A pCW vector harboring rat liver serine dehydratase cDNA was expressed in Escherichia coli. The expressed level was about 5-fold higher in E. coli BL21 than in JM109 cell extract; the former lacked two kinds of proteases. Immunoblot analysis revealed the occurrence of a derivative other than serine dehydratase in the JM109 cell extract. The recombinant enzyme was purified to homogeneity. Staphylococcus aureus V8 protease and trypsin cleaved the enzyme at Glu-206 and Lys-220, respectively, with a concomitant loss of enzyme activity. Spectrophotometrically, the nicked enzyme showed a ~50% reduced capacity for binding of the coenzyme pyridoxal phosphate and no spectral change of circular dichroism in the region 300–480 nm, whereas circular dichroism spectra of both enzymes in the far-UV region were similar, suggesting that proteolysis impairs the coenzyme binding without an accompanying gross change of the secondary structure. Whereas the nicked enzyme behaved like the intact enzyme on Sephadex G-75 column chromatography, it was dissociated into two fragments on the column containing 6 M urea. Upon the removal of urea, both fragments spontaneously refolded. These results suggest that serine dehydratase consists of two folding domains connected by a region that is very susceptible to proteases.

Rat liver L-serine dehydratase (SDH)\(^1\) (deaminase) (EC 4.2.1.13 or threonine dehydratase (deaminase), EC 4.2.1.16) catalyzes the pyridoxal phosphate (PLP)-dependent deamination of L-serine (L-threonine) to yield pyruvate (\(\alpha\)-ketobutyrate). SDH plays an important role in gluconeogenesis because the enzyme activity is remarkably induced by the consumption of high-protein diets, starvation, and other treatments (see Ref. 1 for a review). The purified enzyme is a dimer with a Mr, 34,200 subunit (2–4). PLP binds to Lys-40 to form a Schiff base, and its encompassing amino acid sequence, Ser-Xaa-Lys-Ile-Arg-Gly, is well conserved among SDHs from rat (5), human (6), tomato (7), Escherichia coli (8, 9), yeast (10), and so forth. Moreover, these enzymes have a glycine-rich sequence at a region 100–130 amino acid residues downstream of the PLP binding lysyl residue (9). The importance of this motif in the interaction with the coenzyme was assessed by the finding that substitution of the glycine residues with aspartic acid residues impairs PLP binding to \(E.\ coli\) \(\delta\)-serine deaminase (11, 12). These two conserved sequences suggest that SDHs have evolved from a common ancestral protein (9).

PLP catalyzes a variety of enzyme reactions such as transamination, decarboxylation, isomerization, elimination, and so on (see Ref. 13 for a review). Thus far, the crystal structures of more than 10 PLP enzymes are available (14). These enzymes are mainly from bacterial sources, with the exception of aspartate aminotransferases from chicken and pig livers. On the other hand, PLP enzymes are classified into at least three types of \(\alpha\), \(\beta\), and \(\gamma\) families on the basis of their primary sequences (15). SDH, which catalyzes \(\alpha\),\(\beta\) elimination, belongs to the \(\beta\) family, whereas tryptophanase (14) and tyrosine phenol-lyase (16), although using similar catalyzing reaction mechanisms, are affiliated with the \(\alpha\) family (15). Thus, classification based on the sequence alignment does not always conform to that based on the reaction mechanism. For insight into this problem, we feel that it is vital to accumulate information about the crystal structure of the \(\beta\) family members, which are known to include tryptophan synthase (17) and \(E.\ coli\) threonine deaminase (18). A crucial step toward this goal is to obtain pure enzyme for crystallization. Purification of SDH from rat liver was extremely hard because of its relatively low abundance and the sacrifice of numerous animals (19–23). In this work, we have developed a bacterial expression and purification procedure and characterized the recombinant enzyme. Along with crystal data on other PLP enzymes, our key finding that SDH is specifically cleaved into two fragments by various proteases that can independently refold after denaturation strongly suggests that this enzyme is composed of at least two folding domains.

EXPERIMENTAL PROCEDURES

Materials—Male Wistar strain rats (200 g) and male Japanese white rabbits (2.5 kg) were purchased from Sankyo Labo Service. Biochemical reagents were commercially available and used without further purification.

Plasmid Construction—Plasmid pCWori\(^+\) was described previously (24). The initial portions of \(E.\ coli\) mRNAs are usually rich in A and U, and the expression of foreign DNAs in the bacterium is often facilitated by making the relevant regions rich in A or T. Thus, we introduced three silent mutations in codons 2–5 (i.e., from the native sequence 5'-GCTGCCAAGGAG to the mutated sequence 5'-GCTGCCAAGAG (the introduced changes are underlined)) via polymerase chain reaction mutagenesis. Plasmid pCWori\(^+\) has a NdeI restriction site (CATATG; |
indicates the NdeI cutting site) coincident with the initiation ATG codon

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\(^3\) The abbreviations used are: SDH, serine dehydratase; IPTG, isopropyl-\(\beta\)-D-thiogalactopyranoside; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PLP, pyridoxal 5'-phosphate.
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and multicloning sites of XbaI, SalI, PstI, and HindIII downstream of the NdeI site. A foreign DNA is to be inserted between the NdeI site and any of the cloning sites. SDH cDNA had no NdeI site coincident with the initiation ATG codon and also had no usable restriction site in the 3′ noncoding region. Therefore, a NdeI site and a HindIII site were created by polymerase chain reaction mutagenesis. For this purpose, oligonucleotides 5′-TGGGCTCTGCTAAGATTGCCGACGTG-3′ (the underlined sequence is part of the NdeI site) and 5′-GGATGAAAGCTTGGCACCTGTCG-3′ (the underlined sequence is the HindIII site) were synthesized as the 5′ and 3′ primers, respectively. The latter sequence is derived from the native antiserum strand sequence corresponding to positions 35–59 downstream of the TGA stop codon (5′-GAGCTGGGGACCTGTTTAGC-3′) (3). With these two primers, the SDH cDNA containing the mutations was amplified by polymerase chain reaction. The polymerase chain reaction product was then digested with HindIII to produce a HindIII cut site at the 3′ end. Before ligation of the DNA, pCW Ori + was digested with NdeI, and the NdeI site was filled with the Klenow enzyme. This linearized plasmid was further digested with HindIII to remove the original insert, and the resulting plasmid was ligated to the modified SDH cDNA. This construct was designated pCW-SDH.

**Enzyme Assay**—SDH activity was determined by the dinitrophenylhydrazine method (19). The complete reaction mixture consisted of 50 mM borate-KOH (pH 8.3), 50 mM serine, and 50 μL PLP/enzyme in 0.25 ml. The absorption coefficient of hydrazine is 11.6 mm⁻¹·cm⁻₁, as determined with a 1 cm pathlength at 37 °C. One unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmol of pyruvate per minute at 37 °C.

**Purification of Recombinant SDH**—All operations were carried out at a temperature of 0 °C to 4 °C unless otherwise stated. E. coli carrying pCW-SDH were cultured in 2YT medium (25) containing ampicillin (50 μg/ml) and 75 μg/ml with the recombinant vector pCW-SDH or the control pCW. Other Methods—Protein was determined by the method of Bradford (27) with bovine serum albumin as the standard. SDS-PAGE (28) and immunoblotting (29) were performed as described previously. The monospecific IgG to rat liver SDH was described previously (2) and was used for the experiments shown in Figs. 3 and 6. Densitometry was examined using NIH Image software.

**Expression of SDH cDNA**—E. coli JM109 cells transformed with the recombinant vector pCW-SDH or the control pCW with no insert were cultured in the presence of IPTG. The IPTG-induced SDH activity in a crude extract was found to be about 2 units/mg. The specific activity of SDH purified from rat liver was in the range of 150–989 units/mg (19–23); thus, a 75- to 500-fold purification appeared to be necessary for homogeneity. A plausible cause of the low expression was the instability of the enzyme in this strain. We then resorted to E. coli BL21, which lacks both ATP-dependent Lon protease (31) and OmpT outer membrane protease (32). As expected, the BL21 cell extract was found to have an enzyme activity 5-fold higher than that of the JM109 extract. Fig. 1A shows SDS-PAGE of the extracts. The IPTG-treated JM109 cell extract had a Mr, 35,000 protein corresponding to the subunit of rat liver SDH. This band was more intense in BL21 cells than in JM109 cells (lanes 2 and 4). Immunoblot analysis with the IgG to rat liver SDH indicated that JM109 contained not only the Mrs, 35,000 band but a faint band of Mr, 24,000. This faint band became more evident in an aged preparation (Fig. 1B, lane 4) or in a preparation from JM109 cells grown at 31 °C (data not shown), but it was not found in the BL21 extract. Thus, it is thought that the low expression of the enzyme in JM109 cells results from extensive proteolysis.

**RESULTS**

**Expression of SDH cDNA**—E. coli JM109 cells transformed with the recombinant vector pCW-SDH or the control pCW with no insert were cultured in the presence of IPTG. The IPTG-induced SDH activity in a crude extract was found to be about 2 units/mg. The specific activity of SDH purified from rat liver was in the range of 150–989 units/mg (19–23); thus, a 75- to 500-fold purification appeared to be necessary for homogeneity. A plausible cause of the low expression was the instability of the enzyme in this strain. We then resorted to E. coli BL21, which lacks both ATP-dependent Lon protease (31) and OmpT outer membrane protease (32). As expected, the BL21 cell extract was found to have an enzyme activity 5-fold higher than that of the JM109 extract. Fig. 1A shows SDS-PAGE of the extracts. The IPTG-treated JM109 cell extract had a Mr, 35,000 protein corresponding to the subunit of rat liver SDH. This band was more intense in BL21 cells than in JM109 cells (lanes 2 and 4). Immunoblot analysis with the IgG to rat liver SDH indicated that JM109 contained not only the Mrs, 35,000 band but a faint band of Mr, 24,000. This faint band became more evident in an aged preparation (Fig. 1B, lane 4) or in a preparation from JM109 cells grown at 31 °C (data not shown), but it was not found in the BL21 extract. Thus, it is thought that the low expression of the enzyme in JM109 cells results from extensive proteolysis.
Purification and Some Properties—SDH was purified from E. coli BL21 by the conventional procedure as described under “Experimental Procedures.” Approximately 15 mg of pure enzyme were obtained from a 1-liter culture. The enzyme activity emerged as a single peak on AH-Sepharose or DEAE-cellulose column chromatography. The chromatography deprived PLP of SDH, as found with a colorless preparation. The addition of PLP to the apoenzyme allowed absorption maxima to be restored at 330 and 415 nm, as reported previously for rat liver enzyme (20). Amino acid analysis indicated that the N terminal of the recombinant enzyme was alanine, whereas that of the liver enzyme was acetylated alanine (4, 5). Thus, in E. coli, the N-terminal methionine residue is removed from the enzyme by posttranslational modification, but acetylation of the new terminal residue does not occur. The native and subunit molecular weights were about 66,000 by gel filtration and 34,000 by SDS-PAGE, respectively, indicating that the recombinant enzyme is a dimer like the liver enzyme. The $K_{\text{m}}$ values for serine and threonine of the recombinant enzyme were 67 and 50 $\text{mM}$, respectively, which were almost comparable to those reported for the liver enzyme (20).

**Kinetics of Limited Proteolysis**—To clarify the relationship between proteolysis and inactivation, an apoenzyme was incubated with trypsin as described above, and aliquots of the reaction mixture were withdrawn over time. Trypsin abolished the enzyme activity following pseudo-first order kinetics (Fig. 3A). SDS-PAGE showed the appearance of $M_r$ 35,000 (undigested), $M_r$ 22,000, and $M_r$ 12,000 bands among the lanes were almost equal (left panel) and thus normalized as 100 in each lane. Right panel: $\blacklozenge$, disappearance of the parent band; $+$, appearance of the $M_r$ 22,000 band; $\blacktriangle$, appearance of the $M_r$ 12,000 band. For comparison, the profile of the disappearance of SDH activity by trypsin digestion (A) was depicted again (○). In C, the monospecific IgG to recombinant SDH was used.

**The Sites Digested by Trypsin and S. aureus V8 Protease**—Next we determined the proteolytic site. After treatment with trypsin, the digest was subjected to SDS-PAGE, and the large and small fragments were recovered from the gel. Edman degradation demonstrated that the first 10 amino acid sequences of the large and small fragments were consistent with the N-terminal sequence of the intact protein and the amino acid sequence of a peptide from Ala-221 to Gln-230, respectively.
CD spectra were measured with a 0.5-mm-thick cell in the 280–480 nm region and used was 0.9 mg/ml in nicked SDH, and buffer solution, respectively. The protein concentration used was 1 mg/ml in each case. Absorption spectra of native (solid curve) and trypsin-treated (dashed curve) SDH. The concentration of SDH used was 1 mg/ml in each case. CD spectra were measured with a 0.5-mm-thick cell in the 280–480 nm region (B) and with a 0.2-mm-thick cell in the 200–280 nm region (C). 

Likewise, the cleavage site by S. aureus V8 protease was identified to be between Glu-206 and Gly-207.

Proteolysis Destabilizes PLP Binding—In this section, we studied the spectral change of a nicked enzyme. For this, the recombinant enzyme (the holoenzyme form) was digested with trypsin until a >95% loss of enzyme activity and was dialyzed against two changes of 1 liter of 10 mm potassium phosphate (pH 7.8), 0.1 mm EDTA, and 0.1 mm dithiothreitol at 0 °C over a period of 11 h. A, absorption spectra of native (solid curve) and trypsin-treated (dashed curve) SDH. The concentration of SDH used was 1 mg/ml in each case. CD spectra were measured with a 0.5-mm-thick cell in the 280–480 nm region (B) and with a 0.2-mm-thick cell in the 200–280 nm region (C). Thick, thin, and dotted curves represent the CD spectra of native SDH, nicked SDH, and buffer solution, respectively. The protein concentration used was 0.9 mg/ml in B and 0.2 mg/ml in C.

Interestingly, the holoenzyme previously nicked with trypsin gave no appreciable signal in this region. Similarly, the apoenzyme previously nicked and then reconstituted with PLP did not display a positive ellipticity at either 330 or 415 nm (data not shown). On the other hand, both the intact and nicked holoenzymes exhibited similar CD spectra in the far-UV region; their α-helices were estimated to be 54% and 51%, respectively (Fig. 4C). These results show that proteolysis fairly impairs coenzyme binding without accompanying a gross change in the secondary structure. We further addressed the question of whether the two fragments are separable under nondenaturing conditions. To test this, the trypsin-treated enzyme was applied to a Sephadex G-75 column. The nicked enzyme appeared at the position identical to that of the intact enzyme as monitored with the absorption at 280 nm, suggesting that both fragments still associate with each other.

Refrolding of Large and Small Fragments—Accumulating crystal data have revealed that many PLP enzymes constitute two (or three) folding domains (13). If SDH is made up of distinct folding domains, these domains may be capable of refolding independently under renaturing conditions after denaturation. Thus, a nicked enzyme was denatured with 6 M urea and renatured by dialysis in the same manner as the trypsin fragments (uncut SDH).

Liver Enzyme Is also Susceptible to Trypsin—It has been proposed that protein folding in eukaryotes is cotranslational, whereas in prokaryotes is posttranslational (33). It is possible that the folding of the recombinant enzyme may be different from that of liver enzyme or that the absence or presence of N-terminal blocking may be responsible for the
different folding pattern in the recombinant and liver SDHs. To test this possibility, liver enzyme was partially purified through ammonium sulfate fractionation and gel filtration (Fig. 6A) and subjected to limited proteolysis followed by immunoblot analysis. The preparation that was not treated with trypsin exhibited two bands of $M_\text{r}$ 22,000, and $M_\text{r}$ 24,000 other than an intact band (Fig. 6B, lane 4). Because no protein inhibitor was included in the course of purification, these subbands were considered to be degradation products. Trypsin treatment increased the $M_\text{r}$ 22,000 band accompanying the disappearance of the parent band (Fig. 6B). The result suggests that the liver enzyme is also susceptible to trypsin.

**DISCUSSION**

SDH is widely spread in nature, but its physicochemical properties are considerably different from species to species. For example, rat (20) and sheep liver enzyme (34) is a dimer with Michaelis-Menten kinetics with respect to the substrate, whereas yeast and *E. coli* biosynthetic threonine dehydratase, the first enzyme in the isoleucine synthesis pathway, is a tetramer and is feedback-inhibited by isoleucine and heterotropically activated by valine (8). *E. coli* catabolic threonine dehydratase induced anaerobically in tryptone-yeast extract medium is a tetramer and is allosterically activated by AMP (9). However, *E. coli* D-SDH is a monomer (35, 36). (An appreciable amount of d-serine is present in mammalian brain, which is produced by the racemization of l-serine. The occurrence of d-SDH remains to be determined (37)). The importance of the glycine-rich sequence in SDH was first verified with a catabase amount of D-serine is present in mammalian brain, respectively, at 25 °C for 10 or 20 min and subjected to 13.5% SDS-PAGE followed by Coomassie Blue staining (A) or immunoblotting with anti-recombinant SDH IgG (B).
residues (18). The folding pattern of the N-domain resembles that of the tryptophan synthase β subunit, but the structure of the regulatory domain is rather similar to that of the regulatory serine binding domain in allosteric 3-phosphoglycerate dehydrogenase (48). It remains unclear whether the neck regulatory serine binding domain in allosteric 3-phosphoglycerate the regulatory domain is rather similar to that of the regulatable discussions throughout the work.

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