7, and then loaded onto a HiLoad 26/60 Superdex 200 gel filtration column. The active fractions from the gel filtration chromatographic step were pooled and loaded onto a ResourceQ anion exchange column and further purified. SDS-PAGE analysis revealed three protein bands, two of which correlated with the activity profile of the ResourceQ fractions with molecular weights of ~50 and ~70kDa. The specific activity of isoguanine deaminase increased by approximately 1000-fold, relative to the initial cell lysate. The purification results are summarized in Table 1.

NanoLC electrospray MS/MS analysis was employed to identify the partially purified protein with the ability to catalyze the deamination of isoguanine. The gel from the SDS-PAGE separation was submitted to the Protein Chemistry Laboratory at Texas A&M University for trypsin digestion and analysis. The 70 kDa band was identified as catalase,

and the 50 kDa band was identified as cytosine deaminase from *E. coli* (gi|16128322). The specific peptides that were identified in the mass spectrum are listed in Table 2.

Cytosine deaminase (CDA) from *E. coli* was cloned and inserted into a pET30 expression vector using standard protocols. The CDA transformed cells were grown in the presence of 90 μM dipyridyl supplemented with 1.0 mM Zn^{2+} to diminish the incorporation of iron in the active site (23). To ascertain whether CDA is able to catalyze the deamination of isoguanine, 1.6 μM of the purified protein was incubated with 100 μM cytosine or isoguanine and the UV spectra were obtained before and after addition of the enzyme. The spectra matched that of the two expected products, uracil and xanthine, as illustrated in Figure 2. Xanthine was confirmed by ESI mass spectrometry ($(M+H)^+$ = 153.05 m/z). The activity profiles for the deamination of cytosine and isoguanine from the anion exchange chromatographic step were identical. A structural comparison of cytosine and isoguanine is presented in Scheme 1.

The kinetic constants for the deamination of isoguanine and cytosine with the purified CDA were determined with a direct spectrophotometric assay at 294 and 255 nm using values for $\Delta\varepsilon$ of -6.6×10^3 M^{-1} cm^{-1} and $+2.6 \times 10^3$ M^{-1} cm^{-1}, respectively. The kinetic constants for the deamination of isoguanine by CDA are 49 ± 2 s^{-1}, 72 ± 5 μM, and 6.7 (± 0.3) $\times 10^5$ M^{-1} s^{-1} for the values of k_{cat}, K_m, and k_{cat}/K_m, respectively. Under identical reaction conditions the kinetic constants for the deamination of cytosine are 45 ± 4 s^{-1}, 302 ± 44 μM and 1.5 (± 0.1) $\times 10^5$ M^{-1} s^{-1} for the values of k_{cat}, K_m, and k_{cat}/K_m, respectively, at pH 7.7. The values of k_{cat} are nearly identical for the two substrates but k_{cat}/K_m for the deamination of isoguanine is more than 4-fold greater than for the deamination of cytosine.

To further confirm that CDA is the only enzyme within *E. coli* that is capable of deaminating isoguanine we obtained a strain of this bacterium from the KEIO collection containing a knockout of the gene for cytosine deaminase ($\Delta codA$) (24). The $\Delta codA$ *E. coli* cells were grown to stationary phase and lysed. The rate of deamination of isoguanine was measured by following the decrease in absorbance at 300 nm. Those cells lacking CDA had less than 1% of the isoguanine deaminase activity as the wild type strain.

The three-dimensional structure of cytosine deaminase (PDB code: 1K70) has previously been determined in the presence of an inhibitor that mimics the putative tetrahedral intermediate during the deamination of cytosine (25). In this structure, His-246 and Asp-313 are poised to serve as general acid/base groups to activate the metal-bound water molecule and the amino leaving group. In addition, Glu-217 is positioned to deliver a proton to N3 of the pyrimidine ring. The carbamoyl moiety at N1/C2 is recognized via hydrogen bonding interactions with the side chain of Gln-156. The crystal structure of CDA bound with isoguanine was determined and the molecular interactions with isoguanine are shown in Figure 3 (PDB code: 3RN6). The orientation of isoguanine is nearly identical to that of the cytosine mimic in the previous structure. However, an additional interaction to the substrate is formed via a hydrogen bond between Asp-314 and N7 of the purine ring. The adenine deaminase from cog1816 of the amidohydrolase superfamily (PDB code: 3PAN) also possess an Asp-Asp motif at the end of β-strand 8 within the $(\beta/\alpha)_8$-barrel structure that forms a hydrogen bond with N7 of the purine ring.

We were surprised to find no reports of isoguanine being tested as a potential substrate for CDA. Deamination of both purine and pyrimidine bases by the same protein has, however, been observed in certain tRNA editing enzymes (26). A comprehensive literature search identified a single reference for the deamination of isoguanine by crude extracts of *E. coli* (27). The specific enzyme that was responsible for this transformation has not (until now) been identified in the 60 years since this initial discovery. We have now rescued the orphan

isoguanine deaminase and have demonstrated that this enzyme also catalyzes the deamination of the structurally related base, cytosine. It is of interest to note that the value of k_{cat}/K_m for the deamination of isoguanine by CDA is greater than for the deamination of cytosine. Therefore, in *E. coli* the mutagenic base, isoguanine, can be recycled via the formation of xanthine. It is likely that all of the bacterial cytosine deaminases have the ability to deaminate isoguanine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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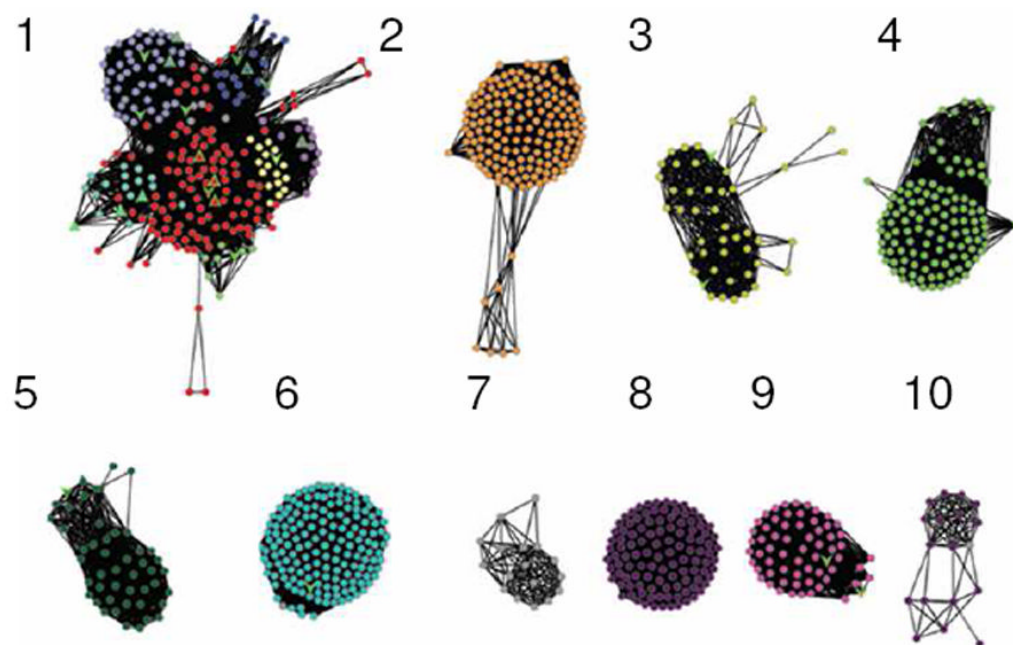27. Friedman S, Gots J. Arch Biochem Biophys. 1951; 1:227–229. [PubMed: 14847690]

Figure 1.
A sequence similarity network representation of the proteins in cog0402. A BLAST network diagram was constructed and rendered with Cytoscape (22). At an E-value cutoff of 10^{-70} cog0402 separates into more than 10 groups, each thought to have a different function. These groups use different active site residues to bind substrates but share identical residues for catalysis and metal binding. The identities of the groups are as follows: **1**: SAH deaminase, **2**: guanine deaminase, **3**: unknown, **4**: 8-oxoguanine/isoxanthopterin deaminase, **5**: unknown, **6**: cytosine deaminase, **7**: unknown, **8**: *N*-formimino-L-glutamate deiminase, **9**: unknown, and **10**: unknown.

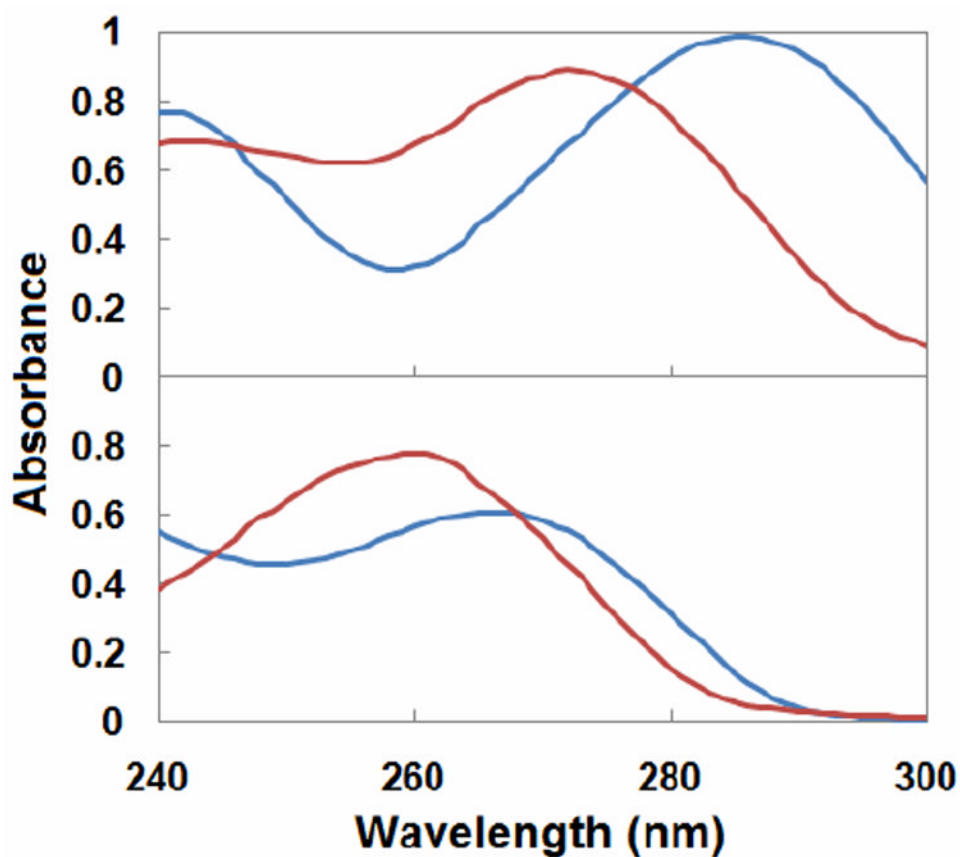**Figure 2.**
Absorption spectra of the reaction mixtures before and after the addition of purified cytosine deaminase to either isoguanine (top panel) or cytosine (bottom panel) at pH 7.7. Isoguanine and cytosine (spectra in blue) were incubated with 1.6 μM CDA for 1.5 hours in 45 mM HEPES, pH 7.7. After the reaction was complete, the UV spectra (shown in red) were collected. The red spectrum in the top panel is consistent with the formation of xanthine (λ_{max} = 272 nm) from isoguanine (λ_{max} = 286 nm). The red spectrum in the bottom panel is consistent with formation of uracil (λ_{max} = 260 nm) from cytosine (λ_{max} = 267 nm).
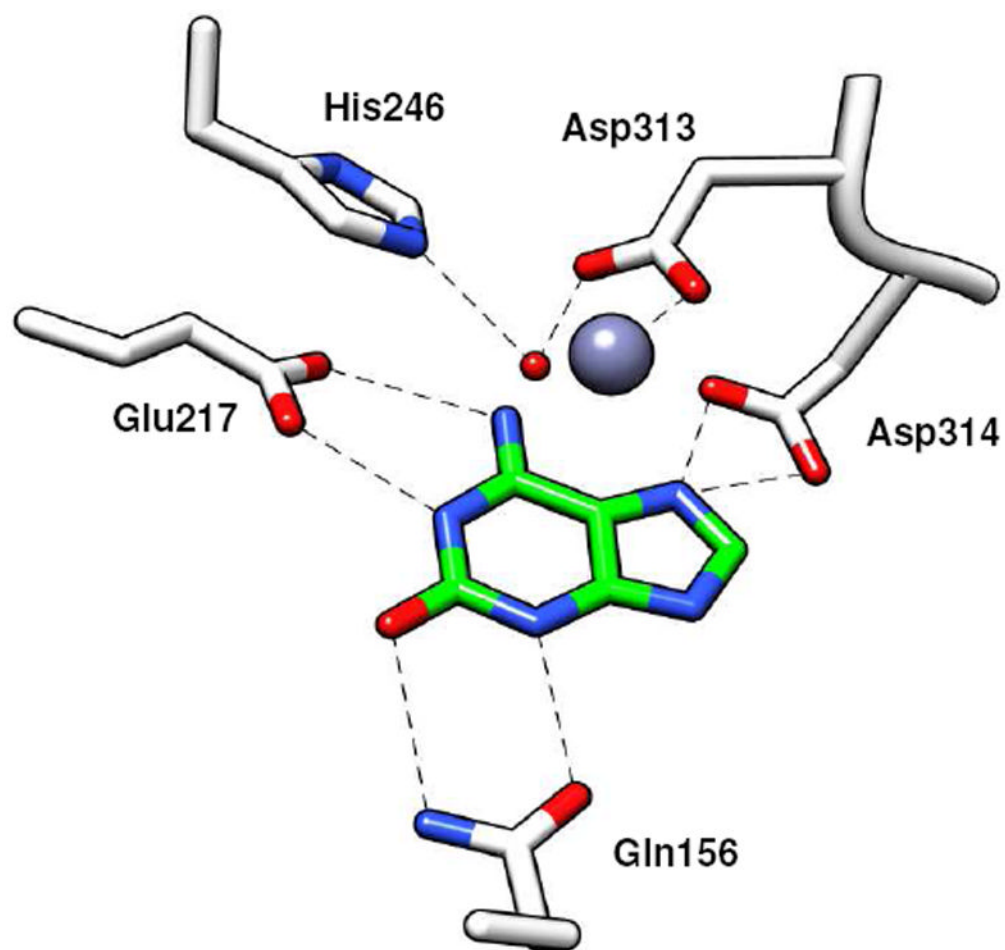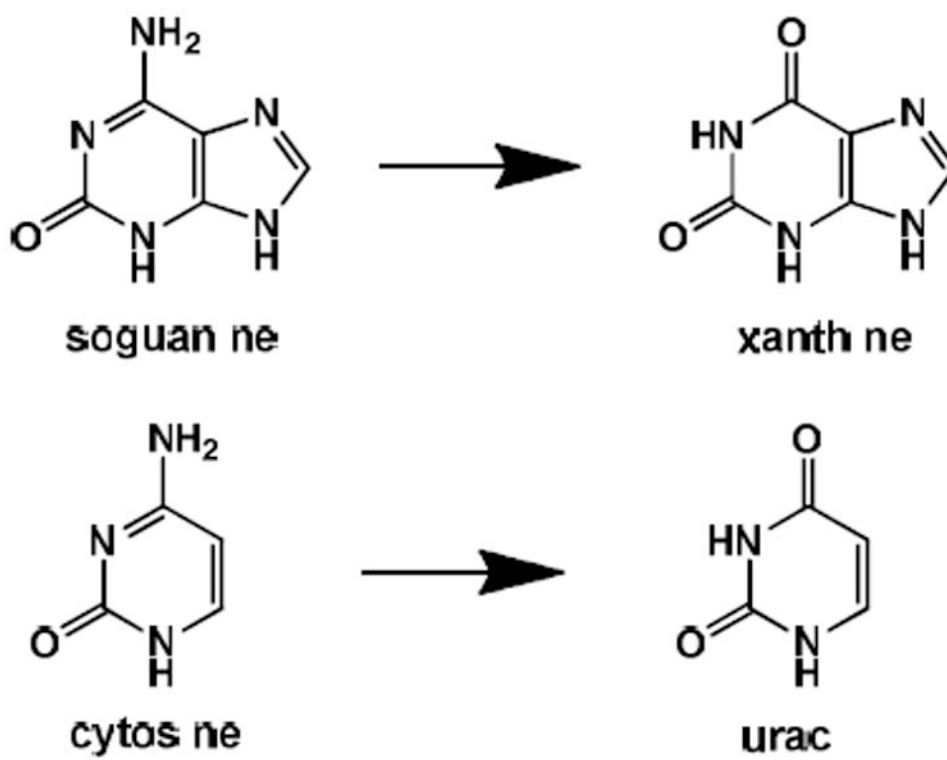
Figure 3.
The structure of isoguanine (in green) bound in the active site of cytosine deaminase.

isoguanine → xanthine

cytosine → uracil

Scheme 1.

Table 1

Purification Scheme for Isoguanine Deaminase[a]

Step	volume (mL)	units (U)	protein (mg)	U/mg
lysate	75	8.6	490	0.018
protamine sulfate	147	6.2	130	0.048
$(NH_4)_2SO_4$	5	5.4	10	0.53
gel filtration	17	2.2	0.63	3.4
anion exchange	4.5	0.97	0.11	14

[a]A Unit (U) is defined as 1.0 μmol/min.

Table 2

Fragments from nanoLC Electrospray MSMS[a]

Position	Observed mass	Actual Mass
3-14	1317.26 (+2)	1315.25
15-30	1822.05 (+3)	1819.02
31-46	1647.20 (+2)	1645.18
95-103	1012.60 (+2)	1010.59
112-122	1323.68 (+2)	1321.67
193-201	1062.06 (+2)	1060.04
227-236	1059.00 (+2)	1056.96
244-262	2058.06 (+3)	2055.04
375-391	1817.46 (+3)	1814.43
405-427	2570.04 (+3)	2567.02

[a]The position indicates the original location of the indicated peptide in the intact protein after cleavage by trypsin. The charge of the fragment is shown next to the observed mass