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Research paper

# Structure-based characterization of canine–human chimeric uricases and its evolutionary implications

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ABSTRACT

Uricase was lost in hominoids during primate evolution, but the inactivation mechanism remains controversial. To investigate the inactivation process of hominoid uricase, chimeric constructions between canine and human uricase were employed to screen the target regions that may contain labile or inactivated mutations in deduced human uricase. Four chimeric uricases were constructed and showed different enzymatic characteristics. Homology modeling, rational site-directed mutagenesis and DNA alignment were used to analyze the changes. Arg119 is conserved in functional mammalian uricases and its side-chains are crucial in maintaining the stability of the β-barrel core. A single CGT (Arg) to CAT (His) mutation at codon 119 that is shared by the human and great ape clade greatly reduces this stability and could cause the loss of uricase activity. We speculate that this missense mutation occurred first and inactivated the uricase protein in humans and great apes and that later the known nonsense mutation at codon 33 occurred and silenced the uricase gene. A single GTC (Val) to GCC (Ala) mutation at codon 296 in canine uricase is regarded as deleterious structural mutation, but such kinds of deleterious mutations have been widely accumulated in extant mammalian uricases. We speculate that a reduction in uricase activity has been an evolutionary tendency in mammals. Moreover, from structure–activity analysis of helix 2 in ancestral primate uricase, we suggest that before the inactivation of hominoid uricase, deleterious structural evolutionary changes had occurred in ancestral primates. The loss of hominoid uricase should be caused by progressive multistep mutations rather than a single mutation event.



## 1. Introduction

Uricase (EC 1.7.3.3) is an enzyme involved in the purine degradation pathway that catalyzes the oxidation of uric acid to allantoin [1]. All five genera of hominoids (human, chimpanzee, gorilla, orangutan, and gibbon) lack active uricase [2–4], whereas most Old World and New World monkeys possess moderated but functional uricase [5–7]. The loss of uricolytic activity results in elevated levels of uric acid in blood; whereas uric acid is considered to be a major antioxidant that may protect against oxidative damage and prolong life span [8]. The inactivated uricase may carry evolutionary advantages in primates [9,10] and may have triggered the emergence of humans [4,11,12]. The mechanisms of inactivation of uricase are different in different hominoid lines [13]. The uricase deficiency in the gibbon lineage was caused by a 13 bp deletion [13] or a nonsense mutation in exon 2 [4]. A single CGA to TGA nonsense mutation at codon position 33 was identified in humans and great apes and proposed to be the inactivation event after the divergence between the human and great ape clade and gibbon [4,13], but the inactivation process and mechanism remains unclear. Christen et al proposed that the loss of uricase gene in hominoids was a multistep process [5], while Friedman et al suggested that a single mutational event inactivated the uricase gene in a hominoid ancestor [6,14].

A total of 304 codons of human uricase have been deduced by eliminating two known nonsense mutations at codon 33 and 187 [4]. However, in the absence of selective pressure, deleterious missense mutations may have accumulated in the human gene [15]. It is difficult to investigate the process of inactivation of hominoid uricase using only the deduced human uricase (dHU) gene. An alternative method is to construct a human chimeric

*Abbreviations:* wCU, wild-type canine uricase; dHU, deduced human uricase; LB, Luria-Bertani; RP-HPLC, Reverse-phase high performance liquid chromatography; SE-HPLC, size exclusion high performance liquid chromatography; AFU, *Aspergillus flavus* uricase; GB, generalized Born; MD, molecular dynamics; RMSD, Root Mean square deviation.

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uricase based on another active mammalian uricase. In this study, wild-type canine uricase (wCU) was selected for chimeric substitution, because of its high catalytic activity [16] and high level of sequence identity with dHU. Thumb chimeric constructions between canine and human uricase were employed to screen the target regions that may contain labile or inactivated mutations in dHU. Four canine–human chimeric uricases were constructed and purified and showed different enzymatic characteristics.

Bioinformatical evaluation was used to analyze the changes. Unlike fungal uricases [17], mammalian uricases are unstable and sparingly soluble below pH 9 [5,18,19]. It is difficult to obtain 3D structures of mammalian uricase by X-ray crystallography. By contrast, homology modeling has become a good alternative for estimating the 3D structures of proteins [20]. To investigate the structure of canine–human chimeric uricases and explain their enzymatic disparities, homology modeling, substrate docking, and molecular dynamics simulations were performed using the X-ray structure of *Aspergillus flavus* uricase (AFU) [21–23].

Two potential crucial mutations were identified based on the chimeric constructions and bioinfomatical evaluation. Rational site-directed mutagenesis was performed to confirm the role of the above missense mutations. The inactivation process of uricase in humans and great apes and the evolution of mammalian uricases are discussed on the basis of the experimental enzymatic characterization, computational structural evaluation and DNA sequence alignment.

## 2. Materials and methods

### 2.1. Materials

The host strain *E.coli* DH5α and Bl21 Star™(DE3) plysS were from Invitrogen. The vector pET3c was purchased from Novagen. Primers, DNA polymerase, DNA marker, T4 DNA ligase, restriction endonucleases *Nde*Iand *BamH*Iwere from Takara. Uric acid and Xanthine Agarose were from Sigma–Aldrich. AKTA Explorer 100 Chromatograph apparatus, SOURCE 15Q 4.6/100 PE were from GE Healthcare Bioscience. High Performance Liquid Chromatography (HPLC) apparatus and Reversed-phase C4 analytical columns were obtained from Agilent. All other reagents were of analytical grade.

### 2.2. Sequence alignments

Multiple protein sequence alignments were generated using Vector NTI –AlignX [24] (Invitrogen) to confirm conserved features between dHU and other active mammalian uricases. The amino acid sequences were extracted from the Genbank, including the uricase from human, baboon, owl monkey, canine, bovine, porcine. SAM-T08 [25] (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html) was used to find out the conserved features between dHU and the whole uricase family.

Multiple DNA sequence alignments were performed with Clustal W [26] to compare uricase gene diversity between inactivated hominoids and other functional mammals. The DNA sequences were extracted from Genbank, including the uricase gene from human, chimpanzee, gorilla, orangutan, gibbon, baboon, rhesus monkey, crabeating monkey, owl monkey [4], canine, bovine, and porcine. Phylogenetic trees were generated with MEGA4.0 [27] by using the neighbor-joining method [28] with 1000 bootstrap replications [29].

### 2.3. Construction of wCU and its mutants

A codon-optimized full length canine uricase gene [30] was designed and synthesized based on the protein sequence of wCU according to the codon bias of *E.coli*. The wCU gene was amplified by PCR and digested by *Nde*I*BamH*Imixture, and then ligated into the multiple cloning site of pET3c vector. The pET3c-wCU was used as the template in next operation. Overlap extension PCR [31] method was employed to generate canine–human chimeric uricase genes and mutated wCU genes. The primers used for splicing and mutation were showed in *Supplemental material one*.

All the recombinant plasmids were transformed into *E.coli* DH5α, then plated on Luria-Bertani (LB) agar containing 0.1 mg/ml ampicillin. An individual transformant was cultivated for preparing the plasmids, respectively, and the mutated expression vectors were sequenced to confirm that the anticipated mutation occurred and no others had been introduced into the sequences (for detail information see *Supplemental material one*).

### 2.4. Expression and purification of wCU and its mutants

The recombinant plasmids were transferred to host strain *E.coli* Bl21 Star™(DE3) plysS. To express one protein, a single colony was inoculated into LB medium containing 0.1 mg/ml ampicillin and grew overnight. The overnight culture was diluted into fresh media and grew at 37 °C. When $OD_{600}$ of the *E.coli* culture reached 0.6, expression was induced by addition of isopropyl β-D-thiogalactoside to the final concentration of 0.4 mM for 5 h. The cells ware harvested and resuspended in lysis buffers as described previously [32]. The suspension was centrifuged and insoluble uricase protein was extracted into buffers containing 0.2 M $Na_2CO_3$, pH10.3. 10% of saturated solution of ammonium sulfate was added to the supernatant and the target protein was salted out. The uricase protein was redissolved and loaded onto a xanthine agarose column. Uricase was eluted with the extraction buffer containing 60 μM xanthine. The elution fraction was then loaded onto SOURCE 15Q 4.6/100 PE. The target fraction was eluted with the extraction buffer containing 0.1 M NaCl.

### 2.5. Protein assays

The protein concentrations of wCU and its mutants were determined using the Bio-Rad protein assay kit with bovine serum albumin as the standard. The homogeneity and molecular mass of the purified protein was determined by SDS-PAGE with a 5% stacking gel and a 15% separation gel as described by Laemmli [33]. The activity of uricase was measured by the decrease in absorbance at 290 nm due to enzymatic oxidation of uric acid as described previously [34]. All enzyme activity and kinetic assays were carried in 0.1 M sodium borate buffer(pH8.6) at 25 °C. An extinction coefficient of 12,300 $M^{-1}cm^{-1}$ for uric acid was used [35]. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of uric acid per min at 25 °C. The initial velocities ($V_o$) of the enzymes were measured with substrate concentrations ranging from $1.5 \times 10^{-5}$ M to $1.0 \times 10^{-4}$ M. The Michaelis–Menten constant ($K_M$) and the maximal velocity ($V_{max}$) were determined by Linerweaver–Burk plot. The catalytic constant ($k_{cat}$) was calculated from the relationship of $k_{cat} = V_{max}/C_{enzyme.}$ The catalytic efficiency was calculated from $k_{cat}/K_M$.

### 2.6. Homology modeling, substrate docking and molecular dynamics simulations

The structures of uricase from *A. flavus* were used as the templates to model the structure of canine–human chimeric uricase. The monomeric structure was generated by using a 1.0 Å resolution X-ray structure (PDB ID: 3L8W [22]) as a template through the SWISSMODEL server. Monomeric chimeric structures were coassembled into a homotetramer model [36] by fitting to the