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Mouse Transthyretin-related Protein Is a Hydrolase which Degrades 5-Hydroxyisourate, the End Product of the Uricase Reaction

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Uric acid is the end product of the purine degradation pathway in humans. It is catabolized to allantoin by urate oxidase or uricase (E.C. 1.7.3.3.) in most vertebrates except humans, some primates, birds, and certain species of reptiles. Here we provide evidence that mouse transthyretin-related protein facilitates the hydrolysis of 5-hydroxyisourate, the end product of the uricase reaction. Mutagenesis experiments showed that the residues that are absolutely conserved across the TRP family, including His11, Arg51, His102, and the C-terminal Tyr-Arg-Gly-Ser, may constitute the active site of mTRP. Based on these results, we propose that the transthyretin-related proteins present in diverse organisms are not functionally related to transthyretin but actually function as hydroxyisourate hydrolases.

Keywords: Hydroxyisourate; Transthyretin-related Proteins; Uric Acid; Uricase.

Introduction

Purines are major components of nucleic acids and nucleotides and are continuously formed and degraded. When nucleotides are degraded to bases and nucleosides, they can be reutilized via purine salvage pathways (Nygaard, 1983) or further degraded. The various purine degradative pathways are unique and differ from other metabolic pathways because they may serve quite different purposes depending on the organism or tissue. Uric acid is the end product of the purine degradation pathway in humans (Wu *et al.*, 1989). It is catabolized to allantoin by uricase (E.C. 1.7.3.3.) in most vertebrates except humans, some primates, birds, and some species of reptiles (Keilin, 1959). In mammals, uricase is localized predominantly in the liver and is associated with peroxisomes as a tetramer with a subunit molecular mass of 32–33 kDa (Pitts *et al.*, 1974).

In *Bacillus subtilis*, mutational analysis of the purine-utilizing system, complemented by functional studies, has led to the identification of 14 *puc* genes and a *gde* gene constituting the purine degradation pathway (Schultz *et al.*, 2001). The functions of many of the *puc* gene products have been assigned and the *pucL* and *pucM* genes have been shown to encode uricase activity. PucL shows some sequence identity, starting from Met171, to several known uricases (Bongaerts *et al.*, 1978). Recent studies have demonstrated that allantoin is not the actual product of the urate oxidase reaction (Kahn and Tipton, 1997); in fact, uricase catalyzes the conversion of urate to 5-hydroxyisourate (HIU), which is then spontaneously converted to allantoin under *in vitro* conditions (Kahn and Tipton, 1997; Kahn *et al.*, 1998). The half-life of HIU at neutral pH is estimated to be about 30 min *in vitro* and non-enzymatic decomposition of HIU generates a racemic mixture of allantoin. However, allantoinase is specific for (S)-allantoin and racemization of allantoin at neutral pH

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Abbreviations: HIU, 5-hydroxyisourate; HIUHase, 5-hydroxyisourate hydrolase; LC-MS, liquid chromatography-mass spectrometer; mTRP, mouse transthyretin-related protein; OHCU, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline; TRPs, transthyretin-related proteins; TTR, transthyretin.

takes almost 10 h, suggesting that non-enzymatic conversion would be far too slow to sustain the pathway (Kahn and Tipton, 1998; Lee and Roush, 1964). These observations led to the discovery of a novel enzyme, designated HIU hydrolase (HIUHase), which facilitates the stereospecific conversion of HIU to (S)-allantoin in soybean (Sarma *et al.*, 1999). We have reported that PucM in *Bacillus* is a HIUHase, and have suggested that all its homologue in prokaryotes and eukaryotes have the same function (Lee *et al.*, 2005). While this paper was in revision catalytic activity of mouse transthyretin-related protein (TRP) was reported (Ramazzina *et al.*, 2006). Here we provide evidence that mTRP also facilitates the hydrolysis of HIU and therefore can be re-designated an HIU hydrolase. These findings prompt us to propose that the TRPs of other organisms function as HIUHase, catalyzing a previously poorly-recognized but important step in the purine catabolic pathway.

Materials and Methods

Cloning of uricase and mTRP The uricase gene was amplified by PCR from the genome of *Bacillus subtilis* 168 (Schultz *et al.*, 2001). The mTRP gene (forward primer: 5′-CGGGATCC ATG GCT ACC GAG AGC AGT C, reverse primer: 5′-ACGC-GTCGAC TTA ACT CCC CCG GTA GGT G) was amplified from a mouse liver cDNA library by PCR using a pair of primers covering the coding sequence of the mature protein. The resulting PCR products (containing *Bam*HI and *Sal*I sites; underlined) were cloned into expression vector pGEX-6p-1 (Pharmacia, Sweden) to produce GST-tagged recombinant proteins in *E. coli.*

Protein expression and purification Plasmids carrying GST-tagged uricase and mTRP were introduced into *E. coli* BL21-DE3 and the transformed bacteria were grown at 30°C. The GST-fusion proteins were purified with Glutathione-Sepharose 4B (Amersham Pharmacia Biotech, UK) in batch absorption mode. The GST moiety was removed with Precision Protease™ as described in the manufacturer's manual. SDS-PAGE analysis revealed that all the proteins were highly pure (~98%; Fig. 2).

Enzyme assays Urate oxidase was assayed by monitoring the change in absorbance at 290 nm due to oxidation of uric acid (Kahn and Tipton, 1998). The assays were carried out aerobically at 30°C unless otherwise specified (Lee *et al.*, 2005). HIU hydrolase activity was assayed by its ability to catalyze the hydrolysis of HIU (Kahn and Tipton, 1998). HIU was generated *in situ* (in a 1-cm path-length cuvette) by addition of 0.06 μM recombinant uricase to 1 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 50 μM urate. Sufficient uricase was used to convert the urate to HIU within 10 min. When the urate oxidase reaction appeared to reach its end-point, as determined by monitoring absorbance at 292 nm, mTRP (0.06 μM) was added to the reaction mixture and disappearance of HIU was monitored at 312 nm.

LC-MS analysis of the reaction products Urate (purity > 99%) was prepared at a concentration of 100 ng/ml in HPLC-grade water. Electrospray ionization mass spectrometry was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., USA) equipped with a Finnigan electrospray source. The system was operated with Xcalibur software (version 1.3 SP2, Thermo Electron, USA). HPLC separations were performed on Finnigan Surveyor™ Modular HPLC Systems (Thermo Electron Co., USA), using a TSK-GEL Amide-80 column (5 μm, 2.0 × 150 mm, Tosoh Co., Japan). Twenty μl of enzyme reaction mixture (incubated for 10 min after adding mTRP) was loaded and eluted isocratically with 60% acetonitrile in 10 mM ammonium acetate (pH 8.0) for 10 min at a flow rate of 0.2 ml/min. Mass analysis was performed with an electrospray ionization (ESI) source in negative ion mode. The spray needle voltage was 5 kV, the ion transfer capillary temperature was 200°C, and the nitrogen sheath gas flow rate was set at 60 arbitrary units and the auxiliary gas flow rate at 5 arbitrary units. The ion trap containing helium damping gas was introduced according to the manufacturer's recommendations. Full-scan mass spectra were obtained in the range m/z 50–300, with 3 microscans and a maximum ion injection time of 300 ms. To identify the reaction products and intermediates, such as HIU, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU), and allantoin, the instrument was operated in selected ion monitoring (SIM) mode together with tandem mass spectrometry (MS/MS). All experiments were controlled by the menu-driven software provided with the system and performed under automatic gain control conditions.

Site-directed mutagenesis and activity assays The mTRP mutants used in this work were amplified from pGEX-6p-1-mTRP plasmid DNA by PCR using appropriate primers, and their sequences were verified by DNA sequencing. Mutant mTRP proteins were expressed as GST-tagged recombinant proteins in *E. coli* using the expression vector pGEX-6p-3 (Pharmacia), and purified with Glutathione-Agarose 4B (Peptron, Korea) in column mode. The GST-tag was removed with PreScission Protease™. The purified mTRP mutants formed single major bands on SDS-PAGE and their activities were assayed as reported previously (Lee *et al.*, 2005); we used 1 μg of each mTRP mutant per reaction mixture and monitored the disappearance of HIU by decrease in absorbance at 312 nm.

Results

mTRP exhibits HIU hydrolase activity TRPs (transthyretin-related proteins) are a group of proteins present in a wide range of animals except humans with sequence similarity to transthyretin (TTR) (Fig. 1). Recently we proved that *Bacillus* TRP is a HIUHase. Mouse TRP has sequence similarity to *Bacillus* TRP but its function is

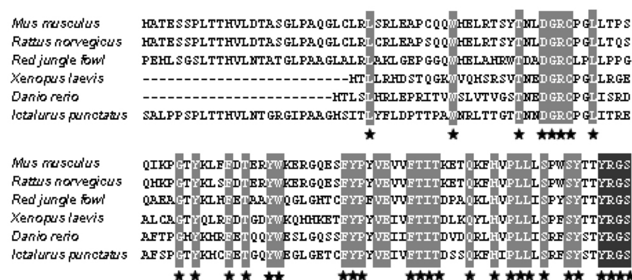

Fig. 1. Multiple sequence alignment of animal transthyretin-related proteins. TRPs from *Mus musculus* (GenBank accession No. AK013117) and orthologues from *Rattus norvegicus* (XM_215112), Red jungle fowl (BX934929), *Xenopus laevis* (AW637709), *Danio rerio* (BI888796) and *Ictalurus punctatus* (BE468943) are compared. The sequences are derived from the GenBank database and the consensus sequences are highlighted and also indicated by an asterisk.

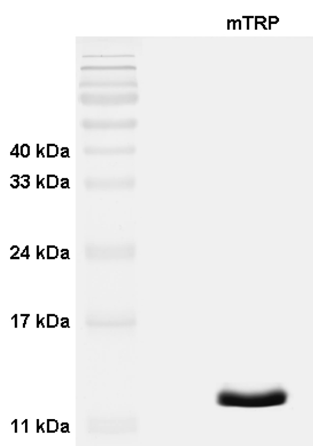

Fig. 2. SDS-PAGE analysis of purified proteins. Lane 1 contains 3 μg of purified protein. M, Molecular-weight standards (PageRuler™ Prestained Protein Ladder); lane 1, mouseTRP. Purified proteins were separated by 15% SDS-PAGE.

unknown. To determine if mTRP indeed facilitates the decay of HIU, we measured its effect on the decomposition of HIU. Production of HIU reached a maximum after a 10 min incubation of urate with purified uricase, after which its level decreased slowly (Lee *et al.*, 2005). As expected, addition of purified mTRP (0.06 μM) greatly accelerated the disappearance of HIU (Fig. 3). We conclude that mTRP is indeed an enzyme facilitating the hydrolysis of HIU.

Mass analysis of reaction products demonstrates that OHCU is produced by mTRP To confirm that mTRP mediates the hydrolysis of HIU, we analyzed the products of the uricase reaction in the absence and presence of mTRP with an LC/MS spectrometer. When urate (m/z 167, $[M-H]^-$) was incubated with uricase alone, the mass

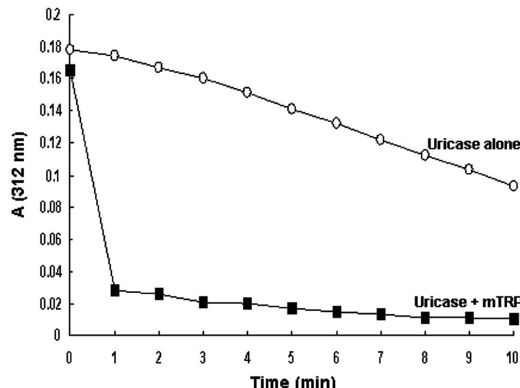

Fig. 3. HIU hydrolase activity of mTRP. Recombinant uricase was pre-incubated with urate for 10 min to generate HIU from urate. After addition of mTRP to the reaction mixture HIUHase activity was monitored by absorbance at 312 nm.

peak corresponding to HIU (m/z 183, $[M-H]^-$) appeared at the expected position (Fig. 4). In addition, there were small mass peaks for OHCU (m/z 201, $[M-H]^-$; the intermediate between HIU and allantoin) and allantoin (m/z 157, $[M-H]^-$; the final product of uric acid decomposition), indicating that a fraction of HIU hydrolyzed spontaneously (Fig. 4). When mTRP was added the HIU mass peak disappeared rapidly and at the same time the OHCU and allantoin mass peaks increased substantially (Fig. 4). We performed independent LC-MS/MS analyses of allantoin, HIU, and OCHU, as standards to compare with the mass peaks from the reaction mixture (data not shown). These results from LC/MS analysis clearly supported our conclusion that mTRP is an HIUHase.

Activities of mTRP mutants Figure 5 shows HIU hydrolysis by various mTRP mutants, including H11N, R51K, R51E, H102N, S118A, and ΔYRGS. The R51E and H11N mutants almost abolished enzyme activity, whereas the conservative R51K substitution failed to affect activity, suggesting that a positive charge is essential at the position of Arg51. Deletion of the conserved C-terminal tetrapeptide also greatly affected catalysis, but Ser118 did not influence the reaction. The activity of another active site substitution, H102N, was reduced more than 10-fold, but this was less than for the other active site substitutions.

Discussion

TRPs are a group of proteins in a wide range of organisms that that have sequence similarity to transthyretin (TTR). TTRs probably evolved by gene duplication in a prevertebrate species; they have been distinguished from TRPs by a phylogenetic analysis based on their sequences (Eneqvist *et al.*, 2003). TRPs are considered precursor

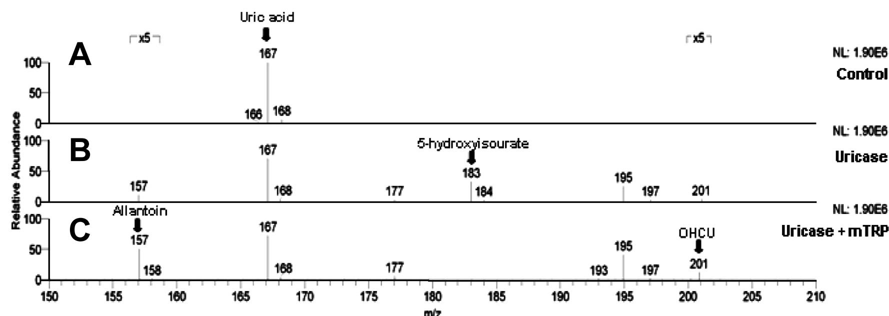

Fig. 4. LC-mass analysis of the products derived from a reaction mixture containing mTRP.

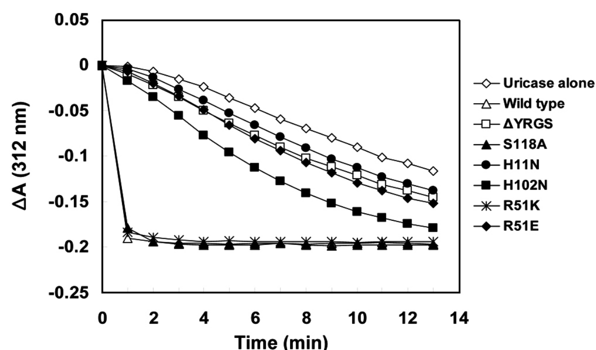

Fig. 5. Hydrolysis of HIU by various mTRP mutants. HIU hydrolysis was calculated as $\Delta A_{312nm} = A_t - A_i$ where A_t is at 312 nm at a given time and A_i is the initial absorbance.

forms of TTRs. but their physiological role remains undefined. It is well-established that TTRs function as transport proteins for thyroid hormone and vitamin A in extracellular fluids; they have thus far only been identified in vertebrates (Eneqvist *et al.*, 2003). Thyroxine binding assays with *E. coli* TRP and hTTR demonstrated that the bacterial TRP did not bind to thyroxine (Eneqvist *et al.*, 2003). In fact, the considerable sequence similarity at the binding site within the TRP family hints that this family of proteins carries out a well-defined function (Eneqvist *et al.*, 2003; Schultz *et al.*, 2001). Thus far, the most important information concerning TRP function has been garnered from mutational studies with *Bacillus subtilis* (Schultz *et al.*, 2001). In several prokaryotes the gene encoding TRP is located within the operon for proteins involved in purine degradation, such as xanthine dehydrogenase, uricase, allantoinase, and ureidoglycolate hydrolase (Eneqvist *et al.*, 2003). In *Bacillus subtilis*, PucM is part of an operon (*pucKJLM*) including urate transport proteins and a putative uricase (PucL). Inactivation of PucL resulted in a uricase-defective phenotype, indicating that the mutant strain cannot utilize guanosine, hypoxanthine, or uric acid as sole source of nitrogen (Schultz *et al.*, 2001). Interestingly, a *pucM* mutant exhibited a similar uricase-defective phenotype (Schultz *et al.*, 2001). Our previous work suggested that PucM could function as a HIUHase that facilitates the conversion of HIU into allantoin, possibly by producing an intermediate, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) from HIU. Interestingly, in plants, TRP expressed sequence tags are predominantly located in roots where urate oxidation takes place, and four TRP sequences were also found in symbiotic rhizobia (Eneqvist *et al.*, 2003; Lee *et al.*, 2004). ESTs coding for these proteins found in the livers of both mouse and fish also indicate that these TRPs may be located in peroxisomes (Wu *et al.*, 1992). However, the physiological relevance of TRPs, urate oxidase activity, and peroxisomes in vertebrates is not yet understood. Moreover, humans and other primates do not have a uricase, which is only found as a nonfunctional pseudogene in the human genome (Wu *et al.*, 1992).

Although more biochemical studies are required to conclude that TRPs are real functional enzyme involved in uric acid metabolism, our current findings nevertheless prompt us to propose that the TRPs in diverse species are HIUHase that catalyze a previously little recognized but important step in the purine catabolic pathway.

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