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Mitochondrial Targeting Signal-Induced Conformational Change and Repression of the Peroxisomal Targeting Signal of the Precursor for Rat Liver Serine: Pyruvate/Alanine: Glyoxylate Aminotransferase

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In the rat liver, two mRNAs for serine:pyruvate (or alanine:glyoxylate) aminotransferase are generated from a single gene by alternative transcription initiation. The longer mRNA encodes a precursor of a mitochondrial enzyme that has a mitochondrial targeting signal at the N-terminus and is translocated into mitochondria. The shorter mRNA encodes a peroxisomal enzyme of mature size that is imported into peroxisomes. We have been interested in the mechanism of selective targeting to mitochondria of the precursor protein that also contains a peroxisomal targeting signal in the molecule. In this study, we examined the effect of the mitochondrial targeting signal on the conformation of the protein and on the function of the peroxisomal targeting signal in the precursor molecule. The results suggest that the mitochondrial targeting signal causes the conformation of the protein to become unfolded and that this conformational change in turn causes repression of the putative peroxisomal targeting signal contained in the precursor protein.

Key words: alternative translocation, conformational change, mitochondrial targeting signal, peroxisomal targeting signal, serine:pyruvate aminotransferase.

In the rat liver, kinetically (*1*) and immunologically (*2*, *3*) indistinguishable serine:pyruvate aminotransferases (SPTs; otherwise known as alanine:glyoxylate aminotransferases, AGTs) are located in two different subcellular organelles, mitochondria, and peroxisomes. Our previous studies showed that two mRNAs encoding the mitochondrial and peroxisomal SPTs are generated from a single SPT gene by transcription from two initiation sites in the first exon (*4*, *5*). The mitochondrial enzyme is synthesized from the longer mRNA, whose transcription is markedly enhanced by administration of glucagon to rats, as a precursor protein containing a mitochondrial targeting signal (MTS) of an N-terminal extension peptide of 22 amino acids (*6*, *7*). The mitochondrial precursor protein synthesized by the longer mRNA is translocated into the mitochondrial matrix *in vivo* and *in vitro*, followed by proteolytic removal of the MTS (*8*). In contrast, the peroxisomal enzyme is synthesized as a product of mature size from the shorter mRNA, which is constitutively transcribed or slightly induced by some stimuli (*9*), and the product is translocated into peroxisomes, being directed by an intramolecular peroxisomal targeting signal (PTS). The structural difference between the mitochondrial precursor SPT (pSPTm) and the peroxisomal SPT (SPTp) is that 22 N-terminal amino acids are included in the former but not in the latter. The precursor of the mitochondrial enzyme, therefore, contains the PTS in addition to the MTS in the molecule. Nevertheless, the precursor protein appears to be exclusively translocated into mitochondria, as judged from the selective induction of mitochondrial SPT on administration of glucagon to rats (*2*). This suggests the existence of some special mechanism that allows selective targeting for mitochondria of the precursor protein having two targeting signals in the molecule.

In this study, we examined the relationship between the conformation of pSPTm and the expression of the PTS function in this precursor protein. Expression in *Escherichia coli* of mutated precursor proteins having mitochondrial targeting sequences of different lengths indicated that the longer the targeting signal is, the higher the sensitivity to protease is. The existence of the mitochondrial targeting sequence seems to cause the precursor protein to take on an unfolded or loosely folded conformation that is easily susceptible to proteolysis. The results of an *in vitro* peroxisomal import experiment indicated that the peroxisomal targeting function is repressed by the presence of the MTS at the N-terminus.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Bacterial expression clones encoding the mutant precursor SPTs with N-terminal mitochondrial targeting sequences of different lengths (pRspt-

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[2] The 1998/1999 catalogue of New England Biolabs Inc. notes that the ER1458 strain is unlikely to be Lon-defective, although the genotype of this strain is described as *lon-* in the same catalogue.

Abbreviations: MTS, mitochondrial targeting signal; pSPTm, precursor of mitochondrial serine:pyruvate aminotransferase; PTS, peroxisomal targeting signal; SPT, serine:pyruvate aminotransferase; SPTm, mitochondrial serine:pyruvate aminotransferase; SPTp, peroxisomal serine:pyruvate aminotransferase.

1AA, 3AA, and 11AA) were constructed as follows. For construction of pRspt3AA, pRspt321 (*10*) containing the nucleotide sequence of full-length pSPTm was digested with *Bsp*EI and *Sca*I, and the resulting 1.5-kb fragment was purified. This fragment was ligated with a pUC8 vector that had been digested with *Hin*dIII, blunt-ended with a Klenow fragment of DNA polymerase I, and digested again with *Psp*AI. The obtained clone, named pRspt3AA, encoded a chimeric protein consisting of 7 N-terminal amino acids of *E. coli* β-galactosidase connected to a precursor of SPT lacking 2 N-terminal amino acids of MTS. For construction of pRspt11AA, pRspt321 was digested with *Xcm*I and *Sca*I, and blunt-ended with a Klenow fragment. The purified 1.5-kb fragment was ligated with a pUC8 vector that had been digested with *Hin*dIII, blunt-ended with a Klenow fragment, and digested again with *Psp*AI. The resulting clone, pRspt11AA, encoded a chimeric protein consisting of 8 N-terminal amino acids of *E. coli* β-galactosidase connected to a precursor of SPT lacking 10 N-terminal amino acids of MTS. For construction of pRspt1AA, the blunt-ended 1.5-kb *Bsp*EI-*Sca*I fragment of pRspt321 was ligated with the *Eco*RI-digested and blunt-ended pUC8 vector. The resulting clone was named pRsptEBS. PCR was carried out using pUC8 plasmid as the template, an upstream primer of 26 nucleotides (5′-TGAGCGTGGGTCTCGCGGTATCATTG-3′) containing the *Bsa*I site of the pUC8 vector, and a downstream primer of 28 nucleotides (5′-GGCCAACATCCGGAACATAGCTGTTTCC-3′) that consisted of 13 nucleotides of a region around the first methionine of β-galactosidase and 15 nucleotides corresponding to the second to sixth amino acids of pSPTm, with an endogenous *Bsp*EI recognition site. The PCR product of the 1.3-kb fragment was purified, digested with *Bsa*I and *Bsp*EI, then ligated with a 2.8-kb fragment of *Bsa*I–*Bsp*EI digest of pRsptEBS. The resultant clone, named pRspt1AA, contained full-length pSPTm under the lactose operon promoter with no extra amino acids at the N-terminus.

pARS45K and pARS43K, clones for *in vitro* transcription of mRNAs for pSPTm and the mature form of SPT, respectively (*11*), were used to prepare the *in vitro* translated products for the *in vitro* peroxisomal import experiment. An *in vitro* transcription clone encoding a chimeric product of pSPTm and urate oxidase was constructed by Dr. Tsuneyoshi Funai as follows. pARS45K was digested with *Apa*I and *Bam*HI, and the resultant 4-kb fragment was isolated. This fragment includes the entire N-terminal MTS of pSPTm and Met1 to Gly352 of mature SPT in addition to a pAM19 vector (Amersham). pARURCD21 (*12*), an *in vitro* transcription clone for rat liver urate oxidase, was also digested with *Apa*I and *Bam*HI, and the resultant 0.5-kb fragment, including a C-terminal region from Pro224 to Leu303 of urate oxidase, was isolated. The 0.5-kb fragment of pARURCD21 was ligated with the 4-kb fragment of pARS45K to generate a clone encoding a chimeric protein containing the MTS of pSPTm and the PTS1 of urate oxidase at the N- and C-termini, respectively. This clone was named pA45K/UO. The clone encoding a chimeric protein consisting of mature SPT and urate oxidase, pA43K/UO, was similarly constructed by the use of pARS43K as the starting plasmid instead of pARS45K.

Protease Digestion of Purified Mitochondrial and Peroxisomal SPTs—Mitochondrial SPT was purified from the livers of glucagon-treated rats as reported previously (*10*). Peroxisomal SPT was purified from isolated rat liver peroxisomes. Briefly, female Wistar rats were fed on a chow containing 2% di-(2-ethylhexyl)phthalate for 14 days as described by Shindo *et al.* (*13*). The livers were excised, and subcellular fractionation was performed as described previously (*2*). A peroxisomal fraction minimally contaminated with mitochondria was prepared by centrifugation of a light mitochondrial fraction at 100,000 ×*g* for 2 h on a 36–57% (w/w) sucrose linear gradient using a RPZ 48T zonal rotor (Hitachi, Tokyo). Subsequent purification procedures of SPTp were the same as those described in a previous report (*10*). Digestion with proteinase K and *Staphylococcus aureus* V8 protease was performed as described previously (*14*).

Expression of MTS-Deleted SPTs in E. coli—*E. coli* DH5 and *E. coli* ER1458, a Lon protease- or some other protease-deficient strain[2] (New England Biolabs, USA), were transformed with pRspt1AA, an expression clone for full-length pSPTm, or the clones encoding MTS-deleted SPTm precursors (pRspt3AA, 11AA, and 22AA). Each transformed bacterium was cultured overnight at 37°C in 10 ml of Luria-Bertani (LB) medium containing 15 mM glucose, in which expression of the encoded protein was repressed by glucose repression of the lactose operon promoter of pUC8. The cultured bacterial suspension was then diluted with 4 volumes of LB medium to decrease the glucose concentration to 3 mM for derepression of the protein expression and was cultured for various periods of time up to 48 h. The SPT activity in the bacterial extract was determined, and expressed products were analyzed by SDS-PAGE and protein staining. A preliminary experiment suggested that SPT is more difficult to transfer by electroblotting than other proteins, probably due to its physical properties as a basic protein of pI 8.0. When we used the gel electrotransferred after SDS-PAGE for the protein staining, SPTs were clearly detected because, although most proteins had been removed, a considerable amount of SPTs still remained in the gel. Electrotransfer of proteins from the gel was carried out at 12 V for 40 min according to the protocol of Millipore (USA).

Analytical Procedures—The SPT activity was determined spectrophotometrically as described previously (*2*). SDS-PAGE was carried out according to Laemmli (*15*). Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce Chemical, USA) with bovine serum albumin as a standard.

RESULTS

Mitochondrial Targeting Signal-Induced Conformational Change of the SPT Molecule—When the *in vitro* transcript from pARS45K encoding the full length of pSPTm was translated *in vitro* with a rabbit reticulocyte lysate, a significant amount of an immunologically SPT-related protein of 43 kDa was synthesized in addition to the expected pSPTm of 45 kDa encoded in the pARS45K clone (Fig. 1, left, lane 1). This product of mature size appeared to be SPTp translated from the third methionine codon of pSPTm mRNA as the initiation methionine due to leaky scanning by ribosomes through the first and second methionine codons (*11*). Indeed, this product was shown to be resistant to proteinase K digestion (Fig. 1, left, lanes 2 and 3), as was SPTp (Fig. 1, right, lanes 2 and 3). Proteinase K

digestion of *in vitro* translated products from pARS45K and pARS43K transcripts was carried out to compare the protease sensitivities of pSPTm and SPTp. As shown in Fig. 1 (upper panels), pSPTm was completely digested with 20 μg/ml proteinase K, whereas most of SPTp was resistant to the protease irrespective of the presence or absence of Triton X-100. A small but significant amount of SPTp was still detected after digestion with 100 μg/ml of proteinase K (Fig. 1, lower panels). When 300 μg/ml of proteinase K was used, SPTp as well as pSPTm were completely digested (data not shown). These results together with previous ones (*11*) suggest that pSPTm is protease-sensitive, probably due to an unfolded or loosely folded conformation, whereas SPTp is protease-resistant and has a folded conformation.

In our previous experiment, the N-terminal amino acid sequence of SPTp could not be determined by Edman degradation, whereas the sequence of SPTm was successfully determined, indicating some modification of the N-terminal amino acid of SPTp purified from peroxisomes. Since it is possible that N-terminal amino acid of *in vitro* translated SPTp was modified immediately after the synthesis and that this modification made SPTp more resistant to protease in the experiment for which the results are shown in

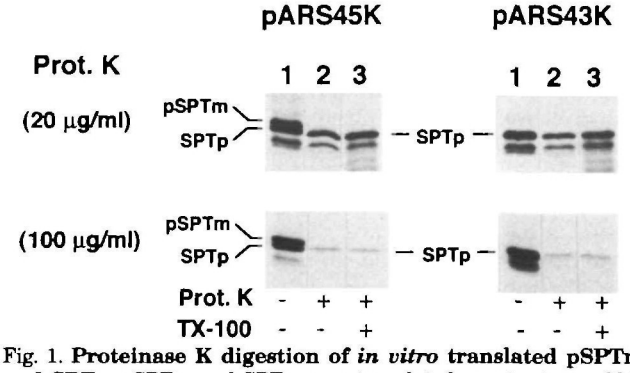

Fig. 1. **Proteinase K digestion of *in vitro* translated pSPTm and SPTp.** pSPTm and SPTp were translated *in vitro* in a rabbit reticulocyte lysate system with *in vitro* transcripts from pARS45K and pARS43K as the templates, respectively, as described previously (*12*). Proteinase K digestion (20 μg/ml or 100 μg/ml for 10 min at 0°C) in the presence or absence of 1% Triton X-100, SDS-PAGE and fluorography were carried out as described previously (*12*). Prot. K, proteinase K; TX-100, Triton X-100.

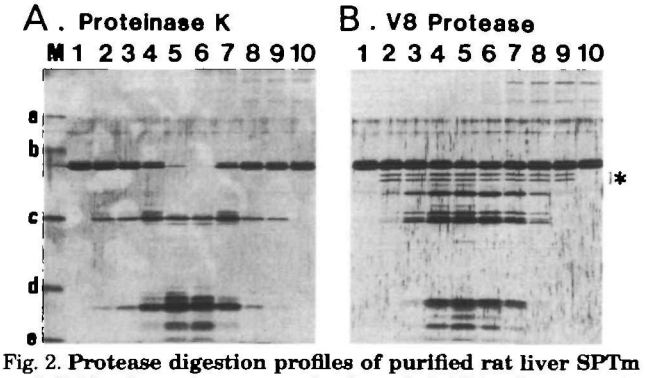

Fig. 2. **Protease digestion profiles of purified rat liver SPTm and SPTp.** One microgram each of purified SPTm (lanes 1–5 in panels A and B) or SPTp (lanes 6–10 in panels A and B) was incubated at 30°C with 2 ng of proteinase K (panel A) or 40 ng of V8 protease (panel B), and the products were detected by SDS-PAGE and silver staining of the gel as described previously (*14*). In lane M, the following proteins were subjected to electrophoresis as size markers: a, bovine serum albumin (68 kDa); b, ovalbumin (45 kDa); c, chymotrypsinogen A (25 kDa); d, ribonuclease A (13.7 kDa) and e, aprotinin (6.5 kDa). The proteases were omitted in lanes 1 and 10 of panels A and B. A: Proteinase K digestion was carried out for 0 min (lanes 1 and 10), 0.25 min (lanes 2 and 9), 0.5 min (lanes 3 and 8), 3 min (lanes 4 and 7), or 10 min (lanes 5 and 6). B: V8 protease digestion was carried out for 0 min (lanes 1 and 10), 2 min (lanes 2 and 9), 15 min (lanes 3 and 8), 60 min (lanes 4 and 7), or 180 min (lanes 5 and 6). Two protein bands marked with an asterisk in panel B are derived from the V8 protease preparation used.

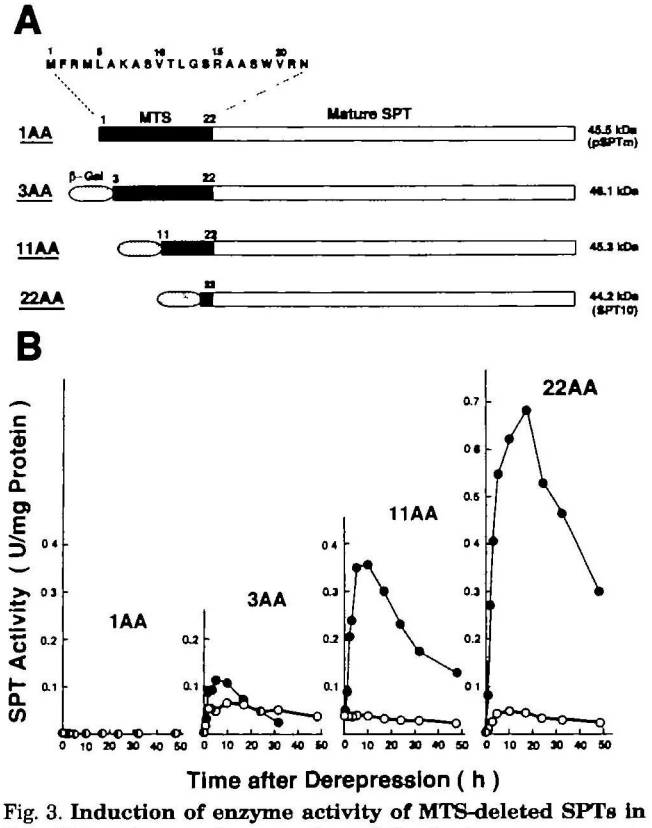

Fig. 3. **Induction of enzyme activity of MTS-deleted SPTs in *E. coli* by glucose derepression of the lactose operon promoter.** Construction of SPT clones with different lengths of MTS, expression of mutated SPTs in *E. coli*, and assay of SPT activity were carried out as described in "EXPERIMENTAL PROCEDURES." A: Construct of MTS-deleted clones. pRspt1AA (1AA) encodes the full length of pSPTm under the lactose operon promoter of *E. coli* with no extra sequence at the N-terminus. pRspt3AA (3AA) encodes a fused protein consisting of 7 N-terminal amino acids of *E. coli* β-galactosidase, 20 amino acids (Arg3 to Asn22) of MTS and mature SPT. pRspt11AA (11AA) encodes a fused protein consisting of 8 N-terminal amino acids of β-galactosidase, 12 amino acids (Thr11 to Asn22) of MTS and mature SPT. pRspt22AA (22AA) encodes a fusion protein (SPT10) consisting of 7 N-terminal amino acids of β-galactosidase, two artificial amino acids (Gly and Trp) inserted (closed box), Asn22 of MTS, and mature SPT (*14*). The hatched box, open box, and shaded ellipse represent mitochondrial targeting signal (MTS), mature SPT of 392 amino acids, and several N-terminal amino acids of *E. coli* β-galactosidase, respectively. B: Induction profile of enzyme activity of MTS-deleted SPTs expressed in *E. coli*. *E. coli* DH5 (open circles) and *E. coli* ER1458 (closed circles) transformed with pRspt1AA, 3AA, 11AA, or 22AA were cultured for various times after derepression of the lactose operon promoter.

Fig. 1, we compared the protease sensitivities of SPTm and N-terminally modified SPTp. Figure 2 shows the time-dependent digestion profile by proteinase K (Fig. 2A) or *S. aureus* V8 protease (Fig. 2B) of purified SPTm and SPTp. No differences in the digestion pattern were observed between SPTm and SPTp with either protease. Thus, the conformations of the mature forms of SPTm and SPTp appear to be indistinguishable by protease digestion, irrespective of the N-terminal amino acid modification of SPT. Therefore, the different protease sensitivities of pSPTm and SPTp shown in Fig. 1 seem to be due to the presence of MTS at the N-terminus of the pSPTm molecule.

We tried to examine the effect of MTS on the conformation of the SPT molecule in an *E. coli* expression system. In our previous study (*14*), SPT10, a recombinant chimeric protein consisting of several N-terminal amino acids of β-galactosidase and mature SPT, was found to have almost the same enzymic properties and functional dimer structure as those of native SPT in the rat liver. As several N-terminal amino acids of β-galactosidase were thus shown to have no inhibitory effect on the activity and the native dimer formation of SPT, we constructed mutated SPTs having different lengths of N-terminal MTS just behind the N-terminal amino acids of β-galactosidase under the lactose operon promoter in a pUC8 vector (Fig. 3A). pRspt1AA contains the full length of pSPTm with no extra amino acids at the N-terminus. pRspt3AA contains a chimeric protein consisting of 7 N-terminal amino acids of β-galactosidase followed by pSPTm lacking 2 N-terminal amino acids. pRspt11AA encodes a chimeric protein that consists of 8 N-terminal amino acids of β-galactosidase followed by pSPTm lacking 10 N-terminal amino acids. pRspt22AA (formerly named pRspt10) encodes a protein (SPT10) consisting of 7 N-terminal amino acids of β-galactosidase followed by 2 artificial amino acids, asparagine 22 of the MTS, and mature SPT (*14*). These four clones were introduced into *E. coli* DH5 or *E. coli* ER1458, a protease-lacking strain, to evaluate the stability of the products in bacteria. Since the lactose operon promoter in the pUC8 vector is repressed by 15 mM glucose in a medium, the expression of the products was induced by decreasing the glucose concentration to 3 mM. The SPT activities of each of the mutated SPTs expressed after induction are shown in Fig. 3B. The longer the MTS contained in the chimeric protein is, the smaller the activities detected in the ER1458 strain of *E. coli* are. Both DH5 and ER1458 strains transformed with pRspt1AA showed no SPT activity at any time after induction. In the DH5 strain, only small activities were detected in pRspt3AA, 11AA, and 22AA. To determine whether the difference in SPT activity among mutated SPTs is due to the amount of enzyme the cells retained or to different specific activities of mutated SPTs, we examined the protein level by direct protein staining, as shown in Fig. 4. Activity and protein analyses showed that the induction profiles of protein coincide with those of SPT activity in the four mutated

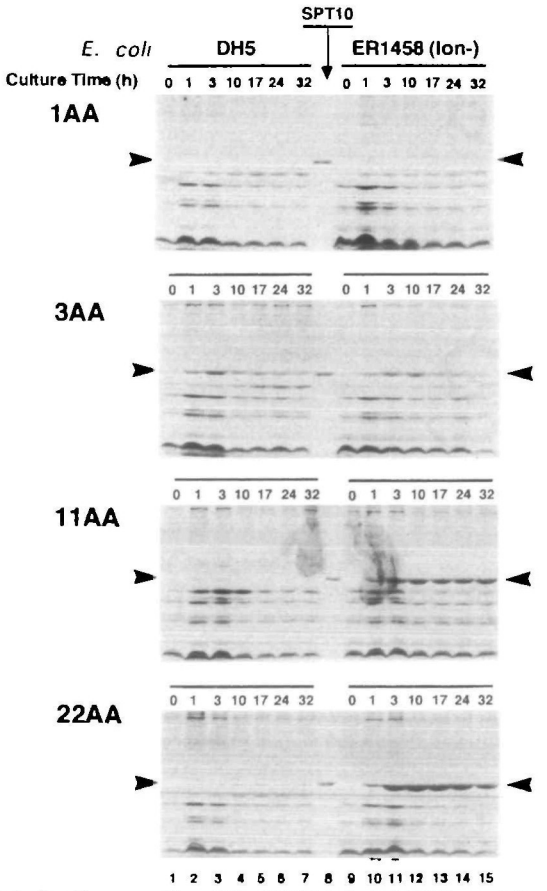

Fig. 4. **Induction profile of the MTS-deleted SPT proteins.** Experimental conditions are the same as those described in the legend to Fig. 3. *E. coli* DH5 (left) and *E. coli* ER1458 (right) transformed with pRspt1AA, 3AA, 11AA, or 22AA were collected at various times after derepression of the lactose operon promoter. The expressed products (20 μg of protein) were analyzed by SDS-PAGE, and proteins on the gel were stained with Coomassie Brilliant Blue R-250 after electroblotting. In the central lane, 1 μg of purified SPT10, the expressed product of pRspt22AA (formerly named pRspt10), was applied. Arrowheads indicate the positions of the expected size of the mutated SPTs.

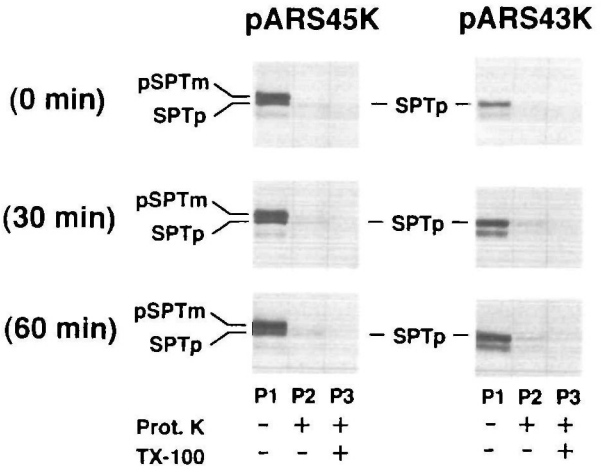

Fig. 5. ***In vitro* import of pSPTm and SPTp into purified peroxisomes.** After *in vitro* transcription of pARS45K or pARS43K with SP6 RNA polymerase and subsequent translation in a rabbit reticulocyte lysate, *in vitro* import reaction with purified peroxisomes was carried out for 0, 30, and 60 min as reported previously (*12*). P1, P2, and P3 represent translated products in the peroxisomal fraction recovered after the second incubation of the import reaction mixture for 10 min at 0°C without additions, with 300 μg/ml proteinase K (Prot. K), and with 300 μg/ml proteinase K and 1% Triton X-100 (TX-100), respectively.

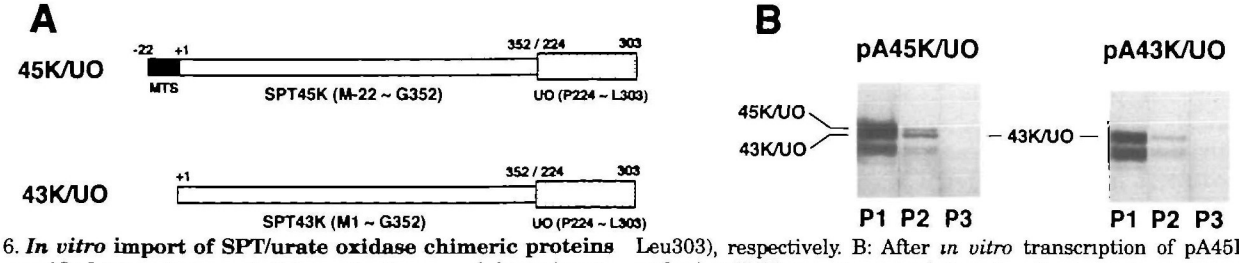

Fig. 6. ***In vitro* import of SPT/urate oxidase chimeric proteins into purified peroxisomes.** A: Schematic diagram of chimeric proteins 45K/UO and 43K/UO. The N-terminal methionine of urate oxidase and that of a mature SPTm are numbered +1. The hatched box, open box, and dotted box represent the MTS of pSPTm (Met-22 to Asn-1), mature SPT from which a C-terminal region was deleted (Met1 to Gly352), and a C-terminal region of urate oxidase (Pro224 to Leu303), respectively. B: After *in vitro* transcription of pA45K/UO and pA43K/UO, *in vitro* translation, *in vitro* import reaction of the translated product into peroxisomes for 1 h at 26°C, and subsequent operations were carried out as described in the legend to Fig. 5. In the left panel, 43K/UO shows a chimeric product translated from the third methionine codon (Met1) of mature SPT. Other symbols are the same as in Fig. 5.

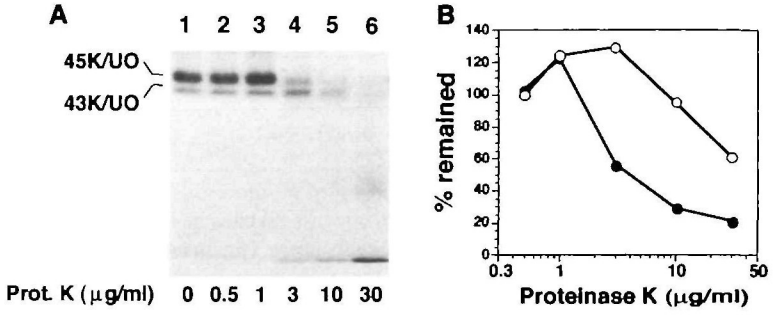

Fig. 7. **Proteinase K digestion of SPT/urate oxidase chimeric proteins.** *In vitro* coupled transcription/translation was carried out at 30°C for 2 h using TNT Coupled Reticulocyte Lysate Systems (Promega Corp., USA) with pA45K/UO plasmid, Tran^{35}S-label (ICN Biomedicals Inc., USA) and SP6 RNA polymerase. A: The proteinase K digestion (0°C, 10 min) was performed at 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 3 (lane 4), 10 (lane 5), and 30 (lane 6) μg/ml of the protease and analyzed by SDS-PAGE and fluorography. B: The amounts of 45K/UO (closed circles) and 43K/UO (open circles) on the gel were separately quantitated by use of FUJIX BAS1000 (Fuji Photo Film, Tokyo), and the digestion profile was graphed.

SPTs after induction, indicating that the specific activities of mutated SPTs are similar to those of native enzyme and SPT10, a bacterially expressed product of pRspt22AA with almost the same specific activity as the native SPT.

Repression of the Peroxisomal Targeting Signal by the Presence of the Mitochondrial Targeting Signal—In the rat liver, administration of glucagon to the animal leads to enhancement of the SPT gene transcription from an upstream initiation site (*4*), and the translated product (pSPTm) is specifically translocated into mitochondria, not into peroxisomes (*2*), then converted to the mature form through the cleavage of MTS (*8*). It is therefore thought that the peroxisomal targeting signal contained in pSPTm is repressed or masked in the cell. We tried to examine, using an *in vitro* peroxisomal import system, whether the PTS in pSPTm is indeed repressed. Figure 5 shows the results of the *in vitro* import of pSPTm (left panels) and SPTp (right panels) into purified peroxisomes. *In vitro* synthesized SPTp was shown to be translocated into peroxisomes as a protease-resistant form (right panels, lane 2) in a time-dependent manner. In lane P1 of the left panels, the intensity of the pSPTm band recovered with peroxisomes is significantly higher than that of the SPTp band. However, in lane 2, the intensity of the proteinase K–resistant band of SPTp is 2- to 3-fold greater than that of pSPTm at all incubation times. These results indicate that the function of PTS in SPTp is more effective than that in pSPTm. Thus, the presence of MTS at the N-terminus may repress the function of PTS in the pSPTm molecule.

To examine whether the repression of PTS by MTS is restricted to the original combination of the targeting signals in SPT, we constructed a clone, named pA45K/UO, that encodes a chimeric protein consisting of an N-terminal portion of pSPTm and a C-terminal portion of urate oxidase (UO), which contains a typical peroxisomal targeting signal, PTS1, at the C-terminus (Fig. 6A). The control plasmid (pA43K/UO) that encodes an SPTp/urate oxidase chimeric protein was also constructed. As shown in Fig. 6B (left panel), the *in vitro* transcription/translation of pA45K/UO also gave two products, named 45K/UO and 43K/UO, which correspond to the translation products from the first and third methionine codons, respectively, as observed in Figs. 1 and 5. The intensities of the protein bands of 45K/UO and 43K/UO in lane P1 were almost the same, but with respect to the proteinase K-resistant band in lane P2, the intensity of 43K/UO band was significantly greater than that of 45K/UO band. These results show that the peroxisomal targeting activity of PTS1 in 43K/UO is more effective than that in 45K/UO, indicating that the function of PTS1 is repressed by the presence of MTS in 45K/UO and that the repression of PTS by MTS is not restricted to the combination of the original PTS and MTS in the pSPTm molecule.

Figure 7A shows the results of proteinase K digestion of two chimeric proteins to examine the difference in their protease sensitivities. The 45K/UO band was markedly decreased by digestion with 3 μg/ml of proteinase K (lane 4), whereas the amount of 43K/UO was not affected by this concentration of protease. Quantification of each band was carried out, and the digestion profile is shown in Fig. 7B. This figure indicates that 45K/UO was 5–10 times more sensitive to proteinase K than was 43K/UO. The activity of MTS to induce the loose conformation of the protein was also observed in SPT/urate oxidase chimeric protein.

DISCUSSION

We showed in this study (Fig. 1) and our previous study (*11*) that the precursor form of mitochondrial SPT (pSPTm) is highly susceptible to exogenously added protease. When mutated SPTs having N-terminal MTS of various lengths were expressed in *E. coli*, both the SPT activity and protein level of the expressed products seemed to depend on the length of MTS contained in the mutated SPTs (Figs. 3 and 4). The induction profiles of SPT activity and the level of protein of mutated SPTs in protease-deficient bacteria coincided well at any time after induction, indicating that the specific activity of the mutated SPTs expressed is similar to that of the authentic enzyme and SPT10 (*14*), although their stabilities are quite different. In the case of pRspt-1AA, both the activity and protein were hardly detectable even just after the induction, suggesting very rapid degradation of the product after its synthesis. To explain these results, we propose that there are two interconvertible conformations of the SPT molecule. One is an unfolded and enzymatically inactive form induced by the presence of MTS or a part of it, and the other is a folded and enzymatically active form shown in the mature SPTm, SPTp, or SPT10, the product encoded by pRspt22AA. It is probable that in pSPTm the equilibrium leans more toward the unfolded form than the folded form, and the unfolded form may be digested easily by protease(s) in *E. coli*. The mature form of SPT (SPTp and SPTm), on the other hand, may lean toward the folded form. The shorter the MTS in the mutated SPT is, the more the equilibrium may shift to the folded form. This idea can explain the alteration of the content of mutated SPTs with no change in their specific activities in bacteria by alteration of the equilibrium between two conformations. Experimental evidence is needed to confirm this idea. Recently, intramolecular chaperone-like activity of the MTS of the F_1-ATPase β-subunit precursor has been reported (*16*), while it has also been reported that MTS plays only a minor role in determining the folding rate of aspartate aminotransferase (*17*).

In our previous studies (*2*, *6*, *8*, *18*), administration of glucagon to rats caused i) specific enhancement of transcription from the upstream initiation site generating pSPTm mRNA, ii) an increased translation of pSPTm in the cytoplasm, and iii) selective translocation of pSPTm into mitochondria. We have been interested in how pSPTm, which has PTS in addition to MTS in the molecule, is translocated selectively into mitochondria. Three possible mechanisms for the selective translocation of pSPTm into mitochondria have been proposed. One is cotranslational import. As the MTS and putative PTS of pSPTm are located at the N-terminus and the C-terminal region of pSPTm, respectively, the MTS translated first may be immediately recognized by the mitochondrial import machinery on the mitochondrial outer membrane before the PTS region is translated. Another possible mechanism is the difference in targeting abilities of the MTS and PTS in the pSPTm molecule. If the targeting ability of MTS is much higher than that of PTS, most of the translated product would be translocated into mitochondria. The third possible mechanism is intramolecular masking or repression of PTS in pSPTm, such as the direct or indirect interaction of MTS with PTS or allosteric inhibition of PTS function by MTS, and so on. In the former two possibilities, PTS has normal targeting activity in pSPTm, whereas in the third possibility, the targeting function of PTS is repressed. To examine whether the PTS function in the pSPTm molecule is repressed, we tried to analyze the *in vitro* import efficiency of *in vitro* translated pSPTm and SPTp into isolated peroxisomes under conditions in which mitochondria have no influence. As shown in Figs. 5 and 6, the PTS function of pSPTm and the 45K/UO chimeric protein was much less than that of mature SPT or the 43K/UO chimeric protein, suggesting that the third possibility is the most likely. The peroxisomal fraction used in the *in vitro* import experiment was highly purified and assumed to contain no significant amount of mitochondria (*19*). Indeed no significant processing of the precursor to the mature form indicative of the presence of mitochondria occurred during the import reaction. The amount of the mature forms (SPTp and 43K/UO) detected in the P2 fraction was almost the same whether the precursors (pSPTm and 45K/UO) or the mature forms (SPTp and 43K/UO) were used for the import reaction (Figs. 5 and 6).

Several studies have shown that there are intramolecular interactions of functional subdomains (*20–24*). It is thought that the interaction of subdomains makes the conformation of the protein more compact and probably more protease-resistant. However, since the presence of MTS in pSPTm leads to a conformation of the protein that is more sensitive to protease, physical interaction of MTS with PTS seems to be unlikely. We recently found that the proline insertion into the C-terminal region of SPTp to affect the secondary structure causes a marked reduction in the peroxisomal targeting activity (Mizuno, T., Ito, K., Ichiyama, A., and Oda, T., unpublished observation). Motly *et al.* (*25*) reported that a C-terminal region of human SPT/AGT of more than 47 amino acids is required for the proper peroxisomal targeting of this enzyme. The peroxisomal targeting signal of SPT thus seems to require the proper conformation around the C-terminal region for its targeting function. We speculate that MTS leads to the repression of PTS by the unfolding of pSPTm or the 45K/UO chimeric protein molecule, especially at the C-terminal region, where the putative peroxisomal targeting signal of SPT or PTS1 of urate oxidase exists (*12*). With respect to the translocation of proteins having both mitochondrial and peroxisomal targeting signals in the molecule, two different results have been reported. Like pSPTm, the selective import of the precursor into mitochondria has been reported in carnitine acetyltransferase (*26*) and alanine:glyoxylate aminotransferase (*27*). On the other hand, the precursors of dienoyl-CoA isomerase (*28*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*29*) are translocated into both mitochondria and peroxisomes, indicating no repression of PTS by the presence of MTS in these molecules. The effect of MTS on PTS function seems to differ depending on the case.

In our previous studies (*6*, *14*), we observed that SPT10 consisting of full-sized mature SPT showed the full enzymic activity and the soluble dimer structure in bacterial cells. However, the 15-N-terminal-amino-acid-deleted SPT, the product encoded in pRspt19 plasmid, showed neither enzymic activity nor the expected size of product in the cell. It is, therefore, probable that the presence or absence of MTS in the SPT molecule affects the stability of the conformation of the protein. In mammalian cells, two protein factors

recognizing MTS have been reported: mitochondrial import stimulating factor (*30*) and presequence binding factor (*31*). It is not yet clear whether these factors interact with MTS of pSPTm and contribute to proper organelle targeting and conformational change of the precursor.

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REFERENCES

1. Noguchi, T. and Takada, Y. (1978) Purification and properties of peroxisomal pyruvate (glyoxylate) aminotransferase from rat liver. *Biochem. J.* **175**, 765–768
2. Oda, T., Yanagisawa, M., and Ichiyama, A. (1982) Induction of serine:pyruvate aminotransferase in rat liver organelles by glucagon and a high-protein diet. *J. Biochem.* **91**, 219–232
3. Yokota, S. and Oda, T. (1984) Fine localization of serine:pyruvate aminotransferase in rat hepatocytes revealed by a post-embedding immunocytochemical technique. *Histochemistry* **80**, 591–595
4. Oda, T., Funai, T., and Ichiyama, A. (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
5. Mori, M., Oda, T., Nishiyama, K., Serikawa, T., Yamada, J., and Ichiyama, A. (1992) A single serine:pyruvate aminotransferase gene on rat chromosome 9q34-36. *Genomics* **13**, 686–689
6. Oda, T., Kitamura, N., Nakanishi, S., and Ichiyama, A. (1985) Cloning and expression in *Escherichia coli* of cDNA for serine: pyruvate aminotransferase of rat liver. *Eur. J. Biochem.* **150**, 415–421
7. Miyajima, H., Oda, T., and Ichiyama, A. (1989) Induction of mitochondrial serine:pyruvate aminotransferase of rat liver by glucagon and insulin through different mechanisms. *J. Biochem.* **105**, 500–504
8. Oda, T., Ichiyama, A., Miura, S., and Mori, M. (1984) Uptake and processing of serine:pyruvate aminotransferase precursor by rat liver mitochondria *in vitro* and *in vivo*. *J. Biochem.* **95**, 815–824
9. Oda, T., Funai, T., and Ichiyama, A. (1997) Induction by peroxisome proliferators and triiodothyronine of serine:pyruvate/alanine:glyoxylate aminotransferase of rat liver. *FEBS Lett.* **418**, 265–268
10. Oda, T., Miyajima, H., Suzuki, Y., and Ichiyama, A. (1987) Nucleotide sequence of the cDNA encoding the precursor for mitochondrial serine:pyruvate aminotransferase of rat liver. *Eur. J. Biochem.* **168**, 537–542
11. Funai, T. and Ichiyama, A. (1995) Fidelity of translation initiation of mRNA for the precursor of rat mitochondrial serine: pyruvate/alanine:glyoxylate aminotransferase. *J. Biochem.* **117**, 1008–1016
12. Miura, S., Oda, T., Funai, T., Ito, M., Okada, Y., and Ichiyama, A. (1994) Urate oxidase is imported into peroxisomes recognizing the C-terminal SKL motif of proteins. *Eur. J. Biochem.* **223**, 141–146
13. Shindo, Y., Osumi, T., and Hashimoto, T. (1978) Effects of administration of di-(2-ethylhexyl)phthalate on rat liver mitochondria. *Biochem. Pharmacol.* **27**, 2683–2688
14. Oda, T., Miyajima, H., Suzuki, Y., Ito, T., Yokota, S., Hoshino, M., and Ichiyama, A. (1989) Purification and characterization of the active serine:pyruvate aminotransferase of rat liver mitochondria expressed in *Escherichia coli*. *J. Biochem.* **106**, 460–467
15. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680–685
16. Hajek, P., Koh, J.Y., Jones, L., and Bedwell, D.M. (1997) The amino terminus of the F_1-ATPase β-subunit precursor functions as an intramolecular chaperone to facilitate mitochondrial protein import. *Mol. Cell. Biol.* **17**, 7169–7177
17. Mattingly, J.R., Jr., Iriarte, A., and Martinez-Carrion, M. (1993) Structural features which control folding of homologous proteins in cell-free translation systems. *J. Biol. Chem.* **268**, 26320–26327
18. Fukushima, M., Aihara, Y., and Ichiyama, A. (1978) Immunochemical studies on induction of rat liver mitochondrial serine:pyruvate aminotransferase by glucagon. *J. Biol. Chem.* **253**, 1187–1194
19. Miura, S., Kasuya-Arai, I., Mori, H., Miyazawa, S., Osumi, T., Hashimoto, T., and Fujiki, Y. (1992) Carboxyl-terminal consensus Ser-Lys-Leu-related tripeptide of peroxisomal proteins functions *in vitro* as a minimal peroxisome-targeting signal. *J. Biol. Chem.* **267**, 14405–14411
20. Kwiatkowski, D.J., Janmey, P.A., and Yin, H.L. (1989) Identification of critical functional and regulatory domains in gelsolin. *J. Cell Biol.* **108**, 1717–1726
21. Way, M., Gooch, J., Pope, B., and Weeds, A.G. (1989) Expression of human plasma gelsolin in *Escherichia coli* and dissection of actin binding sites by segmental deletion mutagenesis. *J. Cell Biol.* **109**, 593–605
22. Enyedi, A., Vorherr, T., James, P., McCormick, D.J., Filoteo, A.G., Carafoli, E., and Penniston, J.T. (1989) The calmodulin binding domain of the plasma membrane Ca^{2+} pump interacts both with calmodulin and with another part of the pump. *J. Biol. Chem.* **264**, 12313–12321
23. Soderling, T.R. (1990) Protein kinases. Regulation by autoinhibitory domains. *J. Biol. Chem.* **265**, 1823–1826
24. Johnson, R.P. and Craig, S.W. (1994) An intramolecular association between the head and tail domains of vinculin modulates talin binding. *J. Biol. Chem.* **269**, 12611–12619
25. Motly, A., Lumb, M.J., Oatey, P.B., Jennings, P.R., De Zoysa, P.A., Wanders, R.J.A., Tabak, H.F., and Danpure, C.J. (1995) Mammalian alanine/glyoxylate aminotransferase 1 is imported into peroxisomes *via* the PTS1 translocation pathway. Increased degeneracy and context specificity of the mammalian PTS1 motif and implications for the peroxisome-to-mitochondrion mistargeting of AGT in primary hyperoxaluria type 1. *J. Cell Biol.* **131**, 95–109
26. Elgersma, Y., van Roermund, C.W.T., Wanders, R.J.A., and Tabak, H.F. (1995) Peroxisomal and mitochondrial carnitine acetyltransferase of *Saccharomyces cerevisiae* are encoded by a single gene. *EMBO J.* **14**, 3472–3479
27. Oatey, P.B., Lumb, M.J., and Danpure, C.J. (1996) Molecular basis of the variable mitochondrial and peroxisomal localisation of alanine-glyoxylate aminotransferase. *Eur. J. Biochem.* **241**, 374–385
28. Filppula, S.A., Yagi, A.I., Kilpelainen, S.H., Novikov, D., Fitz-Patrick, D.R., Vihinen, M., Valle, D., and Hiltunen, J.K. (1998) $\Delta^{3,5}$-$\Delta^{2,4}$-Dienoyl-CoA isomerase from rat liver. Molecular characterization. *J. Biol. Chem.* **273**, 349–355
29. Ashmarina, L.I., Rusnak, N., Miziorko, H.M., and Mitchell, G.A. (1994) 3-Hydroxy-3-methylglutaryl-CoA lyase is present in mouse and human liver peroxisomes. *J. Biol. Chem.* **269**, 31929–31932
30. Mihara, K. and Omura, T. (1996) Cytoplasmic chaperones in precursor targeting to mitochondria: the role of MSF and hsp70. *Trends Cell Biol.* **6**, 104–108
31. Murakami, K. and Mori, M. (1990) Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. *EMBO J.* **9**, 3201–3208