The Primary Structure of Escherichia coli L-Threonine Dehydrogenase*

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The complete primary structure of Escherichia coli L-threonine dehydrogenase has been deduced by sequencing the cloned tdh gene. The primary structure so determined agrees with results obtained independently for the amino acid composition, the N-terminal amino acid sequence (20 residues), and a short sequence at the end of an internal peptide of the purified enzyme. The presence of a predicted Asp-Pro bond at residues 148 and 149 was confirmed by treatment of purified threonine dehydrogenase with dilute acid and subsequent analysis of the resulting cleavage products. The primary structure of L-threonine dehydrogenase from E. coli has been examined for possible homology to other NAD⁺-dependent dehydrogenases; indications are that this enzyme is a member of the zine-containing long-chain alcohol/polyol dehydrogenase family.

At least four different enzymes initiate threonine catabolism in microorganisms. Threonine aldolase (EC 4.1.2.5) catalyzes the conversion of threonine to acetaldehyde and glycine. Alternatively, the action of two distinct threonine dehydratases (EC 4.2.1.16), classified as biosynthetic or biodegradative, forms α-ketobutyrate and NH₃ from threonine. In addition, threonine dehydrogenase (EC 1.1.1.103) catalyzes the NAD⁺-dependent oxidation of threonine to 2-amino-3-ketobutyrate.

The catabolic pathway initiated by threonine dehydrogenase is a major pathway for threonine degradation in Escherichia coli under many growth conditions (Fig. 1). Low levels of this enzymatic activity can be detected under all growth conditions so far examined (1-3). Leucine is an inducer of threonine dehydrogenase (2, 3), and mutations that lead to elevated activity of this enzyme enable cells to grow on threonine as a carbon source (1, 4). In contrast, the other threonine-degrading enzymes show little or no activity under most growth conditions; threonine aldolase has not been detected in cell extracts (3), biosynthetic threonine dehydrogenase activity is inhibited by small amounts of isoleucine (5, 6), and in order to detect biodegradative threonine dehydrogenase activity cells must be grown anaerobically or in the absence of glucose (2, 7).

In the second step of the threonine dehydrogenase-initiated pathway, 2-amino-3-ketobutyrate-CoA ligase (acetyl-CoA:glycine C-acetyltransferase; EC 2.3.1.29) catalyzes the cleavage of 2-amino-3-ketobutyrate with formation of glycine plus acetyl-CoA (1, 4, 5-10). The primary structure of the kbl (2-amino-3-ketobutyrate-CoA ligase) gene and its protein product are known (11). The conversion of threonine to glycine by the coupled action of these two enzymes accounts for the observation that glyA (serine hydroxymethyltransferase gene) mutants can utilize threonine for growth (3, 12, 13). In cells that contain a functional glyA gene, the initial oxidation of threonine to 2-amino-3-ketobutyrate and its subsequent cleavage provide an alternate pathway for serine biosynthesis (14). L-Threonine dehydrogenase activity also initiates a catabolic pathway for threonine utilization in eukaryotic cells (15-22).

Threonine dehydrogenase from E. coli has recently been purified to homogeneity and extensively characterized (23, 24). In this paper, the nucleotide sequence of the structural gene (tdh) for E. coli threonine dehydrogenase is reported. The presence of an acid-labile Asp-Pro bond, predicted from the deduced amino acid sequence, is demonstrated, and the amino acid sequence of threonine dehydrogenase is compared with that of other dehydrogenases.

EXPERIMENTAL PROCEDURES

Materials

Formic acid (98%) and hexafluoroacetone-3H₂O were obtained from Aldrich. Formic acid (85%) was from J. T. Baker Chemical Co. "Ultrapure" guanidine-HCl and [1-14C]jodoacetic acid (specific activity = 6.25 mCi/mmol) were purchased from ICN Biomedicals, Inc.; lysozyme, myoglobin, soybean trypsin inhibitor, and trypsinogen were provided by Sigma. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. Restriction endonucleases, T4 DNA ligase, synthetic oligodeoxynucleotide primers, and DNA polymerase I large fragment (Klenow) were purchased from New England BioLabs, Inc. These reagents were used as specified by the manufacturer. Threonine dehydrogenase was purified to homogeneity from extracts of E. coli mutant strain SBD76 as previously described (23, 24).

Bacterial Strains and Plasmids—Plasmid pDR121 was derived from pOS121 as reported before (9). E. coli strain JM101 (Δpro-loci thi rpsL, hsdH4 endA stbC supE44/F traD36 proA⁺ B⁺lacZ Δ(lacZ)M15) was used for propagation of M13 clones.

DNA Preparations—Plasmid and M13 replicative form DNA were

1. M. Levinthal, unpublished results.
FIG. 1. Threonine dehydrogenase-initiated pathway for threonine utilization. Threonine is converted to glycine and acetyltransferase (glyA) to complete an alternate pathway for CoA ligase (kbl). Glycine can then be converted to serine by serine operon, threonine dehydrogenase (tdh) and 2-amino-3-ketobutyrate isolated by the alkaline lysis procedure of Ish-Horowicz and Burke (25). Plasmid and replicative form DNA were purified by equilibrium centrifugation of a sample of the enzyme, and the pH of this mixture was adjusted to 8.8 with triethylamine. The solution was then saturated with argon, and a 10-fold excess (with respect to protein thiols) of dithiothreitol was added. The sample was again briefly sparged with argon. Reduction of disulfides was allowed to proceed with stirring for 3 h at 25 °C. A 10-fold excess (with respect to dithiothreitol and protein thiols) of solid [1-14C]dithiothreitol was added. The sample was then heated to 110 °C for 5, 10, 20, or 40 min. Hydrolysis was terminated by rapidly cooling the solutions in ice water then freezing them in a dry ice:ethanol mixture and lyophilizing to dryness. In the other procedure, lyophilized samples (240 μg) of the native enzyme were dissolved in 600 μl of 10% (v/v) acetic acid (pH adjusted to 2.5 with pyridine) containing 7 mM guanidine-HCl, and these solutions were incubated for 0, 24, 48, and 96 h at 40 °C. Thereafter, the samples were first neutralized by adding 10 mM NaOH, then dialyzed extensively against distilled water, and finally lyophilized to dryness.

Cleavage of threonine dehydrogenase was monitored by subjecting samples of the acid digests to SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (30) except that a solution of 10% trichloroacetic acid containing 3% sulfosalicylic acid replaced the usual fixative. Myoglobin (Mr = 17,200), lysozyme (Mr = 14,300), soybean trypsin inhibitor (Mr = 20,100), and trypsinogen (Mr = 24,000) served as standard proteins. Radioactive cleavage products of threonine dehydrogenase were detected on polyacrylamide gels by autoradiography on Kodak XAR film; protein bands were visualized by staining with Comassie Brilliant Blue and destaining with a mixture of 7.5% acetic acid, 10% methanol.

Peptide Purification and Sequencing Procedures—Peptide purification and the chromatography of phenylthiohydantoin-amino acid derivatives were accomplished with a Waters Associates (Milford, MA) HPLC system consisting of two M510 pumps, a U6K injector, a model 441 detector, and a model 680 gradient controller; data were plotted and analyzed with the use of a Hewlett-Packard Co. integrator (model 5590A). Manual Edman sequencing of selected peptides was performed by the method described by Sanger et al. (28). Phenylthiohydantoin-amino acid derivatives were separated and analyzed as reported in earlier studies (32). Sequencing of Peptide 2, which was anticipated to have an N-terminal prolinal residue, was carried out after all free α-amino groups in an acid digest were blocked by reaction with o-phthalaldehyde (this derivatized all free α-amino termini). For this purpose, a 1 mg of carboxymethylated threonine dehydrogenase was cleaved by incubating in 1 ml of 0.1 N HCl containing 10% hexafluoroacetone (as described earlier). The sample was then lyophilized to dryness, and the residue was subsequently dissolved in 1 ml of 88% formic acid; this solution was transferred to a sequencing tube and dried under vacuum. The reaction with o-phthalaldehyde was then carried out as described (32) except that 20 μl of the o-phthalaldehyde reagent were added to the sample.

Lyophilized samples of threonine dehydrogenase that had been subjected to mild acid cleavage were dissolved in a minimal volume of 88% formic acid. These solutions were then diluted to 20% formic acid with Solvent A (0.1% trifluoroacetic acid in distilled water) and filtered through a Millipore HV, 0.45-μm filter (Millipore). Acid cleavage products were separated at room temperature by injecting 3–30 nmol of a given sample onto a Beckman C3 Ultrapore HPLC column (4.6 mm × 7.5 cm) equilibrated with Solvent A. Peptide fragments were eluted with a nonlinear gradient of 100% Solvent A to 30% Solvent B, 70% Solvent B, where B consisted of 0.13% trifluoroacetic acid in a mixture of 25% 2-propanol, 75% acetonitrile.

For determination of its N-terminal amino acid sequence, a sample of pure threonine dehydrogenase (2.3 mg of protein in 1.5 ml) was dialyzed exhaustively against water and then lyophilized to dryness. The crude 2.3 mg of threonine dehydrogenase was dissolved in 1 ml of 88% formic acid. These solutions were then diluted to 20% formic acid with Solvent A (0.1% trifluoroacetic acid in distilled water) and filtered through a Millipore HV, 0.45-μm filter (Millipore). Acid cleavage products were separated at room temperature by injecting 3–30 nmol of a given sample onto a Beckman C3 Ultrapore HPLC column (4.6 mm × 7.5 cm) equilibrated with Solvent A. Peptide fragments were eluted with a nonlinear gradient of 100% Solvent A to 30% Solvent B, 70% Solvent B, where B consisted of 0.13% trifluoroacetic acid in a mixture of 25% 2-propanol, 75% acetonitrile.

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Selective Cleavage of an Asp-Pro Bond—Peptide bonds in proteins between the α-carboxyl group of aspartic acid and the imino group of proline have been shown to be uniquely susceptible to cleavage at low pH. For some proteins, cleavage of this bond is highly specific and nearly quantitative under mild conditions of temperature and pH; in other instances, however, more drastic conditions are required and result in only partial hydrolysis with much less specificity. Nucleotide-sequencing data indicate the presence of an Asp-Pro bond between residues 148 and 149 of *E. coli* threonine dehydrogenase. Attempts were made to confirm the presence of this linkage in the enzyme by selective acid cleavage.

Seven different protocols were tested to accomplish optimal cleavage of *E. coli* threonine dehydrogenase by mild acid; the same general pattern of protein bands was seen in every case. Typical results of such experiments are shown in Fig. 4, A and B. Three major bands attributable to threonine dehydrogenase, Peptide 1 (residues 1–148), and Peptide 2 (residues 149–341) were always evident as were also fainter bands corresponding to fragments attributable to nonspecific bond cleavage. The extent of random cleavage varied with the hydrolysis conditions used, as illustrated in Fig. 4, A and B, but in every instance the *R*C values (and thus the molecular weights) of the major bands were the same. As can be seen, the intensity of the threonine dehydrogenase band diminished progressively with increasing time of acid hydrolysis, while those bands designated Peptides 1 and 2 became correspondingly darker. Random bond breakage with the appearance of numerous smaller peptides (*M* < 10,000) increased with time of incubation. We were surprised to observe that simple exposure of this enzyme to mild acid conditions at room temperature (for ~10 min, sample-handling time) caused some partial cleavage (see *lane 1*, Fig. 4A); protein fragments detected under such conditions were not seen when recently purified samples of the dehydrogenase (prepared and stored in 50 mM Tris-HCl buffer, pH 8.4) were immediately subjected to SDS-polyacrylamide gel electrophoresis.

Peptide bonds in proteins involving the α-carboxyl group of aspartic acid are the most acid-labile. Of these, the Asp-Pro linkage is generally the most sensitive (36). Specific cleavage of the Asp-Pro linkage in threonine dehydrogenase would directly yield Peptide 1 (*M* = 16,300) plus Peptide 2 (*M* = 21,000). Most likely, the numerous extraneous bands seen are due to random cleavage at the carboxyl side of several aspartyl residues (there are 22 Asp residues/subunit; 36 Asp + Asn/subunit) in threonine dehydrogenase. The presence of Band B, always observed in these mild acid digests, might possibly be due to hydrolysis of a second especially labile aspartyl bond (besides the Asp-Pro). Relatively rapid cleavage of the Asp-Val linkage between residues 222 and 223, for example, would yield a peptide of *M*~13,000~ (the C-terminal end) plus a fragment with a molecular weight approximately equal to that calculated for Band B (*i.e.* *M* ~24,000). Subsequent cleavage of Band B at the Asp-Pro site would then give Peptide 1 and a small fragment of *M* ~8,000; this would explain our observation that after a given period of treatment at low pH, the band corresponding to Peptide 1 is often more intense than that for Peptide 2. Attempts to purify Band B peptide have failed as it consistently coeluted with native dehydrogenase in HPLC.

Exposure of the gel shown in Fig. 4B to x-ray film clearly showed that the level of radioactivity present in threonine dehydrogenase alkylated with [14C]iodoacetate decreased with time of acid digestion, while the level in Peptide 1 correspondingly increased (see Fig. 4C). Similar results were obtained when the carboxymethylated enzyme was treated with 0.1% trifluoroacetic acid at 110 °C for 0–40 min (data not shown).
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Fig. 3. Nucleotide sequence and encoded polypeptide of the E. coli tdh gene and downstream unidentified reading frame. The nucleotide sequence presented extends from the stop codon of the kb1 gene to the downstream EcoRI site. The amino acid sequence of the N-terminal 20 amino acids of threonine dehydrogenase was also obtained by automated Edman degradation of purified threonine dehydrogenase. These amino acids are shown in boldface and are in exact agreement with the deduced amino acid sequence. The predicted Shine-Dalgarno sequences for the tdh gene and the unidentified reading frame are underlined. The dyad symmetry of the proposed p-independent transcriptional termination sequence is overlined. Note that nucleotide position 1 in this figure is 13,700 nucleotides distal to the upstream EcoRI site.

Fig. 4. SDS-polyacrylamide gel electrophoresis of the peptides released by mild acid treatment of E. coli threonine dehydrogenase. A, native enzyme was dissolved in 10% acetic acid (pH 2.5) containing 7 M guanidine-HCl and incubated at 40°C. Samples were removed after 0, 24, 48, and 96 h (lanes 1–4, respectively). Cytochrome c (Mr, 13,700) and lysozyme (Mr, 14,300) were applied in lane 5; bands were visualized with Coomassie Brilliant Blue. B, enzyme, after reaction with [1-14C]iodoacetate, was incubated at 110°C in 0.1 M HCl containing 10% hexafluoroacetone. Samples (125 μg each) were removed after 0, 5, 10, 20, and 40 min (lanes 2–6, respectively). A mixture of lysozyme (Mr, 14,300), myoglobin (Mr, 17,200), soybean trypsin inhibitor (Mr, 20,100), and trypsinogen (Mr, 24,000) was applied to lanes 1, 7, and 8. The gel was stained with Coomassie Brilliant Blue. C, radioautogram on Kodak XAR film of the gel in B.
The faint level of radioactivity associated with Band B peptide can be explained by a ready cleavage of the Asp-Val bond between residues 222 and 223 as suggested above.

When the apparent molecular weights of Peptides 1 and 2 were estimated from their mobilities on SDS-polyacrylamide gels, values near those calculated from their amino acid compositions were obtained (18,300 and 22,200, respectively, for Peptides 1 and 2 versus 16,300 and 21,000 from their amino acid composition). Since the migration of low molecular weight peptides is often somewhat variable (30) and these two peptide fragments did have the N-terminal amino acid sequence expected (see the following results), the small discrepancy in their estimated molecular weights is not considered significant.

*N*-terminal Sequence of Peptides 1 and 2—When a crude acid digest of carboxymethylated threonine dehydrogenase was fractionated by HPLC on a C18 Ultraprep column, three major protein peaks that coincided with three peaks of radioactivity were detected. No significant radioactivity was observed anywhere else throughout the chromatographic run. By SDS-polyacrylamide gel electrophoresis, one of the three protein/radioactive peaks was shown to be identical with the native carboxymethylated dehydrogenase; a second was found to be a mixture of the carboxymethylated enzyme, Peptide 2, and Band B peptide; and the third comigrated with Peptide 1. Autoradiography of these gels confirmed the identities assigned.

Manual Edman sequencing (five cycles) of purified Peptide 1 gave the following sequence: Met-Lys-Ala-Leu-Ser. This is the N-terminal amino acid sequence of *E. coli* threonine dehydrogenase. Peptide 2 could not be obtained in pure form; it coeluted with carboxymethylated threonine dehydrogenase in each of eight different HPLC gradient systems tested. As a consequence, a crude acid digest was first treated with o-phthalaldehyde to block all primary amino groups, and this mixture was then subjected to manual sequencing. The following sequence corresponding to residues 149–153 of native threonine dehydrogenase was obtained in good yield in each of the five cycles: Pro-Phe-Gly-Asn-Ala.

**DISCUSSION**

Threonine Dehydrogenase—Our results with purified threonine dehydrogenase support the DNA-sequencing data indicating the presence of an Asp-Pro bond that is subject to hydrolytic cleavage at low pH. The enzyme also appears to have a second acid-labile bond (perhaps that between Asp-222 and Val-223). Under the hydrolysis conditions examined, trace quantities of as many as 30 peptides were detected; these are assumed to be products of random cleavage at the carboxyl side of other aspartyl bonds in the molecule. The level of such breakage was to some extent dependent on the conditions used, but no general trend was evident.

In addition to confirming the presence of the Asp-Pro linkage in *E. coli* threonine dehydrogenase, it had been our hope that selective quite specific cleavage of this bond would be a convenient method for breaking the protein roughly in half, thereby significantly enhancing the capability of isolating, sequencing, and assigning the location of “active site” peptides in the molecule. The results presented make such an application quite unlikely. Only a small fraction of the carboxymethylated enzyme is cleaved at the Asp-Pro bond during a typical 40-min incubation in mild acid; most of the protein remains intact and, at best, the yield of Peptide 1 or 2 is only about 5–10% of the starting material. Furthermore, numerous other peptides are formed by random bond cleavage; although they are present in lesser amounts, their yield also increases with longer reaction times.

Homology of L-Threonine Dehydrogenase from *E. coli* with Other NAD*-dependent Dehydrogenases—The NAD*-dependent dehydrogenases constitute a large group of enzymes of which several have been extensively characterized catalytically and structurally. For example, both amino acid sequence and x-ray crystallographic data are available for lactate dehydrogenase, malate dehydrogenase (cytosolic and mitochondrial), glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, and horse liver alcohol dehydrogenase (37).

In addition, amino acid sequence information has been published for many other enzymes in this group. These dehydrogenases as a class are structurally related as they share a common NAD*-binding domain (38). With the primary structure of *E. coli* threonine dehydrogenase now determined, we find this enzyme has considerable sequence homology with the zinc-containing long-chain alcohol/polyol family of dehydrogenases.

Protein amino acid sequence databases were searched through BIONET by the FASTP program of Lipman and Pearson (39) for proteins homologous with *E. coli* threonine dehydrogenase. The same program was used to obtain an initial alignment of the amino acid sequences of threonine dehydrogenase and sheep liver sorbitol dehydrogenase. With this done, the sequences of threonine dehydrogenase, sheep liver sorbitol dehydrogenase, horse liver alcohol dehydrogenase, maize alcohol dehydrogenase, and yeast alcohol dehydrogenase were simultaneously aligned so as to optimize conservation of residues invariant throughout the alcohol/polyol dehydrogenase family (40) while also maximizing matches between threonine dehydrogenase and the other enzymes. In general, the same relative alignment of the alcohol dehydrogenases and sorbitol dehydrogenase, as previously published (40), was retained. Occasionally, relative alignments were changed to accommodate the sequence data for threonine dehydrogenase. The horse liver alcohol dehydrogenase numbering system is used in this paper; residue numbers given are relative to those assigned the horse liver alcohol dehydrogenase sequence.

Fig. 5 shows the results of such amino acid sequence alignments. As is evident, many residues are conserved in all five of these enzymes, while several others are shared among four of the five. The amino acid sequence of threonine dehydrogenase shows between 25 and 28% identity with those of the other four proteins. Threonine dehydrogenase is most similar to sorbitol dehydrogenase and yeast alcohol dehydrogenase (28 and 27% identity, respectively), while it has 25% identity with the sequences of both horse liver alcohol dehydrogenase and maize alcohol dehydrogenase. Furthermore, many of the nonconserved residues have conservative replacements making the overall sequence similarity higher than that suggested by the percent identity between molecules.

Jörnvall et al. (40) recently published an alignment of 16 different alcohol dehydrogenases together with sheep liver sorbitol dehydrogenase (40). They called this group of enzymes the zinc-containing long-chain alcohol/polyol dehydrogenases; all are polypeptides of approximately 350 amino acids (41) and require zinc for catalytic activity (40). Among these 17 enzymes, 22 amino acid residues are strictly conserved. Another 13 residues are highly conserved but not invariant. Examination of the amino acid sequence alignment presented here shows that of these 22 invariant residues, 20 are also found in threonine dehydrogenase (Glu-35 of horse liver alcohol dehydrogenase is replaced conservatively with Asp in threonine dehydrogenase, while threonine dehydrogenase has...
a Leu in place of Pro-31 of horse liver alcohol dehydrogenase. Of the 13 highly conserved residues in these 17 alcohol/polyol dehydrogenases, 6 are shared by threonine dehydrogenase, and 4 others are replaced conservatively. These homologous residues are spread throughout the length of the threonine dehydrogenase molecule and include many amino acids known to be zinc ligands in liver alcohol dehydrogenase. Residue numbers refer to positions in liver alcohol dehydrogenase.

Probably the most interesting similarity between the amino acid sequences of threonine dehydrogenase and the alcohol dehydrogenase/sorbitol dehydrogenase group is that the two zinc-binding sites of horse liver alcohol dehydrogenase (presumed to fill the same role in the other enzymes) are also present in threonine dehydrogenase. Crystallographic studies have shown the ligands to the active site zinc of horse liver alcohol dehydrogenase are Cys-46, His-67, and Cys-174. Like threonine dehydrogenase, this enzyme is more like the alcohol dehydrogenase family as it has cysteine residues at the four positions known to act as ligands for the structural zinc of horse liver alcohol dehydrogenase. However, the identity of the third active site zinc ligand of threonine dehydrogenase is uncertain. We have recently found that threonine dehydrogenase, as isolated, does contain tightly bound zinc, but the stoichiometry has not yet been established.

The significance of the presence of two zinc-binding sites (i.e., active site zinc and structural zinc) in threonine dehydrogenase comparable to the sites of horse liver alcohol dehydrogenase is uncertain. We have recently found that threonine dehydrogenase activity, but EDTA rapidly causes inactivation. The significance of such observations will be the focus of future studies.
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