BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 56–60 (1997)
ARTICLE NO. RC966046

Cloning and Expression of Human Liver Rhodanese cDNA

Noriko Aita, Kazuyuki Ishii, Yousuke Akamatsu, Yuuki Ogasawara, and Shinzou Tanabe[1]
Department of Hygienic Chemistry, Meiji College of Pharmacy, Tanashi 188, Japan

Received December 19, 1996

cDNA for the human rhodanese (thiosulfate; cyanide sulfurtransferase, EC 2.8.1.1) was cloned from a human fetal liver cDNA library. Sequencing of the cDNA revealed an open reading frame that encodes a 297-residue polypeptide with a calculated mass of 33427 daltons. When the rhodanese cDNA was transientlly expressed in *Escherichia coli* and Cos7 cells, the rhodanese activity increased 40-fold and 150-fold, respectively. Sequence homology analysis showed that the human rhodanese is 89.6% identical to bovine, 90.2% identical to rat, 91.2% identical to mouse and Chinese hamster, and 71.4% similar to avian counterparts, respectively, and that rhodanese was highly conserved across evolution.

Rhodanese (EC 2.8.1.1), a thiosulfate; cyanide sulfurtransferase, is a mitochondrial enzyme that is widely distributed in nature (1, 2, 3). Rhodanese has been proposed to play a role in cyanide detoxification and the formation of iron-sulfur proteins and the modification of sulfur-containing enzymes (4, 5, 6). Bovine rhodanese was isolated and crystallized, and its reaction mechanism has been clarified *in vitro* (7). But the biological functions of this enzyme *in vivo* are yet to be understood.

Rhodanese cDNA was isolated from cow (8), rat (9), human (10, 11), chinese hamster (12), and mouse (13). Although human cDNA clone was reported(10, 11), full sequence and the enzymatic activity of the protein encoded by the clone have never been presented. Thus we have cloned the human rhodanese cDNA in order to examine the function of this protein in human cells. In this paper we report the cloning of the human rhodanese cDNA and predicted amino acid sequences, and expression of the cDNA in *E.coli* and in mammalian cells. The result revealed that the cDNA reported previously turned out not to be that of rhodanese but that of 3′-mercaptopyruvate sulfur transferase, a cousin of rhodanese, thus the cDNA clone presently described being the first ever reported for the primary structure of human rhodanese.

MATERIALS AND METHODS

Monoclonal antibody to rhodanese. Bovine liver rhodanese (TypeII SIGMA, St. Louis, MO) was emulsfied in Hunter's TiterMax (CytRx Corporation). The emulsion was injected subcutaneously into Balb/c mice. The mice were boosted twice with the same antigen. Tha last boost was given four days before sacrifice. Spleen cells from the immunized mice were fused to P3/NS1/1-Ag4-1 myelomas using polyethylene glycol 1500 and hybridomas were selected in HAT medium. Hybridoma culture supernatants (SicRhoys) were screened by ELISA.

Screening and isolation of a cDNA clone of human rhodanese. We used to screen transfomants a human fetal liver cDNA library constructed in phage lambda gt11 purchased from Clontech (Paolo Alto, CA, U.S.A.). Approximately $1x10^6$ plaques were screened using monoclonal antibodies (SicRhoys) raised against the bovine rhodanese TypeII. One of the antibodies recognized human rhodanese. Two positive plaques were picked up and purified by three rounds of purification steps. cDNA fragments excised from the phages with *Eco*RI were inserted in pBluescriptII SK-(STRATAGENE Inc. La Jalla, CA) and named pRho1.1.

DNA manipulations. Plasmid DNA isolation, restriction endonuclease analyses, ligations, and gel electrophoresis were performed according to the standard techniques essentially as described by Maniatis et al (14).

Nucleotide sequencing of rhodanese cDNA. The cloned plasmids were further subjected to deletional subcloning. Those derivatives were sequenced by dideoxy nucleotide chain termination method, using dye-primer kit purchased from Applied Biosystems.

Construction of rhodanese expression vectors. From Rho1.1 on pBluescriptII SK-, *Xba*I to *Hin*dIII fragment was transferred to a mammalian expression vector pRc/CMV (STRATAGENE), pRc/CMV/Rho1.1. From Rho1.1 on pBluescriptII SK-, *Nco*I to *Eco*R I fragment was transferred to *E.coli* expression vector pGEX-2T (Pharmacia-Biotech Uppsala, Sweden), adjusting codon frame, pGEX-2T/Rho1.1. They were transformed to *E. coli* XL-I Blue and selected. Plasmids

[1] To whom correspondence should be addressed: Department of Hygienic Chemistry, Meiji College of Pharmacy 1-22-1 Yatocho Tanashi Tokyo 188, Japan.

The nucleotide sequence of human liver rhodanese cDNA reported in this paper has been submitted to the DDBJ / EMBL / GenBank DNA databases with accession number D87292.

Abbreviations used: bp, base pair; IPTG, isopropyl-β-D-tiogalactoside; GST, glutatione S-transferase; MST, 3′-mercaptopyruvate sulfur transferase; SDS, sodium dodecyl sulfate.

0006-291X/97 $25.00

for the transfection were purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Transformation and expression of rhodanese in E. coli. The plasmid was transformed to XL-I Blue. Overnight cultures of bacteria transformed were diluted 10-fold with fresh 2xYT medium supplemented with ampisillin (100μg/ml) and cultured for 6-8hr at 28°C to OD_{600} of 0.9. Expression of human rhodanese was induced with 15μM isopropyl-β-D-tiogalactoside (IPTG) at 28°C for 20hr. The cells were harvested and lysed by 1mg/ml Lysozyme (SIGMA) with extraction buffer contained 50mM Tris-HCl (pH8.0), 1mM EDTA, 10% Sucrose, 1mM DTT, and then digested with DNase I 400μg per gram of *E. coli* and the lysate was clarified by centrifugation at 30krpm for 5min at 4°C in a Beckman SW50.1 rotor. Supernatants were saved as samples for the activity measurement.

Cell culture, transformation, and expression of rhodanese in mammalian cells. Cos7 cells were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% fetal bovine serum (Biocell, Lot No. 4001544). Cells seeded at 2×10^5 cells per dish (30mm FALCON) were transfected with 2μg of pRc/CMV/Rho1.1 using Lipofectamine (Gibco BRL). At the same time, vector plasmid pRc/CMV and mock infection were performed as controls. The rhodanese activity transiently expressed was determined after 48 hr as follows: the cells were washed twice with PBS, and then lysed by incubating in 300μl per dish of the extraction buffer (10mM Tris-HCl, pH8.0, 0.1% Triton X-100, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride) for 15min on ice. The cell lysates were centrifuged at 15 krpm for 10 min at 4°C in a HITACHI RT15S rotor. Supernatants were saved as samples for rhodanese activity measurement.

Rhodanese activity measurements. Rhodanese activity was assayed by the modified method of Sörbo, B. *et al* (3, 15). The reaction mixture contained 50 mM $Na_2S_2O_3$, 50 mM KCN, and 40 mM KH_2PO_4 in 500 μl. Activities were measured by addition of 50 μl sample solution and incubated for 5 min at 25°C. The reactions were stopped by the addition of 250 μl of 15% formaldehyde, and developed by addition of 750 μl of 250 mM $Fe(NO_3)_3$ in nitric acid solution. 5min after addition of ferric nitrate solution, absorbance at 460 nm was measured by U-1000 HITACHI spectrophotometer.

Western blot analysis. Cellular proteins, 30μg each from pRc/CMV or pRc/CMV/Rho1.1 transfected and mock transfected Cos7 cells, were resolved by 12% SDS-PAGE. Following electrophoretical blotting to PVDF filter, the filter was treated with blocking solution, Block Ace (Yukijirushi, Tokyo), followed by incubation in immunological reaction mixture (1μg/ml SicRhoy2 IgG, 3.5×PBS and 0.1% Tween20). Rhodanese was visualized using affinity purified peroxidase labeled goat anti-mouse IgG (H+L) (Kirkegaard & Perry Laboratories Inc.) and ECL Western blotting detection system (Amersham).

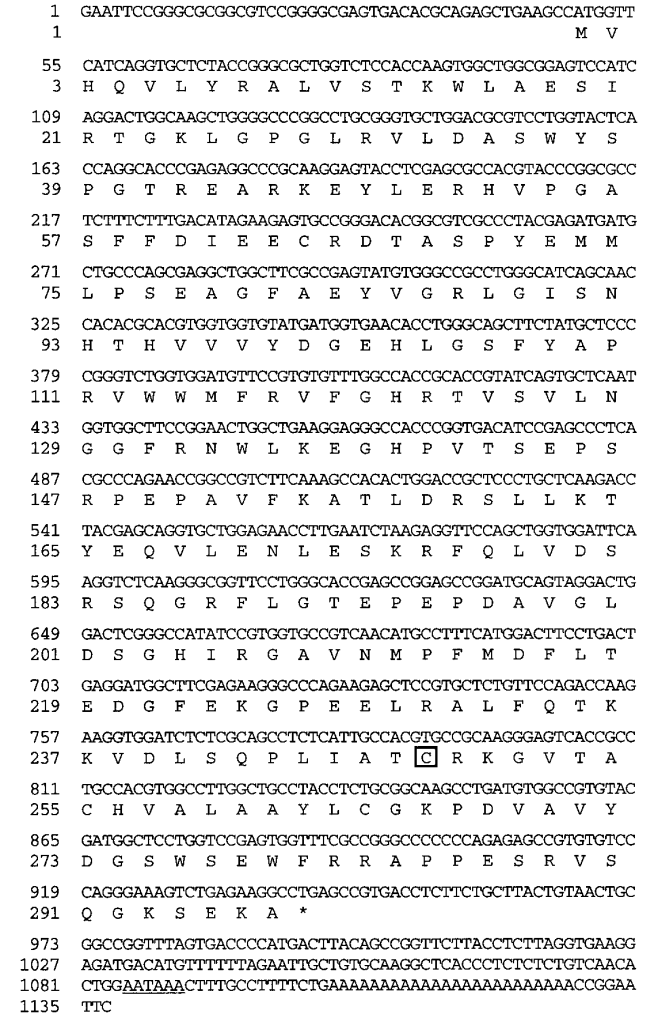

FIG. 1. Nucleotide sequence of the cDNA of human liver rhodanese and the predicted amino acid sequence. The deduced amino acid sequence is shown under the nucleotide sequence in single letter codes. Sequences are numbered on the left, taking the initiation codon as 1. Complete coding sequence and portions of the 5′- and 3′-untranslated region are shown. The open square indicates Cys-248 which is described as the active site of sulfur-rhodanese (see ref.8). AATAAA polyadenylation signal is indicated by underline.

RESULTS AND DISCUSSION

Isolation of a Human Rhodanese cDNA

Two positive clones were isolated from a human fetal liver cDNA library by using a monoclonal antibody against bovine liver rhodanaese. This monoclonal antibody, SicRhoy2, recognized human, mouse and rat rhodanese. But this antibody did not cross-react with 3′-mercaptopyrvate sulfurtransferase (MST; EC 2.8.1.2), a kind of sulfurtransferases similar to rhodanese in physiological properties.

One of the cDNA, designated Rho1.1, appeared to have sufficient length to contain a coding region of rhodanese. The insert of this clone was mapped with restriction enzymes. This insert excised with *Eco*RI was subcloned to pBluescriptII SK-.

Sequence Analysis of Human Liver Rhodanese cDNA

Fig. 1 shows nucleotide and deduced amino acid sequence of human rhodanese obtained by sequencing of the cDNA Rho1.1. The insert contained 1137 bp including an open reading frame, polyadenylation signal and poly(A) tail. The open reading frame contained a predicted amino acid sequence of 297 residues with a molecular mass of 33.4 kDa. As compared in amino acid

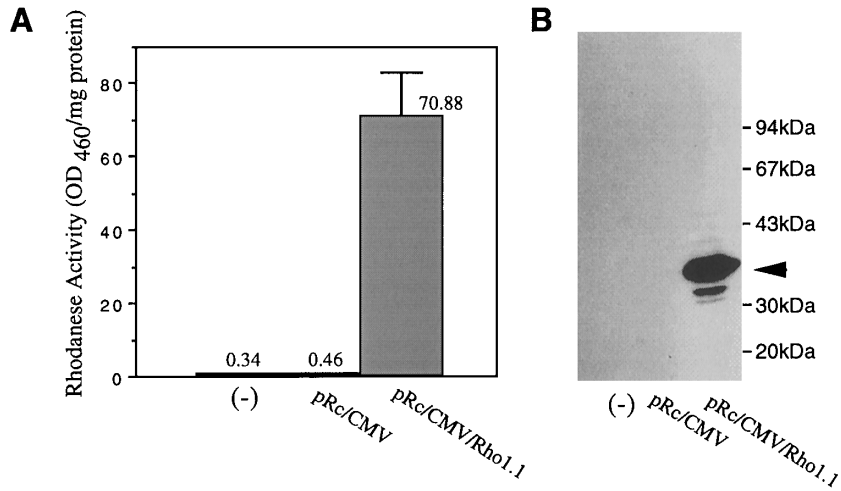

FIG. 2. Transient expression of rhodanese in Cos7 cells transfected with pRc/CMV and pRc/CMV/Rho1.1. (A) Cos7 cells (2×10^5 cells per dish) were transfected with 2mg of pRc/CMV or pRc/CMV/Rho1.1 by the lipofection method. The activities of rhodanese were determined as described in "Materials and Methods." Bars show the average values of three dishes with variations. (−) shows the result of mock-transfection. (B) Western blotting of rhodanese expressed in Cos7 cells. Lysates from Cos7 cells transfected with pRc/CMV and pRc/CMV/Rho1.1, 30μg protein each, were analyzed on SDS-PAGE, followed by immunological staining with SicRhoy2 monoclonal antibody. Relative molecular masses of markers are shown on the right. Arrow indicates the expressed human rhodanese.

sequence the human liver rhodanese is 89.6% identical to the bovine enzyme. The cysteine at 248th residue found in bovine liver rhodanese is presumed to form persulfide bond as the active center (7). In addition some other residues presumed to form active domains were conserved.

For Northern blot analysis of total RNA from HepG2 cells, the insert was used as a probe. A single mRNA band with 1.3 kb was detected (data not shown).

Expression of Rhodanese in E. coli and in Mammalian Cells

In order to determine whether the products expressed in the cells from the cDNA have rhodanese activity, *E. coli* cells were permanently transformed with plasmid pGEX-2T/Rho1.1, and Cos7 cells were transiently transfected with pRc/CMV/Rho1.1, respectively, and the Cos7 cells were harvested 48 hr after transfection.

The human recombinant enzyme fused to GST expressed in *E. coli* showed approximately 40-fold increase in activity relative to vector transformed cells (data not shown). In Cos7 cells transfected with pRc/CMV/Rho1.1 the activity was approximately 150-fold (70.88 ± 11.68 OD_{460}/mg protein) compared to two control cells, i.e. mock and vector pRc/CMV transfected cells (0.34 ± 0.04, 0.46 ± 0.09 OD_{460}/mg protein, respectively). Western blot analysis was shown in Fig.2B. The major band of the products expressed in Cos7 cells is approximately 35 kDa with a minor band showing degradation product.

We have shown the cloning of a cDNA encoding human liver rhodanese and the expression of the cDNA in *E. coli* and Cos7 cells. Furthermore, predicted amino acid sequence of the human rhodanese was compared with those of bovine, rat, chinese hamster, mouse, and avian counterparts (16) in Fig.3. Homology analysis shows that the human rhodanese is 89.6% identical to bovine, 90.2% identical to rat, 91.2% identical to mouse and chinese hamster, and 71.4% similar to avian enzymes, respectively. This comparison showed that the primary structure of rhodanese is highly conserved across evolution, suggesting its important physiological function.

The cloning and sequence of the human liver rhodanese were already reported by Pallini *et al.* (17). They have isolated the clone by using a polyclonal antibody cross-reactive with bovine rhodanese. But the amino acid sequence reported by them agreed quite well with the amino acid sequence of MST, already determined by us (manuscript in preparation). MST catalyzes the transfer of sulfur ion from 3′-mercaptopyruvate (18). Comparison of rhodanese and MST in human showed that the amino acid sequences resemble each other and highly matched (59.7%). Polyclonal antibody against rhodanese may have difficulty in discriminating these proteins, rhodanese and MST, and this ambiguity must have misled them to the conclusion.

Mechanism of localization of the enzyme to mitochondria and the physiological function of rhodanese are not well understood. Our study may contribute to understanding of the physiological roles of these enzymes in cells.

```
Consensus         MVHQVLYRAL VSTKWLAESI R.G..GPGLR VLDASWYSPG TR.ARKEY.E  50
Human             .......... .......... .T.KL..... .......... ..E.....L.  50
Bobine            .......... .........V .A.KV..... .......... ..E.....L.  50
Rat               --........ .......... .S.KV..S.. .......... ..Q.....Q.  48
Mouse             .......... .......... .S.SL..... .......... ..Q.....Q.  50
Chinese hamster   .......... .......... .S.SL..... .......... ..Q.....Q.  50
Avian             -AA.A.G... ..A...S.AV .A.RV.A... .......P.E E.D..Q.FK.  49

Consensus         RHVPGASFFD IEECRDT.SP YEMMLPSEAH FADYVG.LGI SNDTHVVVYD 100
Human             .......... .......A.. .........G ..E...R... ..H....... 100
Bobine            .......... ......KA.. ..V......G ......S... .......... 100
Rat               .......... .......T.. .......... .G....N... .......... 98
Mouse             .......... .......T.. .......... ......S... .......... 100
Chinese hamster   .......... .......T.. .......... ......S... .......... 100
Avian             ..I......N ......KS.. .DF....... ......R..V .......... 99

Consensus         GD.LGSFYAP RVWWMFRVFG HRTVSVLNGG FRNWLKEGHP VTSEPSRPEP 150
Human             .EH....... .......... .......... .......... .......... 150
Bobine            ..D....... .......... .......... .......... .......... 150
Rat               ..D....... .......... .......... .......... .......... 148
Mouse             ..N....... .......... .......... .......... .......... 150
Chinese hamster   ..N....... .......... .......... .......... .......... 150
Avian             ..E..T.... .A.....A.. ..E....... .K..V..... ..A...Q.AE 149

Consensus         AVFKATLDRS LLKTYEQVLE NL.SKRFQLV DSRAQGRYLG TEPEPD.VGL 200
Human             .......... .......... ..E....... ...S...F.. ......A... 200
Bobine            .I.....N.. .......... ..E....... .......... .Q....A... 200
Rat               .......N.. .......... ..Q....... .......... .Q....A... 198
Mouse             .......... .......... ..Q....... .......... ......I... 200
Chinese hamster   .......... .......... ..Q....... .......... ......I... 200
Avian             .....K..KT ....F..AM. .VG..K..V. ...PA..FQ. ..LDQGL--- 196

Consensus         DSGHIRGSVN MPFMNFLTED GFEKSPEELR A.FQ.KKVDL SQPLIATCRK 250
Human             .......A.. ....D..... ....G..... .L..T..... .......... 250
Bobine            .......... .......... .......... .M.EA..... TK........ 250
Rat               .......... V......... .......... .I..D..... .......... 248
Mouse             ........A. .......... .......... .I..D..... .......... 250
Chinese hamster   ........A. .......... .......... .I..D..... .......... 250
Avian             E....P.A.. ...ST....S .H...I..IQ QM.RE..... .K..T..... 246

Consensus         GVTACHIALA AYLCGKPDVA VYDGSWSEWF HRAPPETRVS QGKSGKA     297
Human             ......V... .......... .......... R.....S... ....E..   297
Bobine            .......... .......... I.....F... .......W.. ...G...   297
Rat               .......... .......... .......... R.........  .......   295
Mouse             .......... .......... .......... .Q........  .......   297
Chinese hamster   .......... .......... .......... .Q........  .......   297
Avian             .......... .......... .......... .....QYK.T EL.----    289
```

FIG. 3. Homology alignment of rhodaneses from various species. The amino acid sequence of human rhodanese is compared with those of bovine, rat, mouse, Chinese hamster, and avian. Amino acids are shown in single-letter codes and numbered on the right. The consensus sequence is shown in the top line. Amino acids with no consensus are indicated by dots in the consensus sequence. Identical amino acids to the consensus sequence are shown by dots in each rhodanese sequence. Gaps in the sequence are noted with a hyphen.

ACKNOWLEDGMENTS

We thank Professor Toshiwo Andoh of Soka University for critical reading of the manuscript. This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. Westley, J. (1973) *Adv. Enzymol. Relat. Areas Mol.Biol.* **39,** 327–368.
2. Westley, J. (1980) *in* Enzymatic Basis of Detoxication (Jacoby, W. D., Ed.), Vol.2, pp.245–262 Academic Press, New York.
3. Sörbo, B. H. (1975) *in* Metabolic Pathways, Vol. 12, pp. 433–456, Academic Press, New York.
4. Finazzi Agrò, A., Cannella, C., Graziani, M. T., and Cavallini, D. (1971) *FEBS Lett.* **16,** 172–174.
5. Nishino, T., Usami, C., and Tsushima, K. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1826–1829.
6. Nishino, T. (1986) *Adv. Exp. Med. Biol.* **195B,** 259–262.
7. Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. J. G., Heinrikson, R. L., Keim, P., Weng, L., and Russell, J. (1978) *Nature* **273,** 124–129.
8. Miller, D. M., Delgado, R., Chirgwin, J. M., Hardies, S. C., Horowitz, P. M. (1991) *J. Biol. Chem.* **266,** 4686–4691.
9. Weiland, K. L., and Dooley, T. P. (1991) *Biochem. J.* **275,** 227–231.
10. Durkin, A. S., Nierman, W. C., Zoghbi, H., Jones, C., Kozak, C. A., and Maglott, D. R. (1994) *Cytogenet. Cell Genet* **65,** 86–91.

11. Korenberg, J. R., Chen, X., Adams, M. D., and Venter, J. C. (1995) *GENOMICS* **29,** 364–370.
12. Trevino, R. J., Hunt, J., Horowitz, P. M., Chirgwin, J. M. (1995) *Protein Expression Purif.* **6,** 693–699.
13. Dooley, T. P., Nair, S. K., Gracia IV, R. E., and Courtney, B. C. (1995) *Biochem. biophys. Res. Com.* **216,** 1101–1109.
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.
15. Westley, J. (1981) *Methods Enzymology* **77,** 285–291.
16. Kohanski, R. A., and Heinrikson, R. L. (1990) *Journal of Protein Chemistry* **9,** 369–377.
17. Pallini, R., Guazzi, G. C., Cannella, C., Cacace, M. G. (1991) *Biochem. Biophys. Res. Com.* **180,** 887–893.
18. Jarabak, R. (1981) *Methods in Enzymology* **77,** 291–297.