Phylogenetic articulation of uric acid evolution in mammals and how it informs a therapeutic uricase

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Abstract
The role of uric acid during primate evolution has remained elusive ever since it was discovered over 100 years ago that humans have unusually high levels of the small molecule in our serum. It has been difficult to generate a neutral or adaptive explanation in part because the uricase enzyme evolved to become a pseudogene in apes thus masking typical signals of sequence evolution. Adding to the difficulty is a lack of clarity on the functional role of uric acid in apes. One popular hypothesis proposes that uric acid is a potent antioxidant that increased in concentration to compensate for the lack of vitamin C synthesis in primate species ~65 million years ago (Mya).

Here, we have expanded on our previous work with resurrected ancient uricase proteins to better resolve the reshaping of uricase enzymatic activity prior to ape evolution. Our results suggest that the pivotal death-knell to uricase activity occurred between 20-30 Mya despite small sequential modifications to its catalytic efficiency for the tens of millions of years since primates lost their ability to synthesize vitamin C, and thus the two appear uncorrelated. We also use this opportunity to demonstrate how molecular evolution can contribute to biomedicine by presenting ancient uricases to human immune cells that assay for innate reactivity against foreign antigens. A highly stable and highly catalytic ancient uricase is shown to elicit a lower immune response in more human haplotypes than other uricases currently in therapeutic development.

Keywords: uricase, uric acid, ancestral sequence reconstruction, ancient protein, mammalian evolution, gout therapeutics
Introduction

Uric acid is a small molecule generated mostly by purine metabolism that is subsequently excreted by host organisms after it is oxidized to soluble molecules. The concentration of endogenous uric acid levels are controlled by enzymes upstream and downstream of uric acid in its metabolic pathway, as well as membrane transporters of the small molecule (Ramazzina, et al. 2006; Keebaugh and Thomas 2010). This control is important because uric acid becomes insoluble and forms crystalloids at increasing concentrations, which can quickly cause renal failure in mammals (Kelly, et al. 2001). Even sustained moderate levels of uric acid can also cause crystals to accumulate in avascular tissue (e.g., cartilage, tendons, ligaments) and lead to long-term complications such as gout (Hershfield, et al. 2010).

Apes are unique among nearly all other mammals because they have modified their control of endogenous uric acid levels (Wells 1910). In particular, apes lack a functional form of the enzyme uricase that would otherwise oxidize uric acid. Further, uric acid membrane transporters in ape kidneys have evolved to reabsorb most of the uric acid from the urine at normal physiological conditions and return it back to the blood, while those of non-apes have evolved to excrete most of the small molecule as waste. An evolutionary explanation for modifying the flux of uric acid has remained elusive (Kratzer, et al. 2014).

Uric acid is recognized as an antioxidant and considered to be one of the most important antioxidants in the plasma (Ames, et al. 1981), although its role in oxidative stress has been disputed (Hershfield, et al. 2010). One popular hypothesis posits that the endogenous uric acid observed in apes evolved as a compensation for the lack of vitamin C synthesis in primates (Ames, et al. 1981). It is generally regarded that primates lost the ability to synthesize vitamin C ~65 Mya since neither apes nor monkey nor tarsiers have a functional L-gulono-γ-lactone oxidase (GLO) gene (Lachapelle and Drouin 2011). Our previous studies with resurrected uricases suggested that enzyme activity began to be catalytically reshaped some time between 96-43 Mya. The current study attempts to more accurately determine when this transition began by increasing the species diversity of the uricase phylogenetic tree, and thus increase the number of internal basal nodes for protein resurrection analysis.

In addition to understanding a correlation between uric acid build-up in apes with the loss of vitamin C synthesis in primates, our current study also attempts to determine whether ancient uricases may have potential value as therapeutic enzymes to prevent Tumor Lysis Syndrome.
and/or gouty arthritis in human patients (Najjari, et al. 2021). As a step in this direction, ancient uricases are presented to a diverse panel of human immune-cell proliferation assays.

**Results**

*Biomolecular Characterization of Resurrected Ancestral Uricases*

The eutherian phylogeny inferred from uricase sequences has more than doubled in size over the past couple of years due to the Zoonomia Project (Zoonomia 2020). As seen in Figure 1, these additional sequences provide greater resolution to a uricase phylogeny. In turn, such an increase in phylogenetic articulation can also provide greater accuracy in the inference of ancestral sequences at internal nodes of the tree (Hanson-Smith, et al. 2010; Randall, et al. 2016). We have resurrected ancient uricase enzymes along the evolutionary path that connect humans to their mammalian ancestors ~96 Mya. In agreement with our previous study, Figure 1 demonstrates that ancient uricases were highly active enzymes that slowly and steadily decreased in activity until the enzyme became inactive in the lineage leading to apes ~20 Mya. The substrate affinity ($K_m$, fig. 2A), substrate turnover ($K_{cat}$, fig. 2B) and catalytic efficiency ($K_{cat}/K_m$, fig. 2C) were measured. A few results are worth highlighting. First, the catalytic efficiency of the enzyme slowly decreases from 96-29 Mya (fig. 2C, right-side). This stepwise decrease results in 5-fold lower activity over 60 million years. At this point along the evolutionary trajectory to apes, the enzyme is still overall highly active with an efficiency slightly less than the order of $10^5$. Second, a precipitous decrease in enzyme activity occurs between 29-20 Mya (fig. 2C, left-side). This decrease is caused by a single amino acid replacement (F222S) that results in complete loss of substrate turnover (Kratzer, et al. 2014; Xie, et al. 2016; Duan, et al. 2021; Jiang, et al. 2021). The phenylalanine at position 222 is located on helix $\alpha$4 and stabilizes the dimer–dimer interface via aromatic stacking and hydrophobic interactions, and also has an intrasubunit cation–π interaction with the highly conserved Lys164. Replacement to serine eliminates these interactions and generates an energetic penalty by introducing a hydrophilic residue within this hydrophobic pocket. Since the four active sites of a homotetramer of uricase are the result of head-to-tail monomer/dimer interfaces, the enzyme no longer has stable active sites when serine occupies this position. Third, the last common ancestor of catarrhines (Old World monkey and apes, 29 Mya) had a uricase exhibiting very low substrate turnover (fig. 2B), and yet, its catalytic efficiency remained relatively robust because the improved $K_m$ for substrate binding (by way of its lower value) is able to compensate for a lack of
turnover. This same tradeoff in $K_m$ and $K_{cat}$ is maintained for the ancestor at ~12 Mya located within the Old World monkey clade (fig. 1) as well as the *Macaca mulatta* uricase we characterized ($K_m=13.7, K_{cat}=116, K_{cat}/K_m=1.41 \times 10^5$). Conversely, the uricase of the New World monkey *Aotus nancymaae* that we characterized ($K_m=22.1, K_{cat}=266, K_{cat}/K_m=2.01 \times 10^5$) more closely resembles An43 than An29. This observation is anticipated since the lineage is a direct descendant of node An43.

**Immunogenicity of Ancestral Uricases**

An immuno-cell proliferation assay was used to determine the relative antigenicity of ancestral uricases compared to a leading uricase therapeutic that forms the basis of the FDA-approved Krystexxa®
, which is a pig and baboon chimeric (PBC) (Anderson and Singh 2010; Hershfield, et al. 2010). The Dendritic Cell – T Cell assay utilized a panel of peripheral blood mononuclear cell samples from healthy human donors representing a diversity of 43 Human Leukocyte Antigen (HLA) class II haplotypes. Figure 3 shows that PBC elicited the broadest immune response for the three uricases we assayed. This uricase stimulated >70% of HLA alleles represented in the assay. The An43 uricase fared better than PBC by stimulating just under 50% of the samples. The An96 uricase, however, performed best in the immunogenicity assay by stimulating only ~35% of the samples.

Lastly, we measured the stability of uricases in conditions that mimic the gastrointestinal tract of mammals to assess the potential oral delivery of a therapeutic uricase treatment (Szczurek, et al. 2017; Yun, et al. 2017; Pierzynowska, et al. 2020). Initial creatinine stability assays included the *Candida* uricase, along with PBC and An96 enzymes. *Candida* was very unstable in these conditions, while PBC and An96 were highly stable (fig. 4). In attempt to further stabilize An96, we inserted disulfide bridges into the protein (Craig and Dombkowski 2013; Yainoy, et al. 2019). One variant (Coel) had amino acid replacements at M25C and N287C, while a different variant (DbD3) had replacements at A223C and A130C. As seen in Figure 4, one of the variants was markedly destabilized while the other variant was slightly more stable than An96 by the insertion of a disulfide bridge.

**Discussion**
Tracing the evolutionary history of uricases during placental mammalian evolution demonstrates that this enzyme experienced a slow and sequential reshaping over the course of ~70 million years before its precipitous drop in activity by 20 Mya, crown group of apes. The slow decline in catalytic efficiency prior to apes was achieved by a balance of slower substrate turnover (decrease in $K_{cat}$) and weaker substrate binding (increase in $K_m$). The slow decline and reshaping of enzymatic activity would have allowed other components of the purine metabolism pathway to handle a modified flux of the small molecule. From a physiological perspective, such reshaping is reasonable since we know uricase-knockout mice/rats have a high mortality rate by six weeks post-birth due to complications from renal failure caused by hyperuricemia (Kelly, et al. 2001). The mammalian body cannot handle a rapid and massive increase in uric acid caused by an abrupt lack of uricase activity. In support of this notion, our previous work with resurrected uric acid transporters demonstrated that the transporter URAT1 co-evolved with the reshaping of uricase activity during mammalian evolution (fig. 5). Our current studies were able to add more uricase data points and thus provide greater resolution to the timing of the reshaping events. URAT1 evolved from a high-capacity/low-affinity transporter early in placental evolution to a low-capacity/high-affinity transporter by the predicted origins of the ape lineage (29 Mya). This transition is clearly in motion by 43 Mya as the capacity of the transporter was markedly decreasing while the affinity was conversely increasing, and is also accompanied by a notable decrease in uricase activity. The molecular events that occurred with uricase and URAT1 between 50-30 Mya clearly set the stage for the final blow to uricase activity shortly thereafter. The timing of these events suggest that the co-evolution of uricase and URAT1 did not happen in direct response to the loss of vitamin C synthesis nearly 67 Mya.

While the chemical utility of uric acid as an antioxidant is indisputable, the physiological role for the presumed rise of endogenous uric acid 20 Mya in the crown apes is more speculative. We, along with others, have hypothesized that the increase in uric acid levels promote the biochemical conversion of fructose to triglycerides (Feig, et al. 2008; Johnson, et al. 2008; Johnson, Perez-Pozo, et al. 2009; Johnson, Sautin, et al. 2009; Johnson and Andrews 2010; Johnson, et al. 2011; Lanaspa, et al. 2013; Lyssiotis and Cantley 2013; Choi, et al. 2014; Ciccheri, et al. 2014; Kratzer, et al. 2014; Johnson, et al. 2020). The ability to ingest sucrose from fruits and immediately convert nearly half of the carbohydrate to a long-term storage molecule may have had a tremendous selective advantage for early apes and the increased energy demands required of their large brains.
compared to ancestral monkeys. We anticipate that additional future experiments will help resolve the precise evolutionary and physiological role of increased uric acid that originated nearly 20 Mya in apes.

Regardless of the true evolutionary cause of uricase deactivation, modern humans can suffer from an inability to properly manage endogenous uric acid levels. As such, pharmaceutical companies have spent decades attempting to develop recombinant uricases, as well as inhibitors of uric acid transporters. The race to develop a safe and effective therapeutic uricase has never been more competitive. The launch of Krystexxa© (a PEGylated version of the Pig-Baboon Chimeric) nearly one decade ago has become a textbook case of failure. The fact that most human subjects elicit strong immune responses to the drug (Sundy, et al. 2011; Lipsky, et al. 2014; Nyborg, et al. 2016), along with the fact that the FDA issued a black-box warning, meant most doctors were reluctant to prescribe and administer Krystexxa©. The failure of the drug left a large gap in the gout/hyperuricemia market, so a flood of uricase variants/formulations are currently being developed. Krystexxa© is being reformulated with an improved PEGylated moiety by 3SBIO (Shenyang, China). Selecta Biosciences (MA, USA) is encapsulating a PEGylated Candida uricases in viral particles in hopes of preventing an immune response in human patients (Kishimoto, et al. 2016). China Pharmaceutical University is engineering a uricase that essentially uses the resurrection approach to reactivate the human gene into a functional enzyme (Xie, et al. 2016; Duan, et al. 2021). Horizon Therapeutics (Dublin, IE) is currently co-administering the immune suppressor methotrexate along with PEGylated Pig-Baboon Chimeric uricase in clinical trials and demonstrated that patients have a lower immune response with this combination (Botson, et al. 2020). In 2019, an academic research group reported that they engineered a uricase from the bacteria Arthrobacter that shows greater resistance to general protease digestion compared to other uricases (Shi, et al. 2019). In 2020, Fagan Biomedical Inc. reported its development of a PEGylated canine uricase that has high bioavailability when presented to monkeys (Li, et al. 2020).

There is clearly a lot of competition for a share of the gout/hyperuricemia market. There are two especially interesting developments that have recently occurred during this competition. Both of these developments involve an oral route for uricase delivery that exploits the discovery that the lower intestines plays an important role is lowering serum uric acid levels (Yun, et al. 2017). One approach uses Staphylococcus engineered to export uricase outside of a host bacterial cell. These bacteria have been gavaged (orally administered) in rats and shown to colonize the intestines.
and to release uricase and lower uric acid levels in the intestines (Cai, et al. 2020). The other approach has engineered the Candida uricase to be hyperstable against the pH environment of the digestive tract. Animal studies have demonstrated that oral administration of this uricase can lower serum uric acid levels (Szczurek, et al. 2017; Pierzynowska, et al. 2020) and clinical studies in humans are currently being conducted by Allena Pharmaceuticals (MA, USA). Our results clearly demonstrate that ancestral uricases have superior stability compared to unmodified Candida and PBC uricases when assayed in conditions that simulate the mammalian gastrointestinal tract (fig. 4), and may thus serve as better potential therapeutics.

In total, these therapeutic developments led us to question how immunogenic ancestral proteins would be to human immune cells since these assays serve as a proxy for the safety of a potential therapeutic drug. Figure 3 demonstrates that our most ancestral uricase elicits a substantially lower immune response than the Pig-Baboon Chimeric uricase. PBC was initially developed from two observations: 1) monkey uricases are very unstable when purified (this is also true for both An29 and An12, but not other modern and ancestral uricases), 2) clinical results showing that recombinant pig uricase elicited strong immune responses in human subjects supported the notion that incorporating portions of the monkey sequence into the pig sequence may diminish the immune response in human patients. This notion was highly speculative at the time, but appears to now be supported by the recent discovery of uricase protein expression based on human proteomic analyses (Na, et al. 2019). The read-through of the two premature stop codons in the human transcript does not generate an enzymatically active protein as we previously demonstrated (Kratzer, et al. 2014), but it may ‘teach’ the human immune system that uricase-like peptides/proteins are benign. In total, our assays demonstrate that ancestral uricases are potentially advantageous compared to PBC. In fact, we would conclude that any therapeutic uricase that includes monkey sequence be abandoned because of its long evolutionary history with protein instability, and rather favor other ancestral uricases that are more stable and more human-like in sequence.

It is also noteworthy from these assays that An96 displayed a response index (RI) <0.7, which is comparable to other FDA-approved therapeutics such as Remicade®, Avastin®, Campath®, and many others. Conversely, the RI for PBC was nearly 2.0 which is a warning sign that human immunogenicity will promote complications. With that said, additional experiments will determine whether ancestral uricases are truly useful for future therapeutic development.
To conclude, our results suggest that a substantial rise in endogenous uric acid levels coincided with the origins of ape species in the mammalian lineage 20-30 Mya and was independent of the loss of vitamin C synthesis that occurred in primates ~67 Mya. The rise in endogenous uric acid levels was preceded by a reshaping of the uricase enzyme and the uric acid transporter URAT1. While a growing repertoire of experimental results support one notion that increased levels of uric acid allowed apes to convert fructose to fatty acids, and thus served as a selective advantage, many additional experiments will be required before this hypothesis becomes parlance. We also anticipate that ancient uricases will support the growing idea of evolutionary biomedicine in the near future (Perry 2021; Selberg, et al. 2021).

Material and Methods
Representative DNA and protein sequences for uricase (E.C. 1.7.3.3) were identified from UniProtKB 2021_01 (https://www.uniprot.org/). Homologous DNA and protein sequences were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/), using the nucleotide Basic Local Alignment Search Tool (nBLAST) and the protein BLAST (Altschul, et al. 1990), respectively, with the cut-off threshold of <1 × 10^{-5}. The majority of collected sequences were retrieved from species that were sequenced for the first time by the Zoonomia Project (Zoonomia 2020). These sequences were available only as genomic DNA sequences and thus BLAST searches were conducted against genomes (Table S1). Individual exon sequences were manually concatenated (Fig. S4). Assembled DNA sequences were translated into protein sequences using ExPASy (https://www.expasy.org/). Also, some of the retrieved protein sequences were found to contain superfluous intron sequences at the beginning of the Exon 1 sequence. These intron sequences were manually removed. In contrast, some of the retrieved protein sequences lacked portions of Exon 1. The automated annotation algorithm missed Exon 1 probably due to its short length. For species that had these shortened protein sequences, an inquiry sequence that combines a known Exon 1 sequence and an upstream intron sequence was manually prepared and utilized to find Exon 1 sequences by BLAST genome search. A total of 65 sequences were collected for further analysis.

Sequences were aligned using MUSCLE (ver. 3.8.31) (Edgar 2004). Taxon-specific insertions were manually removed. Trees were constructed using MrBayes (ver. 3.2.6) (Huelsenbeck, et al. 2001), using the GTR+I+Γ model for the DNA sequences and the LG+I+Γ model for the protein
sequences and run at least 1,000,000 generations, with sampling at intervals of 100 generations, and two runs with four chains per run in order to monitor convergence. Twenty-five percent of sampled points were discarded as burn-in. Tree topologies were nearly identical to the species tree published by the Zoonomia Project. When they differed, however, branches were manually edited using Mesquite (ver. 3.61) (Fig. S2 & S3). Ancestral sequences were reconstructed using PAML (ver. 4.8) (Yang 2007) as previously described (Kratzer, et al. 2014) (Fig. S5). The average posterior probability across all sites for all ancestors we inferred (11 total) was greater than 99%.

Uricase-encoding genes were cloned into the pET-21a(+) vector (Life Technologies Corporation) using NdeI and XhoI sites. E. coli BL21 (DE3; New England Biolabs) cells were freshly transformed with uricase-containing vectors. A single colony was used to inoculate a 5-mL overnight culture. This overnight culture was used to inoculate 500 mL of Luria Broth with 100 μg/mL carbenicillin. Cells were grown to an OD$_{600}$ between 0.6 and 0.8 at which point they were induced with 1 mM IPTG. Expression was carried out overnight (16–20 h) at 37 °C with shaking at 250 rpm. After cells were harvested by centrifuge, the pellets were stored at -80 °C. The pellet was lysed and resuspended by 100% BugBuster® protein extraction reagent (Novagen), followed by centrifugation. The lysate was washed with 55% and 10% BugBuster reagent, twice, respectively. The final pellet was resuspended in 0.1 M Na$_2$CO$_3$ buffer at pH 11 with 1 mM PMSF, followed by rocking for 60-90 minutes. Soluble uricases were purified using FPLC (Cytiva) at 4 °C and followed the two-step purification scheme previously described (Kratzer, et al. 2014). The carbonate extraction was loaded on size-exclusion chromatography (HiLoad™ 16/60 Superdex™ 200 prep-grade column; Cytiva) pre-equilibrated with 0.1 M Na$_2$CO$_3$, pH 11. The protein with ~140 kDa was collected for anion exchange chromatography (SOURCE™ 15Q 4.6/100 PE (Cytiva)) pre-equilibrated with 0.1 M Na$_2$CO$_3$, pH 11. The sample was eluted in three different concentrations of NaCl diluted with 0.1 M Na2CO3 buffer (pH=11): 0.15 M, 0.3 M and 0.6 M. The concentration of purified tetrameric uricase was determined by the Quick Bradford Assay (Bio-Rad). Enzymatic curves for all assayed enzymes are provided (supplementary fig. S1, Supplementary Materials online).

The enzymatic activity of purified tetrameric uricase was determined spectrophotometrically by monitoring the decrease of uric acid by following the absorbance at 293 nm (A293). Using 96-well plate and platereader (Molecular Devices). A freshly prepared 200 μM uric acid stock
in 1X sodium phosphate buffer, pH 7.4, was diluted with 1X sodium phosphate buffer (Gibco), pH 7.4, to prepare a range of uric acid concentrations from 0 to 150uM.

The mean from triplicate runs of initial velocities at each uric acid concentration were used to plot a hyperbolic regression curve to determine $K_m$ and $V_{max}$ of the purified uricases (Graphpad Prism 9). The $K_{cat}$ was determined by dividing $V_{max}$ by the concentration of tetrameric uricase used in the kinetics experiments, and the catalytic efficiency ($K_{cat}/K_m$) was calculated by taking the ratio of $K_{cat}$ to $K_m$.

Immunogenicity assays were conducted at ProImmune Ltd (Oxford, UK). Briefly, this Dendritic-cell/T-cell (DC-T) proliferation assay helps to identify the presence or absence of potential T-cell epitopes within antigens. Cell proliferation is determined using the cell dye carboxyfluorescein succinimidyl ester (CFSE), which forms an intracellular fluorescent conjugate following cellular uptake. Fluorescence intensity of CFSE is halved through each consecutive cell division, thus allowing measurement of proliferation as a function of altered fluorescence of CFSE containing cells. Positive controls for cell proliferation include Tuberculin Purified Protein Derivative (PPD) and Keyhole Limpet Hemocyanin (KLH), to which donors are expected to respond through memory recall and naïve responses, respectively. The assay was performed by testing whole uricase proteins following our previous studies (Kratzer, et al. 2014) against a set of 43 healthy human donor cell samples. Detection of proliferation of CD4+ T cells was performed by labelling cells with CFSE co-staining with anti-human CD4 antibody. A panel of peripheral blood mononuclear cell (PBMC) samples was selected from the ProImmune cell bank. Each PBMC sample was HLA (Human Leukocyte Antigen) -typed and stored in liquid nitrogen (vapor phase) prior to use. The panel was selected so that HLA class II alleles known to be highly expressed in the global population were well represented. Donors were predominantly selected by DRB1 allele expression. Alleles used to generate the data are available by request.

To measure stability of uricase against pancreatin, Candida sp. uricase (Sigma-Aldrich, U0880) and purified ancestral and PBC uricases were incubated with pancreatin: 0.25 mg/mL uricase was incubated with 2.5 mg/mL pancreatin (Sigma-Aldrich, P7545) at 37 °C. At indicated time points, 3 uL of the uricase:pancreatin mixture was added to 100 uM uric acid dissolved in simulated intestinal fluid (SIF) buffer (50 mM potassium phosphate, pH 6.8). The decrease in uric acid concentration was monitored by absorbance at 293 nm. The assay was performed at 37 °C. The linear regression was calculated with Graphpad Prism 9. The Disulfide by Design 2.0
A webserver was used to predict the insertion of a single disulfide bridge into the uricase protein (Craig and Dombkowski 2013).

**Data Availability**
All nucleic and amino acid sequences are available by request. Also, HLA allele information is available for the PBMCs used in the immunogenicity assay.

**Supplementary Material**
Supplementary data are available at *Molecular Biology and Evolution* online.

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**Author Contributions**
E.G. conceived the study, Y.H. performed the evolutionary analyses, Z.L. performed the biochemical assays, L.T. generated the uricase variants for the stability assay. All authors contributed to the writing of the manuscript, and editing of the final manuscript. All authors approved the final version of the article.
Fig. 1. Phylogenetic distribution of species used to infer ancestral uricase sequences. Species listed in bold font represent new sequences that were not included in our previous protein resurrection study (Kratzer, et al. 2014). Geological timescale is calculated by TimeTree (Hedges, et al. 2015). The number of amino acid replacements that occur along any given branch is provided in brackets below the branch. The inset table lists the biochemical properties of the resurrected uricase proteins listed in the phylogeny. The list of nonsynonymous substitutions that occurred along internal branches is provided in figure S6.
Fig. 2. The reshaping of uricase biomolecular properties over time. (A) Affinity of the uricase protein for uric acid substrate binding as described by the Michaelis constant ($K_m$). Lower values indicate stronger binding of the substrate. (B) The ability of the uricase protein to turnover (or oxidize) the uric acid substrate as a function of protein concentration and time ($K_{cat}$). (C) Catalytic efficiency measured as $K_{cat}/K_m$ (logscale on the left, truncated scale on the right). Shown in the light gray are the biomolecular properties of ancestral enzymes inferred from nucleic acid sequences (instead of amino acid sequences) for nodes in which the DNA and protein inferences were not identical. The addition of so many species to our phylogeny (fig. 1) lead to different ancestral sequences compared to our previous study (Kratzer, et al. 2014). Only An29 and An20 have identical sequences between the two studies. Regardless of the sequence differences for the other ancestral nodes between the two studies, it is evident that the biomolecular properties are nearly identical for corresponding nodes between the previous and current studies thereby supporting the robustness of the ancestral biomolecular properties at each node.
Fig. 3. Relative immunogenicity of ancestral and chimeric uricases against control proteins known to elicit strong immune responses from human dendritic cells. A panel of 43 HLA type II alleles was used to represent a diverse global pool.
**Fig. 4.** The relative initial velocity of enzyme stability after incubation with pancreatin. The linear regression was performed and (absolute value of) slopes of the best-fit line in which each y-intercept is set at 1.0, which represent velocity of degradation, are presented in order of increasing stability: *Candida* yeast 0.2, DbD3 0.0257 (variant of An96), PBC 0.0064, An96 0.0053 and Coel 0.0048 (variant of An96).
Fig. 5. Relationship between the reshaping of uricase and URAT1 during ancestral placental mammalian evolution leading to modern humans. This graph has been expanded from our previous studies (Tan, et al. 2016). The biomolecular properties of the proteins appear to experience an inflection around 40 Mya and are completed by 20 Mya, the latter of which coincides with the origins of apes (Andrews 2020).
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