Molecular Cloning of a cDNA Encoding Chicken T-protein of the Glycine Cleavage System and Expression of the Functional Protein in *Escherichia coli*

EFFECT OF mRNA SECONDARY STRUCTURE IN THE TRANSLATIONAL INITIATION REGION ON EXPRESSION

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DNA clones encoding chicken T-protein of the glycine cleavage system were isolated from chicken liver Agt10 cDNA libraries. Three overlapping clones provided an open reading frame of 1176 nucleotides that predicts a polypeptide of 392 amino acids (*M*, 42,056) comprised of a 16-residue mitochondrial targeting sequence and a 376-residue mature protein (*M*, 40,292). The amino acid sequence predicted for the mature protein showed 67% identity with that of bovine T-protein. A cDNA encoding mature T-protein was constructed, and the nucleotide sequence just downstream of the initiation codon was modified without amino acid substitution to reduce the free energy of formation for the folded mRNA. Expression plasmids containing these cDNA variants produced large amounts of T-protein in the folded mRNA. Enzymatically active T-protein was obtained and purified to near homogeneity with a yield of about 50%.

The glycine cleavage system is a multienzyme system comprising four proteins named P-protein, H-protein, T-protein, and L-protein and catalyzes the reversible cleavage of glycine. The mechanism of the reaction catalyzed by the system has been fairly well characterized with the chicken system (1-5). P-protein catalyzes the pyridoxal phosphate-dependent carboxylation of glycine and transfer of the remaining methylamine moiety of glycine to the lipoyl prosthetic group of H-protein. T-protein catalyzes the release of ammonia from the intermediate attached to H-protein and the synthesis of 5,10-CH2-H4folate in the presence of H4folate. L-protein is a lipoamide dehydrogenase that catalyzes the reoxidation of the resulting dihydrolipoic acid on H-protein. In eukaryotes, all of the components are synthesized cytoplasmically as precursor forms (6-9), transported into mitochondria, and assembled in the complex after processing. The primary structures of chicken P-protein and H-protein have been determined. Chicken P-protein is composed of 970 amino acid residues (8) and has pyridoxal phosphate at Lys-704 (10). Chicken H-protein is composed of 125 amino acids with a lipoic acid prosthetic group at Lys-59 (11). Recently we have cloned a full-length cDNA encoding bovine T-protein and a partial cDNA for chicken T-protein (9). The predicted primary sequence of bovine T-protein exhibited no sequence homology with other folate-requiring proteins. There is little information about the binding site for H4folate or the association site for H-protein and other components of the glycine cleavage system on T-protein. Since the structural studies require relatively large quantities of enzymatically active protein and since purification and the elucidation of the primary structure of the components of the system have been accomplished mainly with the chicken system, we decided to clone and overexpress chicken T-protein.

In this paper, we describe the cloning of overlapping cDNAs encoding chicken liver T-protein and the expression of mature chicken T-protein in *Escherichia coli*. We modified the nucleotide sequence flanking the initiation codon of the constructed cDNA for mature T-protein without altering the amino acid sequence to reduce the free energy of formation for the folded mRNA. The plasmids constructed were successful in expressing soluble and enzymatically active T-protein in *E. coli* when the expression was conducted at a relatively low growth temperature under low inducing conditions. The availability of large amounts of active T-protein will facilitate our structure/function studies on T-protein.

EXPERIMENTAL PROCEDURES

Materials—The pET-3a vector and the bacterial strain BL21(DE3)pLysS were generous gifts of Dr. Masayuki Yamamoto (Tohoku University, School of Medicine). Restriction endonucleases and other DNA modifying enzymes were obtained from Toyobo (Osaka, Japan), Takara Shuzo (Kyoto, Japan), Nippon Gene (Tokyo, Japan), Seikagaku Kogyo (Tokyo, Japan), or New England Biolabs, Inc. The abbreviations used are: 5,10-CH2-H4folate, methylenetetrahydrofolate; H4folate, tetrahydrofolate; bp, base pair(s); IPTG, isopropyl-1-thio-β-D-galactopyranoside; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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Inc. Radioactive compounds were either from Amersham Corp. or Du Pont-New England Nuclear. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. Chicken liver T-protein was purified by the method described below for recombinant T-protein and stored at -40 °C. All other reagents were obtained commercially.

Preparation of Antibody—A rabbit was immunized by the subcutaneous injection of the purified chicken liver T-protein in Freund's complete adjuvant. Injections (550 μg of protein each time) were given three times at bi-weekly intervals. The IgG fraction was obtained by ammonium sulfate fractionation followed by chromatography on DEAE-cellulose (12). The antibody was further purified by affinity chromatography on a column of Sepharose 4B coupled with chicken T-protein. The specificity of the antibody was checked by Western blot analysis.

Isolation and Sequence Determination of cDNA Clones—Isolation of a partial cDNA clone for chicken T-protein, CT5C, was as described previously (9). To obtain the cDNA encoding the 5’-terminal region of the mRNA, we constructed two cDNA libraries primer-extended apt10 cDNA libraries with the cDNA cloning system Apt10 (Amersham Corp.). Initially, a library was constructed with a synthetic oligonucleotide, 5’ GGTCAGGTACCCGCAAGGCGCCG 3’, corresponding to the 5’-terminal nucleotide sequence of CT5C as the primer. A 685-bp SacI-BglII fragment of BT5A, a cDNA clone for bovine T-protein (9), was labeled with the Multiprime DNA-labeling system (Amersham Corp.) and [α-32P]dCTP and used to screen about 109 recombinant phages. Hybridization was performed under the conditions as described previously (9) except that the final wash of the blots was carried out in 1 x SSC (1 x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). DNA from positive recombinant phages was isolated, and the cDNA inserts were subcloned into a plasmid pGEM-3Z (Promega) and sequenced by the dyeodeoxy chain termination method (13). A clone named CT10 (Fig. 1) was thus isolated. Another library was then constructed with the mixture of two synthetic oligonucleotides, 5’ GGTCAGGTCATCCGGCAGCCCGGC 3’ and 5’ CTCCAGCGGGGAGGCGAGCCTAT 3’, derived from the nucleotide sequence of CT10 as the primer. The library was screened as above and cloning CT5A and CT5B (Fig. 1) was isolated.

Northern Blot Analysis—Total RNA was extracted from chicken liver by the guanidinium isothiocyanate method (14). Poly(A)+ RNA was denatured with formaldehyde, electrophoresed through a 1% agarose gel in the presence of formaldehyde, and transferred to GeneScreen Plus membrane (15). The blot was hybridized with CT5C labeled with [α-32P]CTP by random priming under the condition described previously (9). The filter was washed twice for 5 min at room temperature in 2 x SSC, once for 30 min at 60 °C in 2 x SSC containing 1% SDS, and once for 30 min at room temperature in 0.1 x SSC containing 0.05% SDS. Autoradiography was performed at -80 °C for 5 h with an intensifying screen.

Construction of E. coli Expression Vector for Mature T-protein—The strategy for construction of the expression plasmid is shown in Fig. 2. The plasmid pGEM/CT5C was constructed by cloning CT5C into pGEM-3Z as described previously (9). pGEM/CT5C was then digested with SacI and BglII, and the plasmid containing a 545-bp 3’ fragment of CT5C was isolated. The 243-bp SmaI-PstI fragment of CT10, the 119-bp PstI-BglII fragment of CT5C were isolated and ligated in an one-step reaction into the above mentioned SacI-BglII-digested pGEM/CT5C. The resulting plasmid encoding a full-length cDNA for mature chicken T-protein (pGEM/CT) was verified by DNA sequencing and restriction analysis, and then a 1176-bp SacI-EcoRI fragment was isolated from pGEM/CT and subcloned into a phagemid vector, pTZ18U, generating the plasmid pTZ/CT. An NdeI site carrying in-frame initiator methionine codon at the 5'-end and a BamHI site located downstream of the stop codon were introduced to the mature T-protein cDNA in pTZ/CT by oligonucleotide-directed mutagenesis according to the method of Kunkel et al. (16) with a mutagen phagemid in vitro kit from Bio-Rad. Two oligonucleotides containing several modifications (in boldface), 5’ CCCCCGGTTACAATAT GTCCGGCCCGGA 3’ (the underlined section is the NdeI site) and 5’ TGAGACCGGATCCATTAAAGCGCC 3’ (the underlined section is the BamHI site), were synthesized and used for mutagenesis. The 1141-bp NdeI-BamHI fragment was isolated from the resulting recombinant phagemid, pTZ/CT, and ligated into the NdeI- and BamHI-digested PET-3 (17). This plasmid will be referred to as PET/MCT. The modified cDNA in which the 5’-coding sequence was altered without the amino acid substitution were also constructed from pTZ/CT by site-directed mutagenesis employing oligonucleotides 5’ CATATGTCGGCACCAGAAGGACTGA- TGCCGGCCCGGA 3’ (the underlined section is the NdeI site) and 5’ CATATGTCGGCACCAGAAGGACTGA- TGCCGGCCCGGA 3’ (for PET/MCTT4) or 5’ CATATGTCGGCACCAGAAGGACTGA- TGCCGGCCCGGA 3’ (for PET/MCTB4) and inserted into PET-3a as above. All of the constructs were confirmed by sequencing.

Fig. 1. Restriction map, sequencing strategy, and organization of chicken T-protein cDNA clones. The coding region for mature T-protein is shown as the open box, the coding region for the presequence is dotted, and the noncoding regions are presented by the solid boxes. The solid line represents the poly(A) tail. The arrows indicate the direction and extent of sequencing reactions.

Fig. 2. Construction of the expression vector for recombinant chicken mature T-protein. The procedure for construction of the expression vector is described under "Experimental Procedures." The open boxes and solid boxes are the coding and noncoding regions, respectively, of the cDNA insert. The sites for restriction enzymes are indicated: Ba, BamHI; Bg, BglII; E, EcoRI; N, NdeI; P, PstI; Sa, SacI, Sm, SmaI, bla, β-lactamase gene.
The 5' coding sequences of mRNAs derived from these cDNAs are shown in Fig. 3.

Expression of T-protein in E. coli—Expression plasmids pET-3a, pET/MCT, pET/MCT4, and pET/MCT6 were transfected into E. coli strain BL21(DE3)pLysS, which contained the T7 RNA polymerase gene integrated in the chromosome under the control of the lacUV5 promoter and T7 lysozyme expression plasmid, pLysS (17). The transformants were grown at 37 °C in 10 ml of M9H medium, which is composed of the components of both M9 and H medium (1 g of NaH₂PO₄, 3 g of KH₂PO₄, 15 g of Na₂HPO₄·12H₂O, 4 g of glucose, 1 ml of 1 M MgSO₄, 10 g of Bacto Trypton (Difco), and 5 g of NaCl in 1 liter of water) in the presence of ampicillin (20 µg/ml) and chloramphenicol (30 µg/ml). The culture reached an A₀₉₀ of 0.8, the T7 RNA polymerase was induced by adding IPTG to 1 mM, and the cell growth was continued for 3 h at 37 °C. Preincubation was omitted and the concentration of IPTG was reduced to 25 µM when the culture of BLSl(DE3)pLysS transfected with pET/MCT4 was harvested and sonicated for 5 s. The cells in remaining cultures were harvested and centrifuged at 16,000 g for 30 min, resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 10 µM p-amidinophenylmethanesulfonyl fluoride) of 10% of the original culture volume and incubated for 30 min at 0 °C in a Branson Sonifier 250. The lysate was centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant was collected (referred to as soluble fraction). The pellet was washed once with lysis buffer and extracted with 8 M urea in lysis buffer of the same volume of the soluble fraction (referred to as insoluble fraction). The three fractions were subjected to SDS-PAGE and Western blot analysis.

Purification of Recombinant Chicken T-protein—All purification steps were carried out at 4 °C if not otherwise specified. A 500-ml culture of BL21(DE3)pLysS transfected with pET/MCT4 was harvested after 18 h of growth at 30 °C in the presence of 25 µM IPTG. The cells were suspended in 50 ml of ice-cold buffer A (20 mM Tris-HCl, pH 8.3, 2 mM EDTA, 1 mM DTT, 10 µM p-amidinophenylmethanesulfonyl fluoride, 10% glycerol) and held for 30 min on ice. After freezing at -80 °C, the cells were thawed and sonicated for 30 s twice at 0 °C in a Branson Sonifier 250. The lysate was centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant was collected (referred to as soluble fraction). The pellet was washed once with lysis buffer and extracted with 8 M urea in lysis buffer of the same volume of the soluble fraction (referred to as insoluble fraction). The three fractions were subjected to SDS-PAGE and Western blot analysis.

Isolation and Characterization of cDNA Clones—We have described the isolation of a cDNA clone (CT5C) containing the COOH-terminal half of T-protein from Arthrobacter globiformis (19), 5 units of chicken H-protein (1, 2.5 µg of diaphorase (Boehringer Mannheim) as L-protein, and T-protein. chicken H-protein was determined as described previously (20). The concentration mixture of chicken liver (0.5 µl) was subjected to 10% SDS-PAGE (18), and transferred to Immobilon-P (Millipore) using a semidry electroblot apparatus (Sartoblot II, Sartorius) according to the protocol given by the manufacturer. The filter was blocked for 1 h at room temperature in blocking solution (1% gelatin in TBS, containing 3 M urea at 30 °C overnight. The resulting peptides were fractionated on an ODS-120T column (4.6 × 250 mm, Tosoh, Tokyo, Japan) with acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Elution profile was monitored at 220 nm and compared with that of the authentic chicken liver T-protein processed as above.

Assay of T-protein and Kinetic Analysis—T-protein was routinely assayed in the reverse direction essentially as described previously (5). The reaction mixture (0.5 ml) contained a lysate supernatant (1% gelatin in Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), incubated for 3 h with anti-chicken T-protein antibody (2 µg/ml in blocking solution containing 3 M urea at 30 °C overnight. The resulting peptides were fractionated on an ODS-120T column (4.6 × 250 mm, Tosoh, Tokyo, Japan) with acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Elution profile was monitored at 220 nm and compared with that of the authentic chicken liver T-protein processed as above.

RESULTS

Isolation and Characterization of cDNA Clones—We have described the isolation of a cDNA clone (CT5C) containing a 675-bp insert encoding the COOH-terminal half of chicken T-protein from a Clontech library (9). Since we failed to isolate clones extending to the 5' end of the mRNA from a second library (9), we constructed two chicken liver primer-extended cDNA libraries with an oligonucleotide corresponding to nucleotides 544-567 (Fig. 4) and a mixture of two oligonucleotides corresponding to nucleotides 309-327 and 187-207 (Fig. 4), respectively, as primers. These libraries were screened with a nucleotide fragment of 658 bp containing the 5'-terminal sequence of bovine T-protein. Overlapping cDNA clones designated as CT5A, CT5A, and CT10 were isolated and characterized. The sequence at the 5'-end of the chicken T-protein mRNA, however, was only in a single cDNA clone.
Fig. 4. Nucleotide and predicted amino acid sequences of cDNA for chicken liver T-protein. The nucleotide sequence is numbered from the initiation methionine. Amino acid sequences obtained CT5C is 1228-bp-long and consists of a 19-bp 5'-flanking the first ATG matches the consensus sequence for the protein is present in positions 17-43 in the deduced amino acid sequence. The size predicted for 1-16 is a mature protein of M, 60,000. The positions and sizes of these initial 16 residues yields a mature protein of M, 60,000. The positions and sizes of these initial 16 residues yields a mature protein of M, 60,000.

Expression of Recombinant Mature T-protein in E. coli Cells—Construction of the expression vectors as described under “Experimental Procedures.” The positions and sizes (in kilobases (Kb)) of the RNA markers (Bethesda Research Laboratories) are shown on the left.

Fig. 5. Northern blot analysis of chicken liver mRNA. Poly(A)* RNA (5 µg) was analyzed by Northern blot analysis as described under “Experimental Procedures.” The positions and sizes (in kilobases (Kb)) of the RNA markers (Bethesda Research Laboratories) are shown on the left.

Expression of Recombinant Mature T-protein in E. coli Cells—Construction of the expression vectors as described under “Experimental Procedures.” The positions and sizes (in kilobases (Kb)) of the RNA markers (Bethesda Research Laboratories) are shown on the left.
A kDa

|      | Whole cell lysate | pET-MCT | pET-MCT4 | pET-MCT6 | Purified T-protein |
|------|-------------------|---------|----------|----------|-------------------|
| Lane 1 | Size markers      | S       | S        | S        | I                 |
| Lane 2 | Cell extract (20 μg) | S       | S        | S        | I                 |
| Lane 3 | Pass-through fraction from a DEAE-Sepharose column (5 μg) | S       | S        | S        | I                 |
| Lane 4 | Eluate from a DEAE-Sepharose column (2 μg) | S       | S        | S        | I                 |
| Lane 5 | Eluate from a hydroxyapatite column (2 μg) | S       | S        | S        | I                 |
| Lane 6 | Eluate from a Sephadex G-100 column (2 μg) | S       | S        | S        | I                 |
| Lane 7 | Pooled purified T-protein from chicken liver (1 μg) | S       | S        | S        | I                 |

Fig. 6. Expression of T-protein in E. coli. E. coli strain BL21(DE3)pLysS harboring expression vectors was grown, induced with IPTG, and processed for SDS-PAGE and Western blot as described under “Experimental Procedures.” Panel A, total cellular proteins and soluble and insoluble fractions of E. coli induced by 1 mM IPTG for 3 h at 37°C were analyzed by 10% SDS-PAGE. Samples from 25 μl of each cell culture and 1 μg of purified chicken liver T-protein were loaded. S and I denote soluble and insoluble fraction, respectively. Panel B, the same samples as above but from 0.2 μl of cell culture and 5 ng of purified recombinant T-protein were separated by SDS-PAGE, electrotransferred onto Immobilon-P, and immunostained with anti-T-protein antibody. Panel C, samples from 0.2 μl of each cell culture induced by 25 μM IPTG for 24 h at 30°C and 5 ng of purified recombinant T-protein were treated as above.

kcal/mgl (MCT6). SDA-PAGE and Western blot analysis indicated that the cells transfected with plasmids containing these modified DNAs expressed approximately 40-fold of T-protein compared with the native codon plasmid (Fig. 6, A and B). The recombinant T-protein in E. coli was expressed in a highly insoluble form, and less than 30% was recovered in the soluble fraction. The insoluble T-protein could be solubilized with 8 M urea, but we failed to find the proper conditions for restoring enzymatic activity. We found that the reduction of both growth temperature and concentration of IPTG with prolonged incubation facilitates the recovery of soluble T-protein. When induction was carried out in the presence of 25 μM IPTG at 30°C for 24 h, about 80% of the expressed T-protein remained soluble as shown in Fig. 6C.

Purification and Properties of the Recombinant T-protein—E. coli BL21(DE3)pLysS cells transformed with the plasmid pET/MCT4 were grown at 30°C with 25 μM IPTG. Recombinant T-protein was purified as described under “Experimental Procedures,” and the result of a typical purification was summarized in Table I. One purification procedure yielded approximately 4 mg of pure T-protein from 500 ml of the culture. Fig. 7 shows the Coomassie Brilliant Blue staining of the polypeptide patterns in each purification step. The purified protein (lane 6) had a similar electrophoretic mobility to that of chicken liver T-protein (lane 7) and was recognized by the specific antibody against chicken liver T-protein in immunoblot (not shown).

Table 1: Purification of recombinant chicken T-protein

| Purification step | Total protein | Total activity | Specific activity | Purification | Yield |
|------------------|---------------|----------------|------------------|--------------|-------|
| Cell extract     | 552 mg        | 36,648 units   | 66.4 units/mg    | 1-fold       | 100%  |
| DEAE-Sepharose   | 81.7 mg       | 31,659 units   | 388 units/mg     | 5.84         | 86.4% |
| SP-Sephadex      | 6.64 mg       | 16,683 units   | 2,513 units/mg   | 37.8         | 45.5% |
| Hydroxyapatite   | 4.73 mg       | 13,359 units   | 2,824 units/mg   | 42.5         | 36.5% |
| Sephadex G-100   | 4.10 mg       | 11,676 units   | 2,848 units/mg   | 42.9         | 31.9% |

*Activity of bacterial T-protein was subtracted.

Fig. 7. Purification of recombinant T-protein from E. coli. Cell extract and fractions from each chromatographic step were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, size markers; lane 2, cell extract (20 μg); lane 3, pass-through fraction from a DEAE-Sepharose column (5 μg); lane 4, eluate from a SP-Sephadex column (2 μg); lane 5, eluate from a hydroxyapatite column (2 μg); lane 6, eluate from a Sephadex G-100 column (2 μg); and lane 7, purified T-protein from chicken liver (1 μg).
catalyzed reverse reaction was carried out at varying concentrations of one substrate with saturated concentrations of two other substrates as described under "Experimental Procedures." The results obtained with the native and recombinant T-protein were similar (Table II), yielding \( k_{cat} \) values of 3.9 and 3.3 s\(^{-1}\), respectively. Previously, we reported a \( k_{cat} \) value for the purified chicken T-protein (Table 1 in Ref. 5), but the value was mistyped inadvertently during the copying of the data. The correct value is 5.8 s\(^{-1}\). Overall, the results of these analyses demonstrated that the cloned cDNA directs the synthesis of T-protein that is structurally and functionally equivalent to chicken T-protein.

**DISCUSSION**

We have isolated several partial cDNAs from a commercial chicken liver cDNA library and two primer-extended chicken liver λgt10 cDNA libraries. The composite cDNA sequence is 1228-bp-long and contains an open reading frame that encodes 392 amino acids of the precursor form of chicken T-protein. The deduced amino acid sequence of mature chicken T-protein is compared with that of bovine T-protein (Fig. 9). The overall homology is 67%. Chicken T-protein is a basic protein with a \( pI \) value of 9.8 (2) and contains more arginine residues than bovine T-protein in the NH\(_2\)-terminal half. Consecutive arrays of basic amino acids are found in the COOH-terminal half of both T-proteins. The regions rich in positive charges may contribute to the binding of H\(_2\)folate and/or acidic H-protein.

We used the T7 expression system described by Studier et al. (17) to express mature T-protein in *E. coli*. The pET-3a vector carries a strong T7 promoter (\( \phi 10 \) promoter) and the first 11 codons for the gene 10 protein. We joined the coding sequence for mature T-protein directly to the gene 10 initiation codon as an *NdeI* site that includes the initiation ATG. The resulting plasmid was expected to produce mature T-protein under the direction of the upstream translation signals of gene 10. The result obtained with the plasmid thus constructed (pET/MCT) was unsatisfactory (Fig. 6A). Gross et al. (22) reported that RNA secondary structure in the translational initiation region controls the expression of interferon-\( \beta \) in *E. coli* using the temperature-sensitive \( \lambda \) bacteriophage \( \psi 80 \) promoter. The rate of expression was enhanced when a stem-loop structure located downstream of the initiation codon within the initial binding site of the 30 S ribosomal subunit was modified to a form with reduced free energy. The region just downstream of the initiation codon of the nucleotide sequence for mature T-protein is rich in guanine and cytosine nucleotides. The region is, therefore, likely to form a stable secondary structure (Fig. 3). We substituted several of the guanine nucleotides with adenine nucleotides to reduce the free energy, as estimated according to Zuker and Stiegler (23), with the aid of a computer program. The modified T-protein gene variants MCT1 and MCT6 produced mature T-protein efficiently in *E. coli* (Fig. 6). Although the correlation between the stability predicted by the algorithm of Zuker and Stiegler (23) and the rate of expression has not been examined extensively, the introduction of downstream secondary structure with the relatively low free energy should be considered in the case where the production of the desired protein is disappointingly small and there are no clear reasons (such as the instability of the expressed protein) found for the low expression.

Another problem with the expression of foreign proteins in *E. coli* is the production of aggregated inactive protein that is segregated in inclusion bodies (24). This was the case in the present study, where about 70% of the expressed T-protein at 37 °C and 1 mM IPTG was insoluble and inactive even after it was solubilized with 8 M urea (Fig. 6B). To optimize the production of the soluble T-protein, lower growth temperatures at reduced IPTG concentrations were tested as discussed for the expression of rabbit muscle phosphofructokinase (25). Higher yields of the soluble, enzymatically active T-protein were obtained when the plasmid pET/MCT4 or pET/MCT6 was expressed at 30 °C with 25 \( \mu \)M IPTG. Under this condition, activity of T-protein continued to increase for some time after the immunologically detectable T-protein reached a constant level (not shown). For this reason, longer growth time is necessary for maximum accumulation of active T-protein. Proper folding of the mature form of T-protein, like other mitochondrial proteins (26, 27), may be facilitated by chaperonin. Formation of inactive aggregates of T-protein under the full inducing condition may be the result of the limitation of *E. coli* chaperonin to cope with a large amount of T-protein produced rapidly. It is also possible that chicken T-protein cannot associate with *E. coli* chaperonin, and the folding to the native structure without chaperonin is a slow process that takes place at a relatively low temperature and low concentration of T-protein.

The physical and kinetic properties of the pure recombinant T-protein were determined in this study. Recombinant T-protein has the same molecular weight and the same NH\(_2\)-terminal amino acid sequence as native chicken T-protein. The absence of a methionine residue at the NH\(_2\)-terminus is in full agreement with the predictive rule for the methionine removal from proteins in *E. coli* described by Hirel et al. (28).
Cloning and Expression of T-protein

The kinetic parameters were in the same range as those for the native T-protein (Table II). Thus, recombinant T-protein exhibits quantitatively identical properties with its native chicken counterpart.

Purification of T-protein from animal liver is time-consuming and laborious. Here we have developed a simple method to express and purify milligram quantities of chicken T-protein. The yield of the protein from bacteria is much higher than that from chicken liver mitochondria. The expression system described in this report represents a breakthrough for our future studies, including the determination of three-dimensional structure for the identification of the binding site for folate and other components of the glycine cleavage system.

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Fig. 9. Comparison of the amino acid sequence of chicken and bovine (9) T-protein. Amino acid residues are numbered beginning with NH2-terminal serine of chicken mature T-protein. Vertical lines indicate amino acid identity.