Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 101-107

IDENTIFICATION OF AN AMINO ACID RESIDUE INVOLVED IN THE SUBSTRATE-BINDING SITE OF RAT LIVER URICASE BY SITE-DIRECTED MUTAGENESIS

Masaki Ito[1*], Seiya Kato[1], Masamichi Nakamura[1], Mitiko Go[2] and Yasuyuki Takagi[1]

[1]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, School of Medicine, Toyoake, Aichi 470-11, Japan

[2]Department of Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Received July 16, 1992

SUMMARY: Computer analysis has shown that a conserved amino acid sequence (Leu 160 to Lys 164) of rat liver uricase is also present in other enzymes with purine substrates. The significances of the amino acids in this sequence were studied by site-directed mutagenesis. Replacement of Lys 164 by Glu or Ile resulted in loss of uricase activity and decrease in binding of the competitive inhibitor xanthine. The far ultraviolet circular dichroic spectra of the mutant uricases were identical to that of the wild type protein, indicating that the replacement of Lys 164 by other amino acids did not result in serious modification of the conformation of uricase. These findings suggest that this amino acid is involved in the substrate-binding site of the enzyme.

Uricase(E.C.1.7.3.3), catalyzing the conversion of uric acid to allantoin in the degradation pathway of purines to ammonia has been mainly studied from the two points of view. First, as the enzymes responsible for this degrading process, urease, allantoicase, allantoinase and uricase, have been shown to lose their activities in this order during primate evolution, these enzymes have been used as good experimental systems to study molecular evolution. Second, as the absence of uricase in humans may be the primary reason for metabolic disorders resulting in gouty arthritis and Lesch-Nyhan syndrome (1,2), but its loss seems to result in a

* Present Address: Department of Biology, Saga Medical School, Saga 849, Japan.
Abbreviation used: IPTG, isopropyl-β-D-thio-galactopyranoside.

0006-291X/92 $4.00

high serum level of uric acid, which decreases the incidence of cancer by functioning as a powerful antioxidant or scavenger of radicals (3), this enzyme has been studied from medical view points.

There are reports of the clonings and sequencings of uricase cDNAs from the baboon (4), pig (5), rat (6,7,8) and mouse (4), and genomic analyses of genes encoding uricase in the rat (9), a fly (10) and soybean (11). The amino acid sequences predicted from these nucleotide sequences indicated that uricase has been fairly well conserved during evolution. Therefore, in the present study we examined the amino acid residues involved in the substrate-binding site of rat uricase.

Experimental Procedures

Bacterial strains and construction of recombinant plasmid- E. coli strains BMH71-18 (*mutS*215) and BW313 (*dut*1, *ung*1) were used. Bluescript SK(+) (Stratagene) was employed as a vector, and a cDNA encoding mature rat uricase (302 amino acids), referred to as pRU17, was ligated into this vector at the *Eco*R1 site in the same orientation as the coding frame. The recombinant plasmid obtained encoded a fusion protein carrying an N-terminal extension of 39 amino acids (MTMITPSSELTLTKGNKSWSSTAVAAALELVDPPGCRNS) derived from a linker portion of the vector and is referred to as pRU17BS.

Mutant construction-Site-specific mutagenesis was carried out in an oligonucleotide-directed *in vitro* system by a modification of the method of Kunkel *et al.*(12). Single stranded DNA containing uracil was prepared from phages produced in strain BW313 harboring pRU17BS. The mutagenic oligonucleotide primers employed are shown in Table 1. The oligonucleotide-primed reaction was transfected into BMH 71-18 cells.

Protein preparation-E. coli cells harboring the recombinant plasmid ligated to rat uricase cDNA were grown in 1 liter of LB medium containing 100 μg /ml of ampicillin to the early log phase and then incubated for 2 hrs at 37 °C in the presence of 2 mM isopropyl-β-D-thio-galactopyranoside (IPTG). The cells were then harvested, disrupted with lysozyme and sonication. The recombinant uricase was purified by a procedure described for rat liver uricase (13) with some modifications . The precipitate was washed with 100 mM sodium borate buffer (pH10.0), resuspended in 100 mM sodium carbonate buffer (pH11.0) and shaken for 2 hrs at 4 °C. The mixture was centrifuged at 12,000g for 20 min, and uricase activity was recovered in the supernatant fraction.

Measurements of uricase activity and substrate-binding activity-Uricase activity was assayed at 25 °C by measuring reduction of the absorption at 292 nm due to disappearance of uric acid. One unit of enzyme activity was defined as that reducing 1 n mol of uric acid per minute. For determinations of Km and Ki values, reaction mixtures contained 2 μg of enzyme and 5 - 50 μM uric acid, and 50 μM xanthine, respectively.

The bindings of uricase and its competitive inhibitor xanthine were assayed by gel filtration on an NAP-10 column (Pharmacia). Reaction mixture containing 10 nmol (300 μg) of uricase and 2 nmol of [^{14}C]xanthine (NEN) in 200 μl was incubated at 25 °C for 60 min and applied to the column. The column was then washed with buffer and eluate fractions of 200 μl were collected. [^{14}C]Xanthine bound with the enzyme was recovered in the void volume, reflecting the larger molecular weight of the complex.

Measurement of circular dichroic spectra-Far-ultraviolet dichroic spectra were measured with an automatic spectropolarimeter J600. Spectra were obtained in buffer containing enzyme at a concentration of 300 μg/ml in a cell with an optical path of 2 mm. The mean residue ellipticity [θ] is expressed in unit deg cm^2 dmol^{-1}.

Results and Discussion

Expression of rat uricase in Escherichia coli and its purification -When *E. coli* cells harboring recombinant plasmid pRU17BS were grown in the presence of IPTG, the induced uricase constituted 5 % of the total cell proteins and was concentrated in the insoluble fraction as inclusion bodies. The precipitated uricase was washed, solubilized and purified as described in Experimental Procedures. The preparations obtained during purification are shown in Fig. 1.

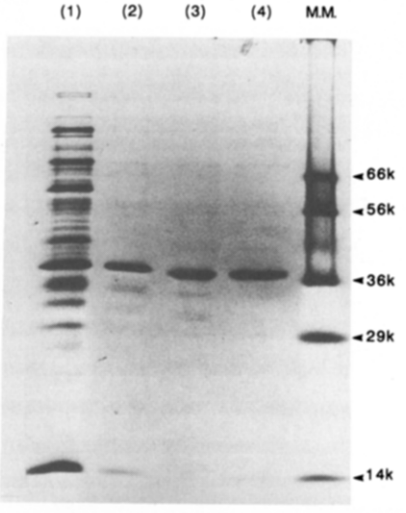

Fig. 1. SDS-polyacrylamide gel electrophoresis of uricase produced in *E. coli*. The uricase preparations described in Experimental Procedures were subjected to SDS-polyacrylamide gel electrophoresis: (1) Cells, (2) Disrupted-cell precipitate, (3) pH 10-precipitate and (4) pH 11-eluate. Molecular weight markers (M.M.) were run in parallel with the preparations. The specific activities (nmol/min/mg) of the fractions were as follows: (1) 73, (2) 200, (3) 1,667, (4) 4,143.

```
                                          *     * * *
Rat      (151-172)  P I I H S G I K D L K V L K T T Q S G F E G
Fly      (195-216)  Q T V I T G I K G L R V L K T T Q S S F V N
Soybean  (147-168)  L Q L T S G I E G L S V L K T T Q S G F V N
```

Fig. 2. Partial alignment of the amino acid sequences of rat (8), fly (10) and soybean (11). Identical amino acid residues are boxed and the residues conserved in HPRT (14) and XGPRT (15) are marked by stars above the sequences. The aligned amino acid positions are shown in brackets (with the first methionine as position 1).

Constructions of mutant uricases-The amino acid sequences of uricases have been deduced from the cDNA nucleotide sequences of enzymes from various sources (4,5,6,7,8,10,11). For determination of the region responsible for enzyme activity, the amino acid sequences of uricases from the rat (8), a fly (10) and soybean (11) were aligned as representative sequences of mammals, invertebrates and plants during evolution, and several conserved regions were found. A computer search showed that of these conserved regions, the sequence of Leu 160 to Lys 164 in rat liver uricase was also presented in human hypoxanthine-guanine phosphoribosyl transferase (HPRT, EC2.4.2.8) (14) and xanthine-guanine phosphoribosyl transferase (XGPRT, EC2.4.2.22) of *E. coli* (15), both of which have a purine as a substrate (Fig. 2). We selected Lys 164 in this sequence for site-directed mutagenesis (12), changing it to Glu or Ile. The oligonucleotide sequences used to direct these site-specific mutations are shown in Table1, and the changes in the mutated cDNAs were verified by sequencing about 200 nucleotides around the mutation site (data not shown).

The cDNAs encoding mutated uricases (K164E and K164I) were expressed in the presence of IPTG in *E. coli* cells. Productions of the mutated uricases at similar levels to that of the wild type protein were observed. Purified preparations of both mutated uricases reacted with anti-rat liver uricase antibody (data not shown) but showed marked reductions of specific activities, to less than 10 nmol/min/mg.

Loss of substrate-binding activity of mutated proteins-The conservation of amino acid sequences in various enzymes with purines as substrates and the loss of enzyme activities of mutated uricases suggested that Lys164 might be involved in

Table 1. Mutagenic primers used. The nucleotide sequence encoding the amino acid sequence from Leu160 to Ser168 is shown. The altered nucleotides and amino acids are indicated by closed circles and underlines, respectively.

mutant	sequence used for construction of mutant uricase
	5′ 3′
wild	CTCAAGGTCTTGAAAACAACCCAGTCT
	LeuLysValLeuLysThrThrGlnSer
K164E	CAAGGTCTTG•GAAACAACCCA
	LysValLeu<u>Glu</u>ThrThr
K164I	AAGGTCTTGA••TCACAACCCAGT
	LysValLeu<u>Ile</u>ThrThrGln

the substrate-binding site of the enzyme. Therefore, we measured the substrate-binding activities of the mutated proteins K164E and K164I. For this purpose, we examined competitive inhibition of the enzyme by xanthine. The Km value of the enzyme for uric acid was calculated to be 8.33 μM and the Ki value of xanthine to be 3.56 μM, indicating that xanthine binds to uricase 2.5- fold more strongly than uric acid.

The bindings of xanthine to wild and mutated uricases were measured by gel filtration. On gel filtration, [^{14}C]xanthine appeared in later fractions reflecting its low molecular weight (Fig. 3, crosses). But with a mixture of wild uricase and [^{14}C]xanthine, the radioactive peak was recovered in the void (earlier) fraction (Fig. 3, closed circles). In the presence of excess cold xanthine, this peak disappeared and the radioactivity was again found in later fractions (Fig. 3, closed triangles). K164E and K164I mutated proteins did not bind with xanthine (Fig. 3, open circles and triangles).

Changes in tertiary structure of mutant proteins were examined by measuring the far ultraviolet circular dichroism spectra. The spectra of the mutant proteins were virtually identical to that of the wild type protein (Fig. 4). Uricases contain 51 ± 0.7 % of α–helix and 19 ± 0.8 % of β-sheet, calculated by the method of Provencher and Gloeckner (16), and change of Lys 164 to Glu or Ile did not cause appreciable alteration in the tertiary structure of the protein. In the present

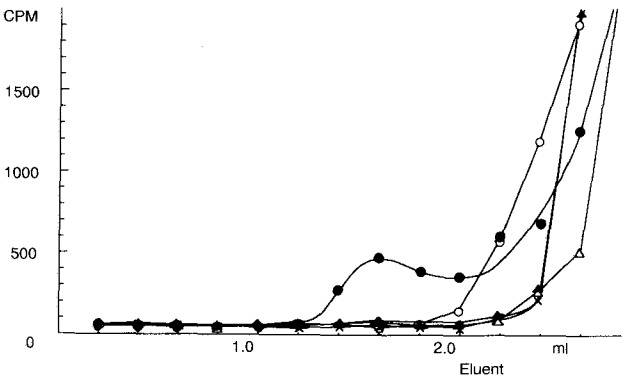

Fig. 3. Measurement of xanthine bound to uricases. The following samples were assayed as described in Experimental Procedures: wild type protein and [^{14}C]xanthine (closed circles); K164E mutant protein and [^{14}C]xanthine (open circles); K164I mutant protein and [^{14}C]xanthine (open triangles); wild type protein, [^{14}C]xanthine and 50-fold excess (100 nmol) of cold xanthine (closed triangles); [^{14}C]xanthine alone (crosses).

experiments using site-directed mutagenesis, we showed that replacement of Lys 164 by Glu or Ile caused reduction of the catalytic activity and the binding activity to the competitive inhibitor xanthine without alteration of the tertiary structure. These results indicated that Lys 164 is envolved in the substrate-binding site of rat liver uricase.

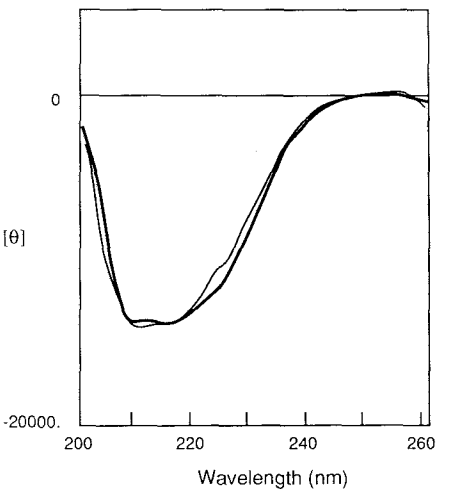

Fig. 4. Comparison of far-ultraviolet dichroic spectra of wild type and mutant uricases. K164E and K164I mutant proteins were examined. The spectrum of K164E (thin line) as representative is compared with that of the wild type protein (bold line).

Acknowledgments

We thank Dr. E. Ichihara for reading the manuscript. This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (01580202, 01480158), a grant from the Fujita Health University, a grant from Science Research Promotion Fund of the Japan Private School Promotion Foundation, and a grant from Japan Foundation for Aging and Health.

References

1. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) Nature326, 292-295.
2. Kuehn, M.R., Bradley, A., Robertoson, E.J. & Evans, M.J. (1987) Nature 326, 295-298.
3. Ames, B.N., Cathcart, B., Schwiers, E., & Hochstein, P. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6858-6862.
4. Wu, X., Lee, C.C., Muzny, D.M. & Caskey, C.T. (1989) Proc. Natl. Acad. Sci. USA 86, 9412-9416.
5. Lee, C.C., Wu, X., Gibbs, R.A., Cook, R.G., Muzny, D.M. & Caskey, T. (1988) Science 239, 1288-1291.
6. Ito, M., Suzuki, M. & Takagi, Y. (1988) Eur. J. Biochem. 173, 459-436.
7. Motojima, K., Kanaya, S. & Goto, S. (1988) J. Biol. Chem. 263, 16677-16681.
8. Alvares, K., Nemali, M.R., Reddy, P.G., Wang, X., Rao, M.S. & Reddy, J.K. (1989) Biochem. Biophys. Res. Comm. 158, 991-995.
9. Ito, M., Nakamura, M. Ogawa, H. Kato, S. & Takagi, Y. (1991) Genomics 11, 905-913.
10. Wallrath, L.L., Burnett, J.B. & Friedman, J.B. (1990) Mol. Cell. Biol. 10, 5114-5127.
11. Nguyen, T., Zelechowska, M., Foster, V., Bergman, H. & Verma, D.P.S. (1985) Proc. Natl. Acad. Sci. USA 82, 5040-5044.
12. Kunkel, T.A., Robert, J.D. & Zakour, R.A. (1987) Methods in Enzymology 154, 367-382 .
13. Watanabe, T., Suga, T. & Hayashi, H. (1977) J. Biochem. 82, 607-609.
14. Edwards, A., Voss, H., Rice, P., Civitello, A., Stegemann, J., Schwager, C., Zimmermann, J., Erfle, H., Caskey, C.T. & Ansorge, W. (1990) Genomics 6, 593-608.
15. Nuesch, J. & Schuemperli, D. (1984) Gene 32, 243-249.
16. Provencher, S. and Gloeckner, J. (1981) Biochemistry 20, 33-37.