ATP-Dependent Degradation of a Mutant Serine:Pyruvate/Alanine:Glyoxylate Aminotransferase in a Primary Hyperoxaluria Type 1 Case

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Abstract. Primary hyperoxaluria type 1 (PH 1), an inborn error of glyoxylate metabolism characterized by excessive synthesis of oxalate and glycolate, is caused by a defect in serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT). This enzyme is peroxisomal in human liver. Recently, we cloned SPT/AGT-cDNA from a PH 1 case, and demonstrated a point mutation of T to C in the coding region of the SPT/AGT gene encoding a Ser to Pro substitution at residue 205 (Nishiyama, K., T. Funai, R. Katafuchi, F. Hattori, K. Onoyama, and A. Ichiyama. 1991. *Biochem. Biophys. Res. Commun.* 176:1093–1099). In the liver of this patient, SPT/AGT was very low with respect to not only activity but also protein detectable on Western blot and immunoprecipitation analyses. Immunocytochemically detectable SPT/AGT labeling was also low, although it was detected predominantly in peroxisomes. On the other hand, the level of translatable SPT/AGT-mRNA was higher than normal, indicating that SPT/AGT had been synthesized in the patient's liver at least as effectively as in normal liver. Rapid degradation of the mutant SPT/AGT was then demonstrated in transfected COS cells and transformed *Escherichia coli*, accounting for the low level of immunodetectable mutant SPT/AGT in the patient's liver. The mutant SPT/AGT was also degraded much faster than normal in an in vitro system with a rabbit reticulocyte extract, and the degradation in vitro was ATP dependent. These results indicate that a single amino acid substitution in SPT/AGT found in the PH1 case leads to a reduced half-life of this protein. It appears that the mutant SPT/AGT is recognized in cells as an abnormal protein to be eliminated by degradation.

SERINE:pyruvate/alanine:glyoxylate aminotransferase (EC 2.6.1.51/EC 2.6.1.44, SPT/AGT)[1,2] is an enzyme whose organelle localization and hormone responsiveness differ with the animal species. In the liver of herbivorous animals, including man, this enzyme is peroxisomal, while in carnivores it is entirely or largely mitochondrial (Noguchi and Takada, 1978; Takada and Noguchi, 1982; Danpure et al., 1990). In rat liver, SPT/AGT is located in both peroxisomes and mitochondria, and only the mitochondrial enzyme is markedly induced by glucagon (Noguchi et al., 1978; Oda et al., 1982). It is well known that in eukaryotic cells each organelle participates in different cellular processes, and proteins involved in these processes are synthesized, predestined to be localized in a relevant subcellular compartment. Therefore, the peroxisomal and/or mitochondrial localization of SPT/AGT suggests that the enzyme in the different organelles participates in different metabolic processes. With respect to the mechanism of directing SPT/AGT to either one of the two organelles in rat liver, we recently demonstrated that transcription of a single SPT/AGT gene from different initiation sites in exon 1 eventually determines the alternative targeting of the expression product to either peroxisomes or mitochondria (Oda et al., 1990; Yokota et al., 1991; Mori et al., 1992).

Primary hyperoxaluria type 1 (PH 1), an inborn error of glyoxylate metabolism characterized by increased oxalate production, has been shown to be caused by a defect in SPT/AGT in peroxisomes in the liver (Danpure and Jennings, 1986). This indicated that an important physiological role of peroxisomal SPT/AGT is to remove glyoxylate, preventing excessive production of oxalate, the injurious end product of metabolism. Glyoxylate is an immediate precursor of oxalate and is produced mainly in peroxisomes in the

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1. *Abbreviations used in this paper*: PH 1, primary hyperoxaluria type 1; SPT/AGT, serine:pyruvate/alanine:glyoxylate aminotransferase.

2. This enzyme has been called serine:pyruvate aminotransferase (SPT, EC 2.6.1.51) or alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44), and both the activities have been assumed to be of physiological importance. In this paper, this enzyme is referred to as SPT/AGT.

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liver. Since SPT/AGT is the enzyme with the unique organelle targeting, phenotypes of cell biological interest are also observed in the deficiency of this enzyme in PH 1. Three phenotypic subgroups of PH 1 have been reported. The majority of PH 1 patients have nearly complete deficiencies of SPT/AGT catalytic activity and SPT/AGT immunoreactive protein, but approximately one-third belonging to the second phenotypic subgroup possess significant levels of residual SPT/AGT activity and immunoreactive protein (Wise et al., 1987; Danpure and Jennings, 1988; Danpure, 1991). In the latter case, it has been reported that the disease appears to be due, at least in part, to a unique protein trafficking defect in which SPT/AGT is erroneously routed to the mitochondrion instead of its normal intracellular location, the peroxisome (Danpure et al., 1989). Among patients with zero SPT/AGT activity, there is another subgroup which has immunoreactive SPT/AGT correctly located within the peroxisomes (Takada et al., 1990; Purdue et al., 1992).

We recently studied a PH 1 case who had had recurrent urinary calculi since the age of 6, reached the end-stage renal failure at age 39, which necessitated hemodialysis, and died at age 46. We cloned SPT/AGT-cDNA from the PH 1 case, and demonstrated a point mutation of T to C at position 613 (relative to A of the initiation ATG codon) encoding a Ser to Pro substitution at residue 205. The T to C conversion created a new SmaI site, which enabled us to demonstrate that the mutation occurs in the patient's gene (Nishiyama et al., 1991). In the liver of this patient, not only the SPT/AGT activity but also the protein detectable on Western blot and immunocytochemical analyses was low, but translatable SPT/AGT-mRNA was clearly detectable. In the present study, we demonstrated that the mutant SPT/AGT is unstable in cells and cell extracts, and decomposes much faster than normal. Although the biological and pathological importance of intracellular degradation systems to eliminate misfolded proteins has long been suspected, this is one of the still relatively few cases in which rapid degradation of mutant protein is demonstrated in a hereditary disease.

Materials and Methods

Liver Samples

Liver specimens obtained on autopsy 5-h postmortem from patients (female, 29 y, and male, 68 y) who died of other diseases and the PH 1 patient were immediately frozen in dry ice and stored at −80°C, as described previously (Nishiyama et al., 1991).

RNA Blot Analysis

RNA blot analysis was performed according to Alwine et al. (1977). Poly(A)-rich RNA was prepared from the PH 1 liver after extraction by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Control poly(A)-rich RNA was prepared from a liver specimen otained on autopsy approximately 5-h postmortem from a patient (29 y, female) who died of a malignant lymphoma. The denatured RNA (5 μg) was electrophoresed on a 1.2% agarose gel and then transferred to a nylon membrane (Hybond-N; Amersham International Plc, Buckinghamshire, England). A ^{32}P-labeled 120-bp NcoI-HpaII fragment of human SPT/AGT cDNA (pHspt12; Nishiyama et al., 1990) was used as a hybridization probe. 4 mo later, β-actin mRNA on the same nylon membrane was determined as an internal standard using a 443-bp HinfI-HinfI fragment of human β-actin genomic DNA (λHa160; Nakajima-Iijima et al., 1985). Both DNA fragments were labeled by the random primer-labeling method (Feinberg and Vogelstein, 1983) with [α-^{32}P]dCTP. Prehybridization and hybridization were performed at 55°C for 20 h in 5× NaCl/Pi/EDTA (1× NaCl/Pi/EDTA: 0.18 M NaCl, 10 mM sodium phosphate [pH 7.4], and 1 mM EDTA) containing 5× Denhardt's solution (Maniatis et al., 1982), 0.5% (wt/vol) SDS, and 100 μg/ml denatured salmon sperm DNA. The membrane was washed twice with 5× NaCl/Pi/EDTA for 15 min each at 55°C, twice in 1× NaCl/Pi/EDTA containing 0.1% (wt/vol) SDS for 10 min each at 55°C, and finally twice in 0.1× NaCl/Pi/EDTA containing 0.1% (wt/vol) SDS for 15 min each at room temperature.

In Vitro Translation

RNA was synthesized in vitro from SPT/AGT cDNA clones isolated from control human liver (pHspt12) (Nishiyama et al., 1990) or the patient's liver (pHOspt2) (Nishiyama et al., 1991) using T7 RNA polymerase (Melton et al., 1984). Linealized template DNA (5 μg) was transcribed at 37°C for 5.5 h with 20 U of T7 RNA polymerase (New England Biolabs, Inc., Beverly, MA) in the presence of 10 mM DTT, 0.5 mM each of ATP, CTP, and UTP, 0.05 mM GTP, 0.5 mM P^1-5′-(7-methyl)-guanosine-P^3-5′-guanosine triphosphate (m^{7}G[5′]PPP[5′]G) (New England Biolabs, Inc.), and 40 U of human placental RNase inhibitor (Takara Shuzo Co., Ltd., Kyoto, Japan) in 40 mM Tris-HCl (pH 7.5) containing 6 mM $MgCl_2$, 2 mM spermidine, and 0.01% (wt/vol) BSA, in a final volume of 50 μl. RNA formed was extracted sequentially with phenol and chloroform, precipitated with ethanol, dried, and then dissolved in 20 μl of diethyl pyrocarbonate-treated water.

Poly(A)-rich RNAs obtained from control liver and the patient's liver (1-μg each) or RNA synthesized in vitro as above were translated in a reticulocyte lysate system in the presence of L-[^{35}S]methionine as described previously (Oda et al., 1981). The translation products were immunoreacted with rabbit anti-rat mitochondrial SPT/AGT serum or rabbit nonimmune serum, and then precipitated by adsorption to Staphylosorb (Mercian Co., Tokyo, Japan). SDS/10% PAGE of the precipitates and fluorography were carried out as previously described (Oda et al., 1981).

Construction of Human SPT/AGT Expression Plasmids

For expression in COS-1 cells, an EcoRI-EcoRI fragment of pHspt12 or pHOspt2 (full-length SPT/AGT cDNAs containing EcoRI linkers at their 5′ and 3′ ends) was blunted with Klenow enzyme, and then directly introduced into a eukaryotic expression vector, pSVL, at the blunted XhoI site. The resultant recombinant plasmids were proliferated in *Escherichia coli* DH5α, and covalently closed circular plasmid DNAs were obtained by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. They were designated as pSVLNOR for normal SPT/AGT and pSVLPH for the patient's SPT/AGT.

For expression in *E. coli* strain JM105 cells, a prokaryotic expression vector, pKK233-2, was digested with HindIII, blunted with Klenow enzyme, and then cut with NcoI. An EcoRI-EcoRI fragment of pHspt12 or pHOspt2 was blunted with Klenow enzyme and then cut at the NcoI site. The NcoI cleavage was carried out to shorten the 5′noncoding region of SPT/AGT cDNA to allow its efficient transcription in *E. coli*. After the NcoI cleavage, the ATG sequence in the NcoI site remained on the coding side of SPT/AGT-cDNA as the initiation ATG codon. The resultant SPT/AGT cDNA fragments were inserted into linealized pKK233-2 by ligation of both the NcoI cohesive terminal and the blunt-ended terminal. The constructed recombinant plasmids were designated as pKKNOR for normal SPT/AGT and pKKPH for the patient's SPT/AGT.

Expression of Human SPT/AGT in COS Cells and E. coli

COS-1 cells were transfected with pSVLNOR or pSVLPH by electroporation as described previously (Yokota et al., 1991), except that 30 μg of the purified recombinant plasmids was cotransfected with 4 μg of pCH110, a plasmid containing a functional *lac Z* gene which is expressed from the SV-40 early promoter. pCH110 was used as an internal standard for monitoring the efficiency of the transient expression. The transfected cells were plated on 35-mm dishes and allowed to grow at 37°C for 48 h in DME supplemented with 10% (vol/vol) FCS, with a medium change at 12 h. The lysate of COS-1 cells was prepared as described previously (Yokota et al., 1991), except that the lysis buffer comprised 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, and 0.02% (wt/vol) sodium azide.

E. coli JM105 was transformed with pKKNOR or pKKPH according to Hanahan (1983). 200 ml of M9CA medium (Maniatis et al., 1982) contain-

ing 50 μg/ml ampicillin, 10 μg/ml streptomycin, and 1 μg/ml thiamine was inoculated with an overnight culture of *E. coli* JM105 transformed with one of the recombinant plasmids, followed by incubation at 37°C with shaking. When the absorbance at 600 nm reached 0.6, chloramphenicol was added to a final concentration of 68 μg/ml, and then the incubation at 37°C was continued overnight. Cells were collected by centrifugation (5,000 *g* for 10 min at 4°C), washed with M9CA medium, and suspended in 50 ml of M9CA medium containing 50 μg/ml ampicillin, 10 μg/ml streptomycin, and 1 μg/ml thiamine. After incubation for 1 h at 37°C, cells were exposed to 1 mM isopropyl-1-thio-β-D-galactopyranoside for 18 h at 37°C to induce transcription from the *trc* promoter, and then immediately chilled in ice and harvested by centrifugation (5,000 *g* at 4°C for 10 min). The pellet was washed with 0.5% (wt/vol) NaCl/0.5% (wt/vol) KCl, and then suspended in 40 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal 5'-phosphate, 1 mM pyruvate, 1 mM PMSF, and 10 μg/ml *trans*-epoxy succinyl-L-leucylamido(3-methyl)butane (E-64C, a thiol protease inhibitor). The suspension was sonicated for 5 min at 200 W using an Insonator (model 200 M; Kubota Manufacturing Co., Tokyo, Japan), and the lysate was clarified by centrifugation (14,800 *g* at 4°C for 10 min). A portion of the *E. coli* suspension was centrifuged without sonication, and the pellet was dissolved in the SDS-gel loading buffer, immediately boiled for 5 min, and subjected to SDS-PAGE.

Preparation of a Liver Extract

A control human liver and the patient's liver (1 g each) were homogenized with 5 vol of extraction buffer (50 mM Hepes [pH 7.4], 150 mM NaCl, 1 mM EDTA, 50 μM pyridoxal 5'-phosphate, 0.02% (wt/vol) sodium azide, and 1% (vol/vol) Triton X-100 containing 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml antipain, 0.7 μg/ml pepstatin A, 1 μg/ml leupeptin, and 10 μg/ml E-64C) in a Potter-Elvehjem homogenizer in an ice bath. A sonicated extract was then prepared from each liver homogenate by sonication using an Insonator (model 200 M; 200 W for 15 min in the cold), followed by centrifugation at 14,800 *g* for 10 min at 4°C.

Western Blot Analysis

A polyclonal antibody against human SPT/AGT was raised in a white New Zealand rabbit (male) weighing 1.5 kg by subcutaneous injection of 0.1 mg of the purified enzyme in complete Freund's adjuvant by the method of Dunbar and Schwoebel (1990). 3 wk and 6 wk later, the rabbit was again immunized with 0.05 mg of the antigen in incomplete Freund's adjuvant. Preimmune serum was obtained just before the first injection of the antigen. Antiserum was obtained 8 wk after the first injection.

Western blot analysis was performed with human liver sonicated extract (10 μg protein), COS-1 cell lysate (100 μg protein), *E. coli* JM105 sonicated extract (37.5 μg protein), or *E. coli* JM105 lysate from equivalent volume of cell suspension to that used for the preparation of sonicated extract, essentially according to Kruger and Hammond (1988), using ^{125}I-labeled protein A. Briefly, electrophoresis was carried out in an SDS/12% polyacrylamide gel, and then the gel was soaked in 25 mM Tris/192 mM glycine/15% (vol/vol) methanol (pH 8.25) for 30 min, followed by electrophoretic transfer (2 mA/cm^2, for 2 h) to an Immobilon Transfer Membrane (Nihon Millipore Ltd., Tokyo, Japan). The membrane was incubated at room temperature for 1 h in a blocking buffer comprising 5% (wt/vol) nonfat dried milk and 0.05% (vol/vol) Tween 20 in PBS, and then placed in diluted rabbit anti-human SPT/AGT serum (1:1,000) in the blocking buffer at 4°C for 16 h with gentle rotation. After washing five times for 10 min each with PBS, the membrane was incubated at room temperature in 20 ml of PBS containing 4% (wt/vol) BSA and 37 kBq of ^{125}I-labeled protein A for 2 h. After washing five times for 10 min each with PBS containing 1% (vol/vol) Triton X-100, the membrane was dried and subjected to autoradiography using x-ray film and an intensifying screen.

Immunoprecipitation of Human SPT/AGT

Immunoprecipitation of human SPT/AGT was carried out using anti-human SPT/AGT serum and Staphylosorb or Pansorbin Cells (Calbiochem-Behring Corp., La Jolla, CA) according to Sambrook et al. (1989). Sonicated extracts from human livers and *E. coli* JM105 cells, and a lysate of COS-1 cells were incubated at 4°C for 10 min with 50 μl of 10% (wt/vol) Staphylosorb or 40 μl of 10% (wt/vol) Pansorbin cells in NET-gel buffer (50 mM Tris-HCl [pH 7.5] containing 150 mM NaCl, 0.1% [vol/vol] NP-40, 1 mM EDTA [pH 8.0], 0.25% [wt/vol] gelatin, and 0.02% [wt/vol] sodium azide) with gentle rocking, in a final vol of 350 μl (in case of Staphylosorb) or 340 μl (in case of Pansorbin cells). After centrifugation (14,800 *g* at 4°C for 5 min), supernatants were incubated at 4°C for 2 h with anti-human SPT/AGT serum in NET-gel buffer, and then 10% (wt/vol) Staphylosorb or Pansorbin cells were added and the incubation was continued at 4°C for 1 h with gentle rocking. The amounts of anti-human SPT/AGT serum and Staphylosorb or Pansorbin cells used in each experiment are given in the legends to the figures. The immune complex adsorbed on Staphylosorb or Pansorbin cells was washed by vigorous vortexing for 2 min each first with NET-gel buffer containing 0.5 M NaCl, then with NET-gel buffer containing 0.1% (wt/vol) SDS and finally with 10 mM Tris-HCl (pH 7.5) containing 0.1% (vol/vol) NP-40. Denaturation of the proteins in the immunoprecipitate and analysis by SDS/12% PAGE were performed as described previously (Oda et al., 1981). In a preliminary experiment in which [^{35}S]methionine-labeled SPT/AGT in the COS-1 cell lysate (850 μg protein) was immunoprecipitated using 50 μl of 10% (wt/vol) Staphylosorb, essentially the same radioactivity was recovered in the 43-kD band (representing SPT/AGT) with any amount of anti-human SPT/AGT serum between 1 and 100 μl.

Pulse-Chase Experiment

Experiment with COS-1 Cells. COS-1 cells transfected with pSVLNOR or pSVLPH were cultured for 48 h as described above, and then washed twice with 2 ml of prewarmed (37°C) methionine-free DME. The cells were further incubated for 20 min at 37°C in 2 ml of methionine-free DME, followed by pulse-labeling with 740 or 1,110 kBq (20 or 30 pmol) of Expre^{35}S^{35}S protein labeling mix (a mixture of [^{35}S]methionine and [^{35}S]cysteine) for 20 min at 37°C in 0.5 ml of fresh methionine-free DME. The cells were then washed twice with prewarmed (37°C) DME supplemented with 10% (vol/vol) FCS, to which unlabeled L-methionine and L-cysteine were added to 225 μg/ml and 375 μg/ml, respectively, and incubated at 37°C in 2 ml of the same medium for various periods of time. The incubated cells were washed with PBS, stored at −80°C, and lysates were prepared as described above.

Experiment with E. coli JM105 Cells. *E. coli* JM105 cells bearing pKKNOR or pKKPH were cultured overnight in a small scale (2 ml of medium) as described above, and then washed twice with 1 ml each of M9CA medium supplemented with a mixture of all the L-amino acids (except methionine) at 20 μg/ml each, 50 μg/ml ampicillin, 10 μg/ml streptomycin, and 1 μg/ml thiamine. The washed cells were suspended in 0.6 ml of the same medium and incubated for 10 min at 37°C, and then isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM to induce the transcription of transfected pKKNOR and pKKPH. After incubation with shaking for 30 min at 37°C, 1.85 MBq (50 pmol) of L-[^{35}S]methionine was added and the incubation was continued for additional 5 min. Unlabeled L-methionine was then added to 100 μg/ml, and 100-μl aliquots of the cell suspension were incubated in separate Eppendorf tubes (Brinkman Instruments Inc., Westbury, NY) for various periods of time with shaking at 37°C. The cells were harvested, washed with M9CA medium, resuspended in 100 μl of M9CA medium, and stored frozen in dry ice until all the samples were ready for sonication. The cell suspension was then sonicated in an Eppendorf tube three times for 10 s each at 8 W with the aid of an Ultrasonic Processor (model 7040; SEIKO Instruments & Electronics Ltd., Tokyo, Japan), and resultant sonicated extract was subjected to the reaction with anti-human SPT/AGT serum.

Degradation In Vitro with Rabbit Reticulocyte Extract

^{35}S-labeled normal and mutant human SPT/AGT were synthesized from respective cDNAs in a T7 RNA polymerase-reticulocyte lysate system essentially as described above, with following changes. pAM19 containing EcoRI-EcoRI fragment of pHsptI2 or pHOspt2 was linearized with BamHI, purified by treatment with 100 μg/ml proteinase K for 30 min at 37°C in the presence of 0.5% SDS and used as template DNA. Spermidine was omitted from the transcription buffer, and after the transcription, the template DNA was digested with 300 μU/ml of RNase-free DNase I (Pharmacia, Uppsala, Sweden) for 15 min at 37°C. The concentration of synthesized RNA was determined spectrophotometrically by absorption at 260 nm, and the size of the RNA was checked by electrophoresis through agarose gel. A 0.2-μg aliquot of the RNA formed was translated for 90 min at 25°C with 10 μl of rabbit reticulocyte lysate for in vitro translation, in the presence of 1.85 MBq of Expre^{35}S^{35}S protein labeling mix in a methionine-free translation cocktail (Mori et al., 1979), in a final vol of 25 μl. The translation was stopped by adding 40 μg/ml of cycloheximide and 5 mM each of L-methionine and L-cysteine. The degradation of ^{35}S-labeled SPT/AGT was then followed in a reaction mixture comprising 3 μl

of the translated SPT/AGT, 5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, and 30 μl of rabbit reticulocyte extract for protein degradation in 50 mM Tris-HCl (pH 7.8), in a final volume of 60 μl. When energy requirement of the SPT/AGT degradation was to be examined, ATP was omitted from the reaction mixture, and 20 mM 2-deoxyglucose and 25 U/ml of hexokinase were added. After incubation at 37°C for various periods of time, a 10-μl aliquot of the reaction mixture was removed, mixed with 20 μl of SDS-gel loading buffer, and immediately boiled for 5 min. One half of the sample was then subjected to SDS/10% PAGE and autoradiography for estimation of the radioactivity in the 43-kD band.

Immunocytochemical Staining of Human SPT/AGT

Preparation of Tissues. Frozen tissue blocks of biopsy and autopsy liver samples were put into ice-cold fixative I, that consisted of 4% (wt/vol) paraformaldehyde (Merck, Darmstadt, Germany), 0.2% (vol/vol) glutaraldehyde (Nacalai Tesque Inc., Kyoto, Japan), 0.01% (wt/vol) $CaCl_2$, and 0.15 M cacodylate-HCl buffer (pH 7.4) (Yokota, 1990), and then slowly thawed. The tissue blocks were embedded on the specimen folder of a Vibratome (Oxford Labs., City, CA) in agarose, cut into 200-μm thick sections in a trough filled with fixative I, and then fixed for 2 h at 4°C. The sections were cut into about 1 mm × 1 mm cubes and divided into three groups. The first and second groups were fixed again in 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate-HCl (pH 7.4) buffer for 1 h at 4°C. The third group was further fixed in fixative I for an additional 1 h at 4°C. The first group was then postosmicated with 1% (wt/vol) reduced osmium tetroxide, dehydrated in a graded ethanol series, and then embedded in Epon (TAAB, London, England). The second group was stained for catalase by means of the alkaline diaminobenzidine reaction (LeHir et al., 1979), postosmicated, dehydrated, and then embedded in Epon. The third group was dehydrated in a graded dimethylformamide series and then embedded in LR White (Nippon Bio-Rad Laboratories, K. K., Tokyo, Japan) at −20°C. Polymerization of LR White was carried out under UV light at −20°C for 24 h (Yokota, 1990). Ultra-thin sections were cut with an LKB Ultrotome (LKB, Broma, Sweden) equipped with a diamond knife. Thin sections of the first group were used for morphological observation. Diaminobenzidine staining of peroxisomal catalase was observed using thin sections of the second group.

Immunoelectron Microscopic Procedures. Thin sections from the third group were mounted on uncoated nickel grids. Immunostaining procedures were based on the method of Roth (1982). The sections were incubated in 1% (wt/vol) BSA for 5 min. This was followed by overnight incubation with 50 μg protein/ml each of anti-human SPT/AGT serum, anti-rat mitochondrial SPT/AGT serum, anti-rat liver catalase serum or nonimmune serum at 4°C. After washing with PBS, the sections were incubated with a protein A-gold probe (15 nm in diameter) for 30 min at room temperature. The sections were then washed with distilled water, air-dried, and contrasted with 2% (wt/vol) uranyl acetate for 3 min and with 4% (wt/vol) lead citrate for 20 s. After carbon coating, the sections were examined under a Hitachi H600 electron microscope at an accelerating voltage of 75 kV. To evaluate the intracellular labeling density distribution, 10 micrographs of hepatocytes were taken. The surface areas of the peroxisomes, mitochondria, and cytoplasm in the positive pictures, enlarged to a final magnification of 40,000, were estimated with a semi-computing system, and then the number of gold particles on each area was determined (Yokota, 1986). The labeling density was expressed as the number of gold particles per square micron (Bendayan et al., 1980).

Other Methods

RNA was determined spectrophotometrically with the assumption that an absorbance reading of 1.0 at 260 nm corresponds to a concentration of 40 μg/ml. The β-galactosidase activity used to monitor the transfection efficiency was assayed using a TKO-100 Dedicated Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) essentially according to a technical bulletin for the β-galactosidase assay by use of the fluorometer (contributed by W. A. Braell). The reaction mixture comprised 160 μl of a reaction cocktail (25 mM Tris-HCl [pH 7.5], 125 mM NaCl, 2 mM $MgCl_2$, 12 mM β-mercaptoethanol, and 0.3 mM 4-methylumbelliferyl-β-D-galactoside) and 40 μl of COS-1 cell lysate (20 μg protein). The reaction was carried out at 37°C for 30 min and was stopped by adding 50 μl of 25% (vol/vol) TCA, followed by cooling on ice. The samples were centrifuged for 2 min at room temperature in an Eppendorf microfuge. The resulting supernatant (100 μl) was added to 2 ml of glycine-carbonate reagent (133 mM glycine and 83 mM Na_2CO_3 [pH 10.7]), and then the fluorescence (excitation at 380 nm, emission at 460 nm) was read with a TKO-100 Mini Fluorometer (Hoefer Scientific Instruments). 1 U of the β-galactosidase activity was expressed arbitrarily as the activity that produced 1 pmol of 4-methylumbelliferone in 30 min at 37°C. Under these conditions the β-galactosidase activity determined in COS-1 cells transfected with pSVLNOR or pSVLPH was ~50 U per 20 μg protein of cell lysate. The β-galactosidase activity obtained in COS-1 cells into which pSVLNOR or pSVLPH was cotransfected with pCH110 ranged from 400 to 1,200 U per 20 μg protein of cell lysate, but the variation in the enzyme activity at each time point in one pulse-chase experiment was within 20% of the average. The protein concentrations in extracts from human livers, COS-1 cells and *E. coli* JM105 cells were measured by the Bradford method (Hammond and Kruger, 1988).

Materials

E-64C, a thiol protease inhibitor, was kindly donated by Research Laboratories, Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). COS-1 cells were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Male New Zealand white rabbits were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan), and a nuclease-treated rabbit reticulocyte lysate for in vitro translation was prepared as described by Pelham and Jackson (1976), A rabbit reticulocyte extract for in vitro protein degradation was prepared according to Fagan et al. (1986). A reticulocyte extract used in preliminary experiments for in vitro protein degradation was kindly donated by Drs. K. Tanaka and A. Ichihara (Institute for Enzyme Research, University of Tokushima, Japan). [α-^{32}P]dCTP (111 TBq/mmol) and Expre^{35}S^{35}S protein labeling mix (37 TBq/mmol) were obtained from DuPont-New England Nuclear (Boston, MA), L-[^{35}S]methionine (37 TBq/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO), ^{125}I-Protein A labeled with Bolton-Hunter reagent (1.77 MBq/μg) from ICN Biochemicals Inc. (Costa Mesa, CA), ^{14}C-methylated protein mixture (185 kBq/ml), DNA-labeling system and pAM19, a plasmid vector with SP6 and T7 promoters, from Amersham International Plc (Buckinghamshire, England), pSVL, a eukaryotic expression vector containing the SV-40 late promoter, pCH110, a plasmid coding for β-galactosidase activity, pKK233-2, a prokaryotic expression vector, and *E. coli* JM105 from Pharmacia LKB Biotechnology (Tokyo, Japan), *E. coli* DH5α from Life Technologies Oriental, Inc. (Tokyo, Japan), *E. coli* ER1458, a lon- strain, from New England Biolabs, Inc. (Beverly, MA), NcoI, EcoRI and other restriction enzymes from Toyobo Co., Ltd. (Osaka, Japan), a 443-bp HinfI-HinfI fragment of human β-actin genomic DNA (λHa160) and PMSF from Wako Pure Chemical Industries Ltd. (Osaka, Japan), pepstatin A and antipain from Peptide Institute, Inc. (Osaka, Japan), and leupeptin, aprotinin, 16S- and 23S-ribosomal RNAs from *E. coli* and hexokinase from yeast from Boehringer Mannheim Yamanouchi, Inc. (Tokyo, Japan).

Results

RNA Blot Analysis

On RNA blot analysis with poly(A)-rich RNA, a single band of SPT/AGT mRNA was observed in both control and patient liver. The size of the patient's SPT/AGT mRNA was the same as that of the 1.7-kb SPT/AGT mRNA from control human liver. On densitometric analysis, the level of SPT/AGT mRNA in the patient's liver (Fig. 1, lane *1*) was found to be approximately three times higher than that in control human liver (Fig. 1, lane *2*). On the other hand, the level of 2.0-kb human β-actin mRNA used as an internal standard was almost the same among control and patient liver poly(A)-rich RNAs (Fig. 1, lanes *3* and *4*).

In Vitro Translation

In vitro translation was carried out in a rabbit reticulocyte lysate system, and the products were analyzed by SDS/PAGE after immunoprecipitation with anti-rat mitochondrial SPT/AGT serum or non-immune serum. As shown in Fig. 2, an immunoprecipitated band of approximately 43 kD was observed as the translation product from both the control human liver (Fig. 2, lane *3*) and patient liver (Fig. 2, lane *5*)

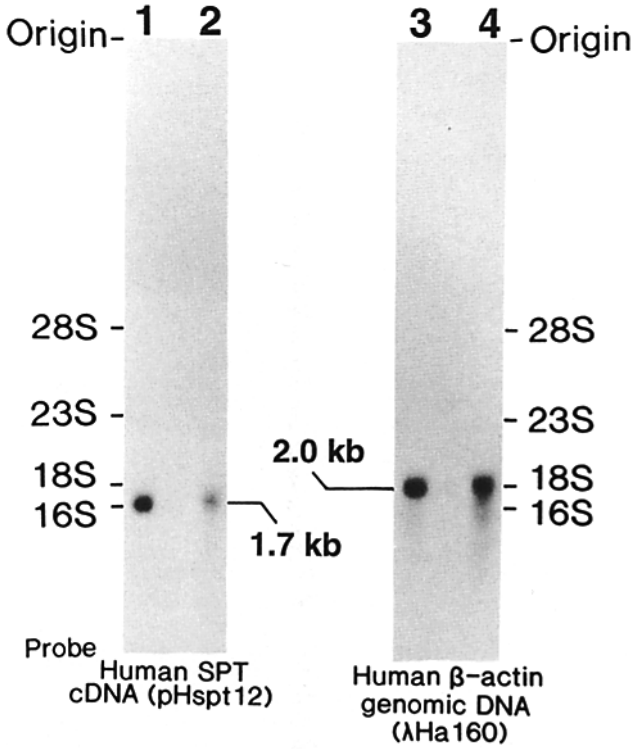

Figure 1. RNA blot analysis. The experimental procedures are described under Materials and Methods. Lanes *1* and *3*, poly(A)-rich RNA from the PH 1 liver; lanes *2* and *4*, control liver poly(A)-rich RNA. Human liver (28S and 18S) and *E. coli* (23S and 16S) ribosomal RNAs were used as size markers.

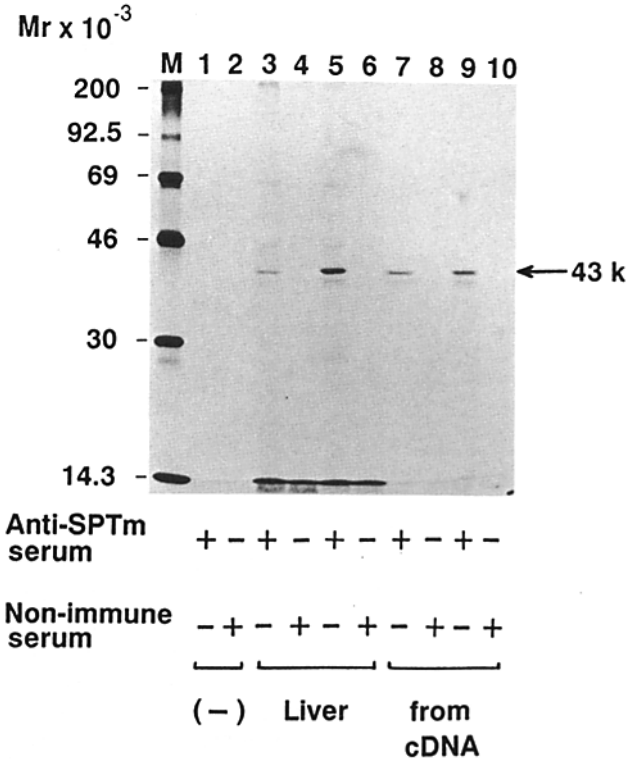

Figure 2. In vitro translation. The experimental procedures are described under Materials and Methods. Cell-free translation was performed without exogenous mRNA (lanes *1* and *2*), with poly(A)-rich RNA isolated from a control liver (29-yr-old woman who died of a malignant lymphoma) (lanes *3* and *4*) or the liver of the PH 1 patient (lanes *5* and *6*), or with capped mRNA synthesized using T7 RNA polymerase from SPT/AGT cDNA clones isolated from a control human liver (pHspt12) (lanes *7* and *8*) or the patient's liver (pHOspt2) (lanes *9* and *10*). The translation products were reacted with nonimmune serum (even-numbered lanes) or anti-rat mitochondrial SPT/AGT serum (odd-numbered lanes). The molecular size markers (lane *M*) were ^{14}C-labeled myosin (200 kD), phosphorylase b (92.5 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), and lysozyme (14.3 kD) from Amersham International Plc. (*SPTm*, rat mitochondrial SPT/AGT).

poly(A)-rich RNAs. The immunological cross-reactivity of the translation product for human SPT/AGT with the anti-rat mitochondrial SPT/AGT antibody had been demonstrated previously (Nishiyama et al., 1990), and the patient's liver SPT/AGT was also shown to react with the anti-rat mitochondrial SPT/AGT antibody in this study. The amount of the 43-kD product translated from the patient's liver poly(A)-rich RNA was even higher than that from the control.

Cell-free translation was also carried out with RNA synthesized in vitro from the T7 RNA polymerase promoter in SPT/AGT cDNA clones isolated from control human liver (pHspt12) and the patient's liver (pHOspt2). The translation products from the synthesized RNAs reacted with the anti-rat mitochondrial SPT/AGT antibody (Fig. 2, lanes *7* and *9*) and have the same molecular mass as that of the in vitro translation product from liver poly(A)-rich RNA. We previously showed that the isolated clones, pHspt12 and pHOspt2, have a cDNA insert which encompasses the whole coding region of human SPT/AGT mRNA, and this coding region encodes a protein of approximately 43 kD (Nishiyama et al., 1990, 1991). Therefore, the results of the in vitro translation suggest that the same translation initiation ATG codon defines an open reading frame of 392 codons in both normal and patient SPT/AGT mRNA. In addition, RNA synthesized from pHOspt2 directed the in vitro translation at least as effectively as RNA synthesized from pHspt12, as judged from the time course of translation and the effect of the amount of RNA used (data not shown). These results together suggested that the patient's SPT/AGT-mRNA was translatable. This suggestion was also supported by the results from pulse labeling in COS cells and *E. coli* described below. It was thus indicated that SPT/AGT had been synthesized in the patient's liver at least as effectively as in the control liver.

Western Blot Analysis and Immunoprecipitation of Human SPT/AGT

Despite the suggestion that the mutant SPT/AGT had been synthesized in the patient's liver, Western blot analysis carried out with not only the anti-rat mitochondrial SPT/AGT antibody but also the anti-human SPT/AGT antibody failed to detect the mutant SPT/AGT, when 10 μg protein of extract from the patient's liver was subjected to analysis (Fig. 3, lane *3*). The mutant SPT/AGT was detectable when as much as 500 μg protein of the liver extract was subjected to Western blot anlysis (Fig. 3, lane *9*). In contrast, a single band of 43 kD was clearly observed for the control human liver (Fig. 3, lane *2*) when 10 μg protein of liver extract was applied. When 500 μg protein of the control liver extract was applied,

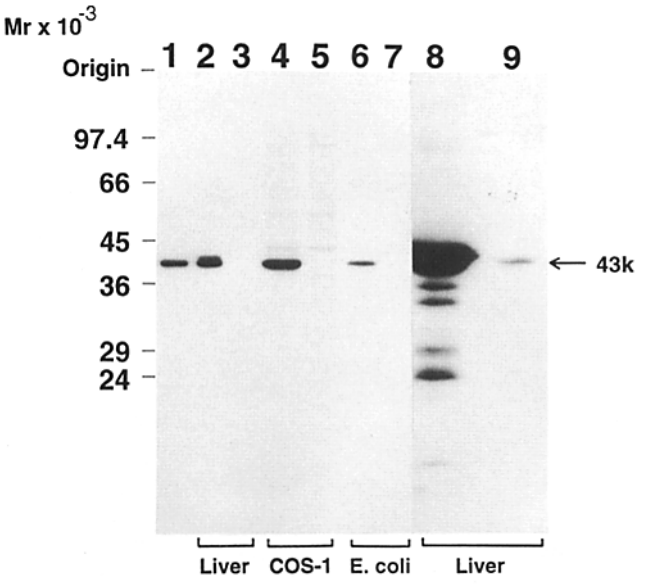

Figure 3. Western blot analysis. The experimental procedures were as described under Materials and Methods, except that 500 μg protein of liver extracts was subjected to the analysis where indicated. Blots were probed with a rabbit antibody raised against normal human SPT/AGT. Signals were developed using ^{125}I-labeled protein A from Staphylococcus aureus. 20 ng protein of purified human SPT/AGT (lane *1*), 10 μg protein of a sonicated liver extract from a 29-yr-old control subject (lane *2*) and the PH 1 patient (lane *3*), 100 μg protein of a COS 1 cell lysate transfected with pSVLNOR (lane *4*) and pSVLPH (lane *5*), and 37.5 μg protein of a sonicated extract of *E. coli* JM105 cells transformed with pKKNOR (lane *6*) and pKKPH (lane *7*) were analyzed by SDS/12% PAGE. In lanes *8* and *9*, 500 μg protein of the liver extracts from the control and the PH 1 patient was applied, respectively. The Western blot analyzes of 10 μg (lanes *2* and *3*) and 500 μg (lanes *8* and *9*) liver extracts were carried out in separate experiments. The molecular size markers were phosphorylase b (*97.4 kD*), BSA (*66 kD*), ovalbumin (*45 kD*), glyceraldehyde phosphate dehydrogenase (*36 kD*), carbonic anhydrase (*29 kD*), and trypsinogen (*24 kD*) from Sigma Immunochemicals (St. Louis, MO).

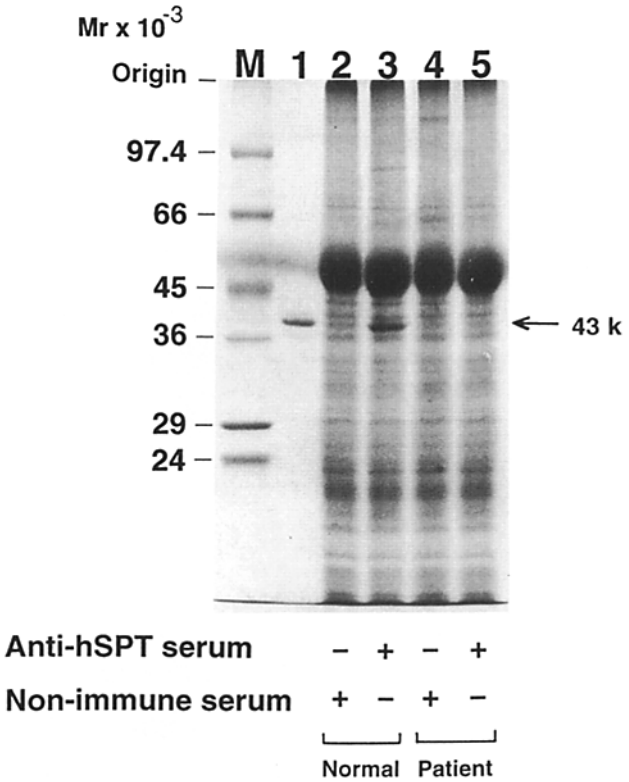

Figure 4. Immunoprecipitation of liver SPT/AGT. The immunoprecipitation procedures are described under Materials and Methods. Sonicated liver extracts (6 mg protein each) from a 29-yr-old control subject (lanes *2* and *3*) and the PH 1 patient (lanes *4* and *5*) were first incubated with 40 μl of 10% (wt/vol) Pansorbin cells in a final vol of 340 μl. After centrifugation, supernatants were immunoreacted with 50 μl of anti-human SPT/AGT serum (lanes *3* and *5*) or nonimmune serum (lanes *2* and *4*), and then immunoprecipitated with 40 μl of 10% (wt/vol) Pansorbin cells. The immune complexes adsorbed on Pansorbin cells were subjected to SDS/12% PAGE, followed by Coomassie brilliant blue R-250 staining. When 100 μl of 10% (wt/vol) Staphylosorb was used for immunoprecipitation, the result was the same as that obtained with Pansorbin cells, except that nonspecific adsorption on Pansorbin was slightly lower than that on Staphylosorb. The molecular size markers (lane *M*) were those given in the legend to Fig. 3. Lane *1*, 7.5 μg of purified human SPT/AGT. (*hSPT*, human SPT/AGT).

the 43-kD band of normal human SPT/AGT became gigantic (Fig. 3, lane *8*). The bands corresponding to normal and patient SPT/AGT (Fig. 3, lanes *8* and *9*) were cut out and the radioactivity was measured. The radioactivity of the normal SPT/AGT band was 35 times higher than that of the patient's SPT/AGT.

In another experiment, COS-1 cells transfected with pSVLNOR or pSVLPH were cultured for 48 h and then pulse labeled with [^{35}S]methionine/cysteine for 20 min. A cell lysate was then prepared and subjected to Western blot analysis. The COS-1 cells transfected with pSVLPH synthesized the immunoprecipitable 43-kD protein (SPT/AGT) as effectively as the cells transfected with pSVLNOR, as judged on pulse labeling with [^{35}S]methionine/cysteine, but no discernible amount of the mutant SPT/AGT was accumulated in the cells, while normal SPT/AGT was distinctly detectable (Fig. 3, lanes *4* and *5*). Likewise, no accumulation of the mutant SPT/AGT was observed in *E. coli* JM105 cells bearing pKKPH (Fig. 3, lanes *6* and *7*). Transformed *E. coli* ER1458, a lon- strain, also failed to accumulate discernible amount of the mutant SPT/AGT, as judged by immunoperoxidase method (data not shown). Misfolded proteins often form insoluble aggregates, especially when overproduced in prokaryotic cells, but in experiments with *E. coli* both normal and mutant SPT/AGTs were expressed only to a level which was detectable by Western blot analysis. In addition, when transformed *E. coli* (JM105 and ER1458) was dissolved in SDS-gel loading buffer without sonication and subjected to Western blot analysis without centrifugation, the same results as those with sonicated supernatants (Fig. 3, lanes *6* and *7*) were obtained. In experiments with COS-1 cells, no aggregates of the mutant SPT/AGT were detectable immunocytochemically. These results suggested, although not proved, that the failure in detecting the immunoreactive mutant SPT/AGT was not entirely due to failure to extract the enzyme.

We also tried to recover the mutant SPT/AGT from a sonic extract of the patient's liver by reaction with the anti-human SPT/AGT antibody in solution. SPT/AGT was immunopre-

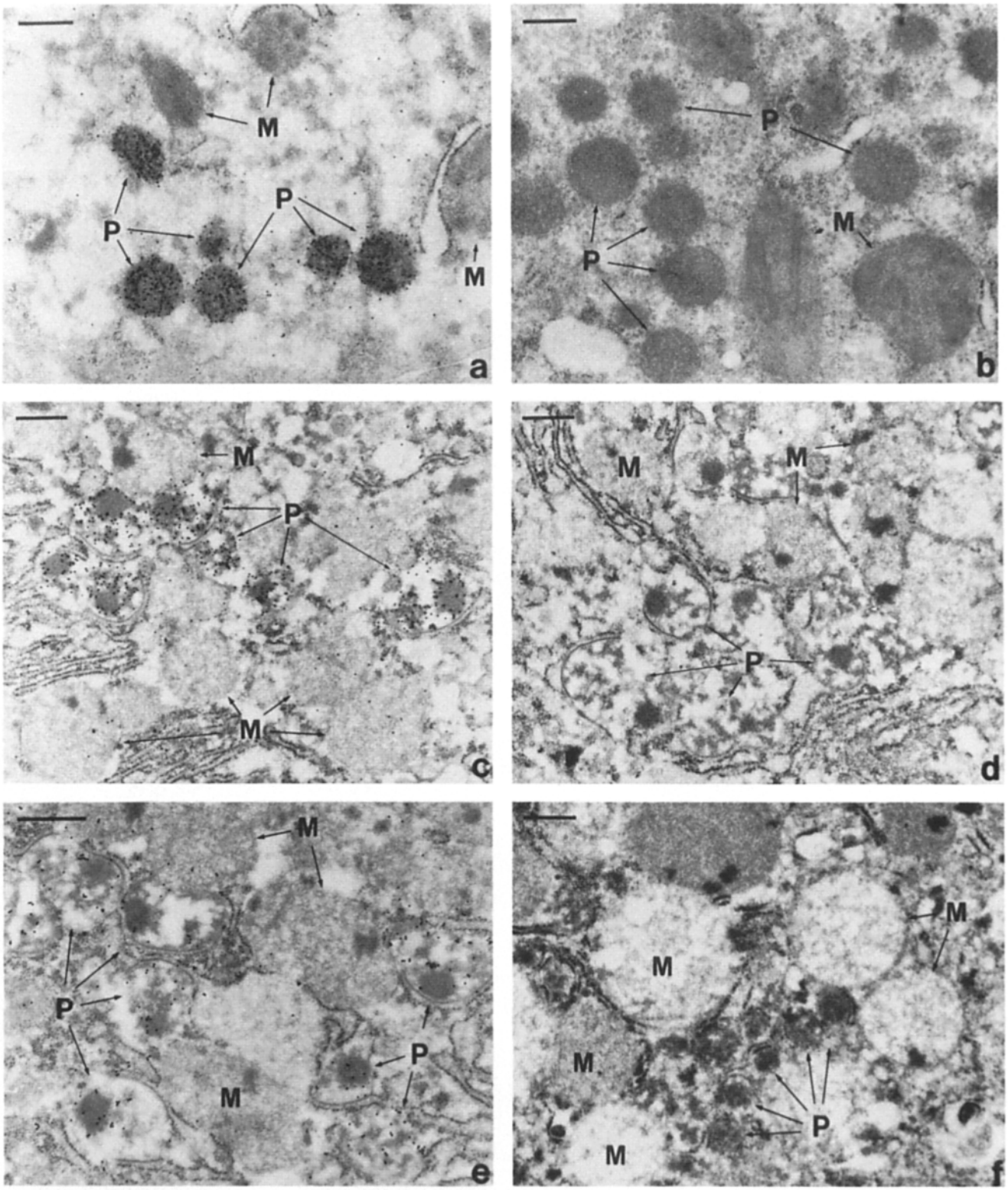

Figure 5. Protein A-gold immunocytochemistry of SPT/AGT in livers. The experimental procedures are described under Materials and Methods. Anti-human SPT/AGT serum was used for *a*, *c*, and *e*, and non-immune serum for *b*, *d*, and *f*. (*a* and *b*) Sections from the liver obtained on open biopsy from a 57-yr-old man suffering from gastric cancer; (*c* and *d*) sections from the liver obtained on autopsy 5-h postmortem from a 29-yr-old control subject; (*e* and *f*) sections from the liver of the PH 1 patient. The liver of the PH 1 patient was obtained on autopsy 5-h postmortem. (*M*, mitochondria; *P*, peroxisomes). Bars, 0.5 μm.

cipitated from a sonic extract of the control liver and recovered as the 43 kD product (Fig. 4, lane *3*), but no specific band of 43 kD was detected when the sonic extract of the patient's liver was subjected to immunoprecipitation (Fig. 4, lane *5*). These findings indicated that the patient's liver contained an extremely low level of the mutant SPT/AGT, probably due to rapid degradation.

Immunocytochemical Staining of SPT/AGT in Control and Patient Liver

The intracellular localization of mutant SPT/AGT in the patient's liver was studied immunocytochemically using anti-human SPT/AGT serum and anti-rat mitochondrial SPT/AGT serum. Fig. 5 shows the results obtained with anti-human SPT/AGT serum. Quantitative analysis of the labeling density was carried out in a separate experiment involving anti-rat mitochondrial SPT/AGT serum.

In a fresh liver preparation, gold particles showing catalase were present exclusively in peroxisomes. Other cell organelles such as mitochondria and ER were consistently almost negative for catalase. The labeling densities (gold particles/μm^2) in mitochondria and the cytoplasmic matrix were calculated to be 0.39% and 0.48% of that in peroxisomes, respectively (Table I). When sections from the same preparation were incubated with either anti-rat mitochondrial SPT/AGT serum or anti-human SPT/AGT serum, heavy labeling with gold particles was observed almost exclusively in peroxisomes. Few gold particles were present in mitochondria and the cytoplasmic matrix (Fig. 5 *a*, and Table I).

In postmortem preparations of liver, preservation of the hepatocyte ultrastructure was considerably poor, most mitochondria being markedly swollen and having lost their cristae. Some mitochondria contained electron-dense material (Fig. 5, *c–f*). Most peroxisomes showed partial membrane disruption. The intact region of the membrane was associated with the endoplasmic reticulum membrane (Fig. 5, *c–e*). The peroxisomal matrix aggregated to form a flocculent configuration which was never observed in the peroxisomes of a fresh liver preparation. Nevertheless, gold particles showing the antigenic sites for catalase were almost confined to peroxisomes in autopsy liver preparations from patients who died of other diseases (Table I). Gold particles showing the SPT/AGT antigens sites were also detected mainly in peroxisomes, but a few particles were scattered outside the peroxisomes (Fig. 5 *c*). The labeling densities of SPT/AGT in mitochondria and the cytoplasmic matrix were

Table I. Labeling Densities for Catalase and SPT/AGT in Mitochondria and Cytoplasmic Matrix Relative to Those in Peroxisomes

	Catalase		SPT/AGT	
	Mitochondria	Cytoplasmic matrix	Mitochondria	Cytoplasmic matrix
	(%)		(%)	
Biopsy (control)	0.39	0.48	0.59	0.49
Postmortem (control)	3.0	2.4	15.0	8.5
Postmortem (PH 1)	1.4	1.3	25.6	14.4

Labeling densities (gold particles/μm^2) are expressed as percentages of the density in peroxisomes of each liver sample. Values are the mean of 10 determinations on different electron micrographs of hepatocytes in the same liver specimen. Experimental procedures are described under Materials and Methods.

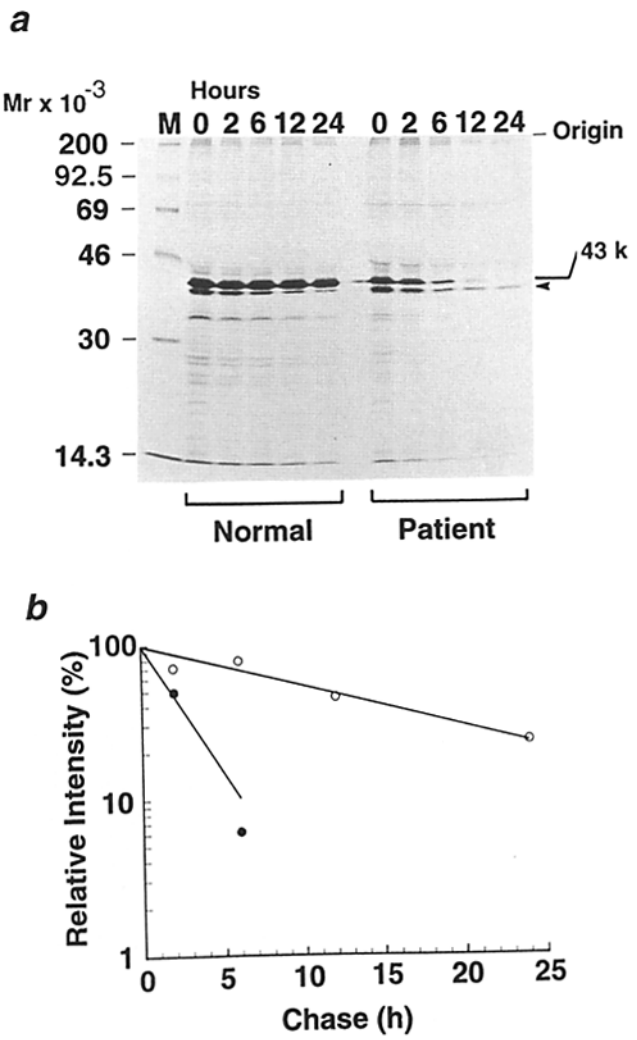

Figure 6. Pulse-chase experiment with COS-1 cells. The experimental procedures are described under Materials and Methods. (*a*) COS-1 cells were labeled with [^{35}S]methionine/cysteine for 20 min. At time zero, unlabeled L-methionine and L-cysteine were added in excess, and then the incubation was continued for the indicated periods. The COS-1 cell lysate (850 μg protein) was first incubated with 50 μl of 10% (wt/vol) Staphylosorb in a final vol of 350 μl. After centrifugation, the supernatant was immunoreacted with 10 μl of anti-human SPT/AGT serum and then immunoprecipitated with 50 μl of 10% (wt/vol) Staphylosorb. The immune complex was analyzed by SDS/12% PAGE. This experiment was carried out twice (in one experiment all procedures after the transfection were performed in triplicate) with almost identical results. The molecular size markers were those given in the legend to Fig. 2. (*Arrowhead*) A minor band with a molecular mass of approximately 37 kD. (*b*) Autoradiograms were scanned with a Shimadzu high-speed TLC scanner CS-920 (Shimadzu Corp., Kyoto, Japan) and the densitometric readings at 595 nm are expressed as percentages of the density at time zero. (○) Normal human SPT/AGT; (●) mutant SPT/AGT of the PH 1 patient. Each point represents the average of triplicate determinations.

15.0 and 8.5% of that in peroxisomes, respectively (Table I). In the liver of the PH 1 patient, in which gold labels for catalase were concentrated in the peroxisomes (Table I), gold particles showing SPT/AGT antigenic sites were scarce on the whole but were detected mainly in peroxisomes (Fig. 5 *e*). The labeling density relative to that in peroxisomes were approximately 25.6% in mitochondria and 14.4% in the cytoplasmic matrix. The mitochondrial labeling density was higher than the cytoplasmic background, but the ratio of the labeling density in mitochondria to that in the cytoplasmic matrix was about the same in the control autopsy sample and the PH 1 sample. No gold particles were noted in any of the control sections in which non-immune serum was used instead of anti-SPT/AGT serum (Fig. 5, *b*, *d*, and *f*). The immunocytochemical analysis suggested that the mutant SPT/AGT had been synthesized in the patient's liver, predestined to be localized in peroxisomes. It also appeared from the experiment that SPT/AGT leaks from peroxisomes more readily than catalase during the postmortem cell destruction (c.f. Table I). Several peroxisomal matrix enzymes have been shown to vary in how easily they leak out when isolated rat liver peroxisomes are subjected to mechanical damages (Alexon et al., 1985).

Degradation Rate of Normal and Patient SPT/AGT

The degradation rate of ^{35}S-labeled SPT/AGT was analyzed in an attempt to reveal whether or not the low content of immunoreactive SPT/AGT in this PH 1 patient is due to instability of the mutant SPT/AGT in the cells. The COS-1 cells and *E. coli* JM105 used in this experiment did not produce SPT/AGT by themselves. In this experiment, pSVLNOR or pSVLPH was cotransfected into the COS-1 cells with pCH110, a plasmid coding for β-galactosidase activity, to monitor the efficiency of the transient expression. The β-galactosidase activity in cell lysate was almost the same between the COS-1 cells transfected with pSVLNOR and pSVLPH, indicating that pSVLNOR and pSVLPH were almost equally transfected. As shown in Fig. 6, the pulse-chase experiments with COS-1 cells demonstrated that normal SPT/AGT is relatively stable and is degraded with an apparent half-life of approximately 11 h. However, the protein concentration in COS-1 cell lysate increased with time, and after 24 h, it reached a 1.5 to 1.9 times higher level than that at time zero. It thus appears that the pulse-labeled SPT/AGT was diluted by the growth and multiplication of COS-1 cells with time and the half-life of normal SPT/AGT was underestimated. On the other hand, the decay of the patient's SPT/AGT in COS-1 cells was distinctly rapid. Approximately 90% of the labeled SPT/AGT was lost within 6 h, with an apparent half-life of approximately 1.8 h, and after 24 h, the labeled 43-kD band was no longer detectable. During the degradation of the patient's SPT/AGT, no immunoprecipitable products with higher molecular masses, such as the conjugates with other proteins, were detectable on SDS-PAGE analysis, but a minor band with a smaller molecular mass (37 kD) was observed throughout the incubation period, in addition to the major band (43 kD) of normal and patient SPT/AGTs (Fig. 6 *a*, *arrowhead*). The 37-kD protein appeared to be derived through translation from a downstream AUG codon rather than a degradation product of the 43-kD SPT/AGT.

The pulse-chase experiment with transformed *E. coli* also demonstrated the rapid decay of the mutant SPT/AGT. As shown in Fig. 7, an almost equal amount of immunoprecipitable SPT/AGT was synthesized during 5-min pulse labeling with [^{35}S]methionine in both *E. coli* JM105 cells bearing pKKNOR and pKKPH. Thereafter, normal SPT/AGT was stable and rather increased about 2.5-fold in the course of chase incubation for unknown reasons. On the other hand, pulse-labeled patient's SPT/AGT was rapidly degraded, with an almost 60% decrease during the first 1 h.

Tanaka et al. (1983) demonstrated that two distinct nonlysosomal ATP-dependent proteolytic systems exist in reticulocytes; one requires ubiquitin and the other is independent of ubiquitin. We then examined degradation of the patient's SPT/AGT in a rabbit reticulocyte extract system with special attention to the ATP-dependency (Fig. 8). [^{35}S]labeled nor-

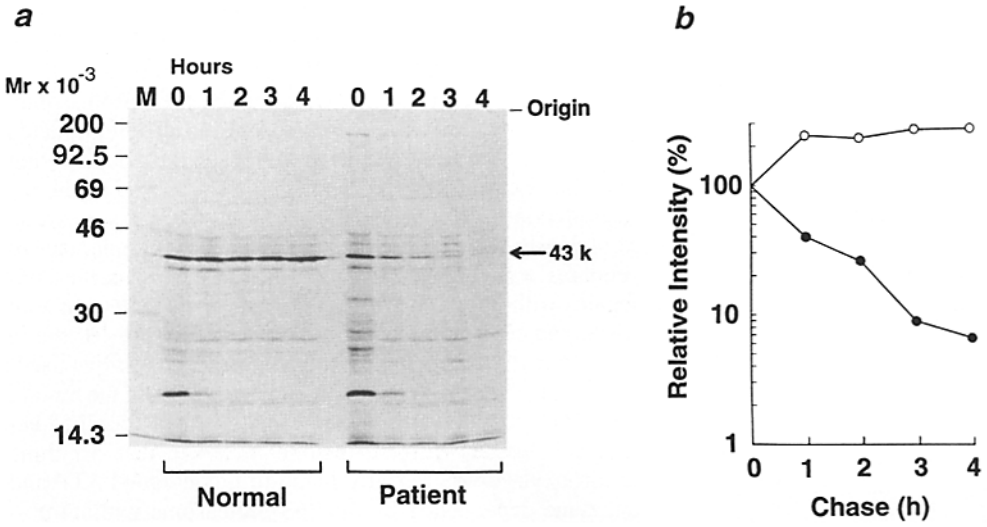

Figure 7. Pulse-chase experiment with *E. coli* JM105. The experimental procedures are described under Materials and Methods. (*a*) Transformed *E. coli* JM105 had been labeled with [^{35}S]methionine for 5 min. At time zero, unlabeled L-methionine was added in excess, and then the incubation was continued for the times indicated. The sonicated extract (100 μl) was first incubated with 50 μl of 10% (wt/vol) Staphylosorb in a final vol of 350 μl. After centrifugation, the supernatant was immunoreacted with 10 μl of anti-human SPT/AGT serum, followed by immunoprecipitation with 50 μl of 10% (wt/vol) Staphylosorb. The immune complex was analyzed by SDS/12% PAGE. Three separate experiments were performed with essentially the same results. The molecular size markers were those given in the legend to Fig. 2. (*b*) Autoradiograms were scanned with a Shimadzu high-speed TLC scanner CS-920. The relative densitometric readings at 595 nm were plotted. (○) Normal SPT/AGT; (●) mutant SPT/AGT of the PH 1 patient. Each point represents the average of three separate experiments.

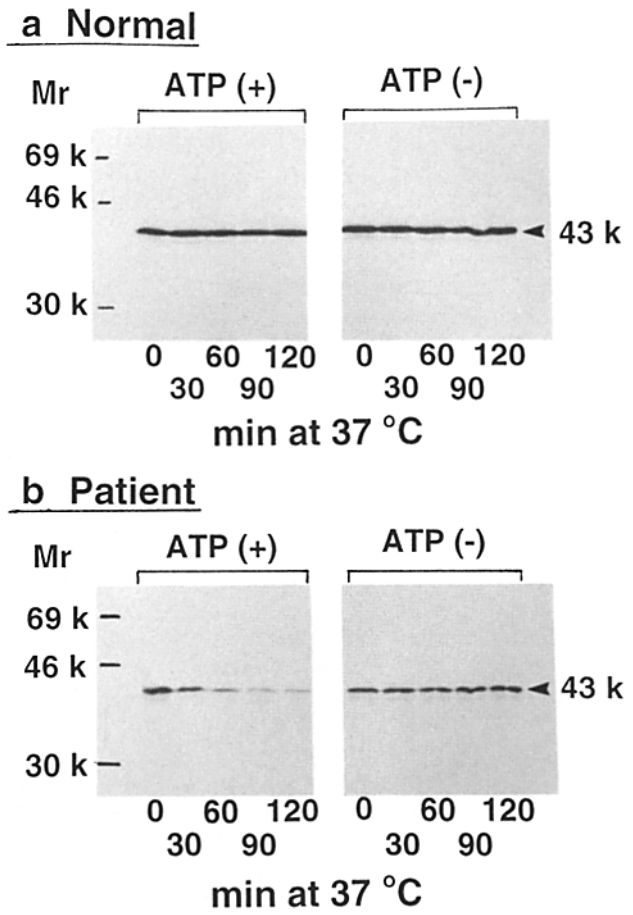

Figure 8. ATP-dependent degradation of mutant SPT/AGT in rabbit reticulocyte extract. ^{35}S-labeled normal human SPT/AGT (*a*) and the patient's SPT/AGT (*b*) were synthesized from respective cDNAs in a T7 RNA polymerase–reticulocyte lysate system, and subjected to the reaction with reticulocyte extract for protein degradation in the presence or absence of ATP at 37°C for the indicated periods. Detailed experimental procedures are given under Materials and Methods. *ATP (+)*: The reaction for degradation was carried out in the presence of 5 mM ATP. *ATP (−)*: 20 mM 2-deoxyglucose and 25 U/ml of hexokinase were added instead of ATP to deplete ATP from the reaction mixture. The molecular size markers were those given in the legend to Fig. 2.

mal and mutant human SPT/AGTs synthesized from respective cDNAs were incubated with rabbit reticulocyte extract for protein degradation in the presence of ATP or under conditions of ATP depletion established by adding 2-deoxyglucose and hexokinase instead of ATP. Normal human SPT/AGT was stable and almost all the ^{35}S-labeled enzyme was retained after 120-min incubation in either the presence or the absence of ATP. In contrast, the patient's SPT/AGT decreased to a very low level after 90–120-min incubation for degradation in the presence of ATP, and the degradation was distinctly dependent on ATP.

Discussion

This study is the first to provide information as to the molecular mechanism responsible for the SPT/AGT deficiency in primary hyperoxaluria type I due to rapid degradation of mutant SPT/AGT. The PH 1 patient presented had decreased SPT activity (~1% of the normal level) and his SPT/AGT gene had a homozygous point mutation in the coding region, as demonstrated previously (Nishiyama et al., 1991). In an attempt to purify the mutant SPT/AGT from the patient's liver by the same procedures as those described previously for normal human SPT/AGT (Nishiyama et al., 1990), protein(s) with the low SPT activity behaved in both phosphocellulose and hydroxylapatite column chromatographies quite differently from normal SPT/AGT. In addition, no band with a molecular mass of 43 kD was detected in fractions with the SPT activity on SDS/PAGE analysis (data not shown). Since some other aminotransferases are known to exhibit weak SPT activity, it is highly possible that the low SPT activity detected in the patient's liver is not entirely that of the mutant SPT/AGT. Western blot analysis performed in this study demonstrated that not only the activity (<1% of normal) (Nishiyama et al., 1991) but also the immunoreactive protein (~2.8% of normal) was severely decreased (c.f. Fig. 3). RNA blot analysis and in vitro translation of mRNA from the patient's liver indicated that the enzyme deficiency is due to neither a defect in transcription of the SPT/AGT gene nor one in the translation of the message. Estimation of the decay rate of SPT/AGT in COS-1 cells and *E. coli* JM105 revealed that the patient's SPT/AGT was unstable in the cells and rapidly degraded as compared with normal SPT/AGT. In COS-1 cells, the half-life of the patient's SPT/AGT was about 6 times shorter than that of normal SPT/AGT (c.f. Fig. 6 *b*). The patient's SPT/AGT was also degraded rapidly in vitro in rabbit reticulocyte extract in an ATP-dependent manner. From these results, we suspect that the enzyme defect in this patient is accounted for, at least in part, by the instability and intracellular rapid degradation of the mutant SPT/AGT. This is likely to be due to a point mutation in the patient's SPT/AGT gene leading to a substitution of Ser to Pro at residue 205 of SPT/AGT.

Nonlysosomal, energy- and ubiquitin-dependent processes are supposed to be involved in selective removal of unnecessary proteins with a rapid turnover, such as cyclin (Glotzer et al., 1991) and c-Mos (Nishizawa et al., 1992) which are closely related to cell cycle progression, and of abnormal proteins generated in cells (Rechsteiner, 1991). In these processes, ubiquitin serves as a signal for degradation of proteins, and a novel ATP-dependent protease complex with an apparent sedimentation coefficient of 26S (26S proteasome) appears to be responsible for recognition of ubiquitinated proteins and their subsequent ATP-dependent degradation (Matthews et al., 1989; Goldberg, 1992). In this study, the degradation of the mutant SPT/AGT in reticulocyte extract was clearly ATP dependent. However, no immunoreactive products with higher molecular masses, such as the conjugates with ubiquitin, were detectable during the course of its degradation in either the transfected COS cells or the in vitro reticulocyte lysate system, under the conditions used. Although ubiquitin-dependency of degradation of the mutant SPT/AGT in eukaryotic cells has to be further examined before conclusion, it is worthwhile to notice that ornithine decarboxylase was recently found to be degraded ATP and antizyme dependently by the 26S proteasome without ubiquitination (Murakami et al., 1992). The observation that the patient's SPT/AGT was also degraded in *E. coli* would be of great interest, if it means that the mutant enzyme is recognized as an abnormal protein to be degraded in the two

phylogenetically distant cells. However, since we have not yet studied in detail the degradation in *E. coli*, we would like to reserve any discussion on the bacterial degradation before elucidation of the underlying mechanism.

With respect to the degradation of the mutant SPT/AGT in vitro, no degradation occurred in reticulocyte lysate for translation, probably because, at least in part, the lysate contained 40 μM hemin. In reticulocyte lysate, hemin appears to promote protein synthesis by preventing the inactivation of a specific factor (eif-2) through the mediation of a heme-dependent kinase (Datta et al., 1978; Ranu et al., 1978; Tahara et al., 1978), but hemin has been also shown to inhibit the ATP-dependent degradation of abnormal and normal proteins. 50% inhibition of the degradation of [$^{14}CH_3$](apo)-hemoglobin, [^{125}I]BSA, and [^{3}H]casein has been achieved by 12, 25, and 40 μM hemin, respectively (Etlinger and Goldberg, 1980; Haas and Rose, 1981; Tanaka et al., 1983). In the present study, the hemin concentration in the reaction mixture for degradation of ^{35}S-labeled SPT/AGT was lowered to 0.8 μM by minimizing the carry-over from the in vitro translation mixture.

It is known that peroxisomal proteins are synthesized on free polysomes, released into the cytosol, and then transported into peroxisomes (Lazarow and Fujiki, 1985). On the other hand, functional proteolytic systems such as the ATP-dependent 26S proteasome have been found in the cytosol (Tanaka et al., 1992), but proteolytic systems in peroxisomes have not been clearly demonstrated. In the case of the PH 1 patient, immunocytochemically detected SPT/AGT antigenic sites were scarce on the whole but were concentrated predominantly in peroxisomes, suggesting that mutant SPT/AGT retained the capacity of targeting the proper organelle. However, it still remains to be clarified where the mutant enzyme is degraded rapidly, i.e., in the cytosol during the transport to peroxisomes or in the peroxisomes after transport. Preliminary results obtained to date demonstrated the mutant SPT/AGT degradation in the cytosol of transfected COS cells, although they have not yet excluded a possibility that the mutant protein is also degraded in peroxisomes. If the degradation occurs only or mainly in the cytosol, then it should be answered whether the mutant enzyme is degraded during the normal transport into peroxisomes or is degraded rapidly partly because the enzyme stays longer in the cytosol due to impaired or inefficient transport. Kinetic studies on transport into peroxisomes of the mutant SPT/AGT in comparison with the kinetics of its degradation will give us an answer, but for this purpose characterization of the proteolytic system(s) responsible for the degradation of the mutant SPT/AGT is indispensable as a prerequisite.

Another major question still remains to be answered is whether or not the mutant SPT/AGT has enzyme activity and what kind of conformational change is caused by the Ser to Pro substitution at residue 205. We have been especially anxious for the purification of the mutant enzyme, but since *E. coli* transformed with the recombinant plasmid, pKKPH did not accumulate the mutant SPT/AGT, we have not been successful at the purification. Continued trials to purify the mutant SPT/AGT are under way.

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References

Alexson, S. E. H., Y. Fujiki, H. Shio, and P. B. Lazarow. 1985. Partial disassembly of peroxisomes. *J. Cell Biol.* 101:294–304.

Alwine, J. C., D. J. Kemp, and G. R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. USA.* 74:5350–5354.

Bendayan, M., J. Roth, A. Perrelet, and L. Orci. 1980. Quantitative immunocytochemical localization of pancreatic secretory proteins in subcellular compartments of the rat acinar cell. *J. Histochem. Cytochem.* 28:149–160.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Danpure, C. J. 1991. Molecular and clinical heterogeneity in primary hyperoxaluria type 1. Am. *J. Kidney Dis.* 17:366–369.

Danpure, C. J., and P. R. Jennings. 1986. Peroxisomal alanine:glyoxylate aminotransferase deficiency in primary hyperoxaluria type I. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 201:20–24.

Danpure, C. J., and P. R. Jennings. 1988. Further studies on the activity and subcellular distribution of alanine:glyoxylate aminotransferase in the livers of patients with primary hyperoxaluria type 1. *Clin. Sci.* 75:315–322.

Danpure, C. J., P. J. Cooper, P. J. Wise, and P. R. Jennings. 1989. An enzyme trafficking defect in two patients with primary hyperoxaluria type 1: peroxisomal alanine/glyoxylate aminotransferase rerouted to mitochondria. *J. Cell Biol.* 108:1345–1352.

Danpure, C. J., K. M. Guttridge, P. Fryer, P. R. Jennings, J. Allsop, and P. E. Purdue. 1990. Subcellular distribution of hepatic alanine:glyoxylate aminotransferase in various mammalian species. *J. Cell Sci.* 97:669–678.

Datta, A., C. deHaro, and S. Ochoa. 1978. Translational control by hemin is due to binding to cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 75:1148–1152.

Dunbar, B. S., and E. D. Schwoebel. 1990. Preparation of polyclonal antibodies. *In* Methods in Enzymology: Guide to Protein Purification. M. P. Deutscher, editor. Academic Press, Inc., San Diego, CA. 663–670.

Etlinger, J. D., and A. L. Goldberg. 1980. Control of protein degradation in reticulocytes and reticulocyte extracts by hemin. *J. Biol. Chem.* 255:4563–4568.

Fagan, J. M., L. Waxman, and A. L. Goldberg. 1986. Red blood cells contain a pathway for the degradation of oxidant-damaged hemoglobin that does not require ATP or ubiquitin. *J. Biol. Chem.* 261:5705–5713.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

Glotzer, M., A. W. Murray, and M. W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature (Lond.).* 349:132–138.

Goldberg, A. L. 1992. The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. *Eur. J. Biochem.* 203:9–23.

Haas, A. L., and L. A. Rose. 1981. Hemin inhibits ATP-dependent ubiquitin-dependent proteolysis: role of hemin in regulating ubiquitin-conjugate degradation. *Proc. Natl. Acad. Sci. USA.* 78:6845–6848.

Hammond, J. B. W., and N. J. Kruger. 1988. The Bradford method for protein quantitation. *In* Methods in Molecular Biology: New Protein Techniques. J. M. Walker, editor. Humana Press, Clifton, NJ. 25–32.

Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. *J. Mol. Biol.* 166:557–580.

Kruger, N. J., and J. B. W. Hammond. 1988. Immunodetection of proteins on "Western" blots using ^{125}I-labeled protein A. *In* Methods in Molecular Biology: New Protein Techniques. J. M. Walker, editor. Humana Press, Clifton, NJ. 409–417.

Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.

LeHir, M., V. Herzog, and H. D. Fahimi. 1979. Cytochemical detection of catalase with 3,3'-diaminobenzidine: a quantitative reinvestigation of the optimal conditions. *Histochemistry.* 64:51–66.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 545 pp.

Matthews, M., K. Tanaka, J. Driscoll, A. Ichihara, and A. L. Goldberg. 1989. Involvement of the proteasome in various degradative processes in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 86:2597–2601.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active mRNA and

RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.

Mori, M., S. Miura, M. Tatibana, and P. P. Cohen. 1979. Cell-free synthesis and processing of a putative precursor for mitochondrial carbamyl phosphate synthetase I of rat liver. *Proc. Natl. Acad. Sci. USA.* 76:5071–5075.

Mori, M., T. Oda, K. Nishiyama, T. Serikawa, J. Yamada, and A. Ichiyama. 1992. A single serine:pyruvate aminotransferase gene on rat chromosome 9q34-q36. *Genomics.* 13:686–689.

Murakami, T., S. Matsufuji, T. Kameji, S. Hayashi, K. Igarashi, T. Tamura, K. Tanaka, and A. Ichihara. 1992. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature (Lond.).* 360:597–599.

Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunaga. 1985. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA.* 82:6133–6137.

Nishiyama, K., G. Berstein, T. Oda, and A. Ichiyama. 1990. Cloning and nucleotide sequence of cDNA encoding human liver serine-pyruvate aminotransferase. *Eur. J. Biochem.* 194:9–18.

Nishiyama, K., T. Funai, R. Katafuchi, F. Hattori, K. Onoyama, and A. Ichiyama. 1991. Primary hyperoxaluria type I due to a point mutation of T to C in the coding region of the serine:pyruvate aminotransferase gene. *Biochem. Biophys. Res. Commun.* 176:1093–1099.

Nishizawa, M., K. Ozaki, N. Furuno, N. Watanabe, and N. Sagata. 1992. The 'second-codon rule' and autophosphorylation govern the stability and activity of Mos during the meiotic cell cycle in *Xenopus* oocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2433–2446.

Noguchi, T., and Y. Takada. 1978. Peroxisomal localization of serine:pyruvate aminotransferase in human liver. *J. Biol. Chem.* 253:7598–7600.

Noguchi, T., Y. Minatogawa, Y. Takada, E. Okuno, and R. Kido. 1978. Subcellular distribution of pyruvate (glyoxylate) aminotransferase in rat liver. *Biochem. J.* 170:173–175.

Oda, T., A. Ichiyama, S. Miura, M. Mori, and M. Tatibana. 1981. *In vitro* synthesis of a putative precursor of serine:pyruvate aminotransferase of rat liver mitochondria. *Biochem. Biophys. Res. Commun.* 102:568–573.

Oda, T., M. Yanagisawa, and A. Ichiyama. 1982. Induction of serine:pyruvate aminotransferase in rat liver organelles by glucagon and a high-protein diet. *J. Biochem.* 91:219–232.

Oda, T., T. Funai, and A. Ichiyama. 1990. Generation from a single gene of two mRNAs that encode the mitochondrial and peroxisomal serine:pyruvate aminotransferase of rat liver. *J. Biol. Chem.* 265:7513–7519.

Pelham H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.

Purdue, P. E., M. J. Lumb, J. Allsop, Y. Minatogawa, and C. J. Danpure. 1992. A glycine-to-glutamate substitution abolishes alanine-glyoxylate aminotransferase catalytic activity in a subset of patients with primary hyperoxaluria type-1. *Genomics.* 13:215–218.

Ranu, R. S., I. M. London, A. Das, A. Dasgupta, A. Majumdar, R. Ralston, R. Roy, and N. K. Gupta. 1978. Regulation of protein synthesis in rabbit reticulocyte lysate by the heme-regulated protein kinase: inhibition of interaction of Met-tRNA$_f^{Met}$ binding factor with another initiation factor in formation of Met-tRNA$_f^{Met}$-40S ribosomal subunit complexes. *Proc. Natl. Acad. Sci. USA.* 75:745–749.

Rechsteiner, M. 1991. Natural substrates of the ubiquitin paroteolytic pathway. *Cell.* 66:615–618.

Roth, J. 1982. The protein A-gold (pAg) technique: a qualitative and quantitative approach for antigen localization on thin sections. *In* Techniques in Immuncytochemistry. G. R. Bullock, and P. Petrusz, editors. Academic Press, New York. 108–133.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *In* Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1.1–I.47.

Tahara, S. M., J. A. Traugh, S. B. Sharp, T. S. Lundak, B. Safer, and W. C. Merrick. 1978. Effect of hemin on site-specific phosphorylation of eukaryotic initiation factor 2. *Proc. Natl. Acad. Sci. USA.* 75:789–793.

Takada, Y., and T. Noguchi. 1982. Subcellular distribution, and physical and immunological properties of hepatic alanine:glyoxylate aminotransferase isoenzymes in different mammalian species. *Comp. Biochem. Physiol.* 72B:597–604.

Takada, Y., N. Kaneko, H. Esumi, P. E. Purdue, and C. J. Danpure. 1990. Human peroxisomal L-alanine:glyoxylate aminotransferase: evolutional loss of a mitochondrial targeting signal by point mutation of the initiation codon. *Biochem. J.* 268:517–520.

Tanaka, K., L. Waxman, and A. L. Goldberg. 1983. ATP serves two distinct roles in protein degradation in reticulocytes: one requiring and one independent of ubiquitin. *J. Cell Biol.* 96:1580–1585.

Tanaka, K., T. Tamura, T. Yoshimura, and A. Ichihara. 1992. Proteasomes: protein and gene structures. *New Biol.* 4:173–187.

Wise, P. J., C. J. Danpure, and P. R. Jennings. 1987. Immunological heterogeneity of hepatic alanine:glyoxylate aminotransferase in primary hyperoxaluria type 1. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 222:17–20.

Yokota, S. 1986. Quantitative immunocytochemical studies on differential induction of serine:pyruvate aminotransferase in mitochondria and peroxisomes of rat liver cells by administration of glucagon or di-(2-ethylhexyl)-phthalate. *Histochemistry.* 85:145–155.

Yokota, S. 1990. Cytochemical and immunocytochemical study on the peroxisomes of rat liver after administration of a hypolipidemic drug, MLM-160. *Eur. J. Cell Biol.* 53:112–121.

Yokota, S., T. Funai, and A. Ichiyama. 1991. Organelle localization of rat liver serine:pyruvate aminotransferase expressed in transfected COS-1 cells. *Biomed. Res.* 12:53–59.