Proteolytic Degradation of Calf Thymus Terminal Deoxynucleotidyl Transferase*

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A high molecular weight preparation of terminal transferase containing 58,000- and 44,000-dalton peptides has been purified from calf thymus glands. The relationship of these terminal transferase peptides to the low molecular weight form was established with an immunoblot procedure using rabbit antibody directed against the homogeneous calf thymus low molecular weight terminal transferase (32,000 daltons). The 58,000- and 44,000-dalton enzyme species are each shown to be enzymatically active by renaturation in situ after electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate. These results suggest that the homogeneous terminal transferase previously described is derived from the higher molecular weight species by proteolysis during fractionation. Controlled degradation of the high molecular weight calf thymus terminal transferase with trypsin produces fully active enzyme containing α- and β-peptides similar to those found in the 32,000-dalton species. Isoelectric focusing experiments show a decrease of isoelectric pH of the enzyme with proteolysis.

Terminal deoxynucleotidyl transferase purified to homogeneity from calf thymus glands was originally shown to have a native $M_r = 32,000$ by equilibrium sedimentation and two peptides of $M_r = 26,500$ (β-peptide) and 8,000 (α-peptide) when analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate (1). Rabbit antibody prepared against this homogeneous enzyme preparation (2) precipitates a single 58,000-dalton peptide from cell extracts of human and mouse leukemia lines labeled with radioactive amino acids (3). A high molecular weight enzyme (62,000-dalton peptide) was also purified from human leukemic lymphoblasts (4). Various molecular weight forms of terminal transferase have been described from human leukemic cells (5) and from calf, pig, rat, and mouse thymus glands (6).

The immunoreactive peptides of terminal transferase preparations can be analyzed directly on electrophoretic blots of sodium dodecyl sulfate-polyacrylamide gels in the procedure described by Towbin et al. (7). Using this procedure we found a 58- to 60,000-dalton peptide to be the major immunoreactive peptide present in crude terminal transferase preparations from calf thymus, rat thymus, mouse thymus, chicken thymus, cat thymus, human 8402 cells, and mouse P1798 cells (8).

These results suggested that our original preparation of homogeneous calf thymus terminal transferase might be a proteolytically degraded form of the 58,000-dalton peptide, providing a satisfying generality in that terminal transferase from human, bovine, rat, chicken, cat, and mouse sources are immunologically related and have similar conserved polypeptide molecular weights.

In this presentation we demonstrate that a calf thymus gland terminal transferase preparation containing only the high molecular weight peptides can be converted to the active low molecular weight terminal transferase (containing α- and β-peptides) by degradation with trypsin. The 32,000-dalton terminal transferase produced by trypsin degradation has the same isoelectric pH as the homogeneous low molecular weight terminal transferase.

**EXPERIMENTAL PROCEDURES**

**Assay for Terminal Transferase—**Enzyme activity was assayed using d[α³²P]TP as initiator and [³H]GTP as substrate, as previously described (9). One enzyme unit is defined as the amount of enzyme required to polymerize 1 nmol of [³H]GTP into acid-insoluble material in 1 h. Immunoreactive peptides of terminal transferase were detected using the immunoblot procedure described by Towbin et al. (7). The specific reagents and the procedure used were as previously described (8).

**Purification of High Molecular Weight Terminal Transferase from Calf Thymus Glands—**High molecular terminal transferase was purified from 2 kg of frozen calf thymus glands. In addition to monitoring enzyme activity, enrichment of the high molecular weight terminal transferase peptides during purification was monitored by the immunoblot procedure. All purification steps were carried out at 4 °C.

**Crude Extract—**Calf thymus glands were homogenized in a Waring Blendor in 8 liters of 0.04 M potassium phosphate buffer at pH 7.4 with 0.04 M NaCl, 1 mM phenylmethanesulfonyl fluoride, and 1% dimethyl sulfoxide. The homogenate was centrifugated by centrifugation for 10 min at 5000 rpm in an HG-4 rotor in a Sorvall RC-3 centrifuge. The supernatant was filtered through 4 layers of cheesecloth, providing 8.3 liters of crude extract.

**Protamine Sulfate Precipitation of Nucleic Acid—**The nucleic acid in the crude extract was precipitated by addition of 410 ml of 3% protamine sulfate solution to the extract with mixing, followed by removal of the precipitate by centrifugation for 15 min at 5000 rpm in an HG-4 rotor in a Sorvall RC-3 centrifuge. The supernatant solution was filtered through 4 layers of cheesecloth, providing 7.7 liters of clear protamine sulfate supernatant.

**Ammonium Sulfate Fractionation—**Solid ammonium sulfate was added to the protamine sulfate supernatant to 30% saturation, and the solution was allowed to stand for 1 h at 4 °C. The precipitate formed was removed by mixing in 75 g of Hypflo-Supercel (diatomaceous earth)/liter and then filtered through a pad of Hypflo-Supercel on S & S no. 410 filter paper on a Buchner funnel using gentle suction. Solid ammonium sulfate was added to the filtrate to 35% saturation and mixing was continued for 30 min. The solution was allowed to...

1 The abbreviations used for nucleotides and polynucleotides are those suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) (1970) J. Biol. Chem. 245: 5171-5175.
stand for 2 h at 4 °C. The precipitate formed was collected by centrifugation for 15 min at 8500 rpm in a GSA rotor in a Sorvall RC-5 centrifuge, and redissolved in 1 M potassium phosphate at pH 7.4 and 10 mM 2-mercaptoethanol. The redissolved ammonium sulfate fraction was clarified by centrifugation at 8500 rpm in a GSA rotor yielding 90 ml of clarified 30–55% saturated ammonium sulfate fraction.

**Isouleinuclease-Sephrose Column**—The clarified ammonium sulfate fraction was divided into two halves, and each half was loaded directly onto an isouleinuclease-4B (11) column (2.6 x 30 cm) previously equilibrated with 1 M ammonium sulfate in 0.2 M potassium phosphate at pH 7.4 containing 10 mM 2-mercaptoethanol. Each column was washed with 500 ml of column buffer and protein was eluted from the column buffer, and 10% glycerol as a broad peak at pH 7.4 and 10 mM 2-mercaptoethanol. The active fractions eluting from the isouleinuclease-Sephrose columns were pooled, producing 306 ml of isouleinuclease-Sepharose fraction.

**Phosphocellulose Chromatography**—The isouleinuclease-Sepharose fraction was dialyzed overnight against two 4-liter changes of 0.05 M potassium phosphate at pH 7.4 in 0.25 M NaCl, 50 mM Hepes buffer at pH 7.5, and 10% glycerol. The fractions containing terminal transferase activity were pooled, and the volume was 68 ml. To remove 2-mercaptoethanol protein was precipitated by addition of solid ammonium sulfate to 60% saturation, and the precipitates were redissolved in 45 ml of 0.5 M NaCl in 25 mM potassium phosphate at pH 7.4, 10 mM 2-mercaptoethanol, and 10% glycerol.

**Gel Filtration on Sepharose 6B**—The ammonium sulfate-concentrated phosphocellulose fraction was fractionated on a Sepharose 6B column (2.6 x 90 cm) in 0.5 M NaCl, 10 mM 2-mercaptoethanol, 25 mM potassium phosphate at pH 7.4 and 10% glycerol. The fractions containing terminal transferase activity were pooled, and the volume was 68 ml. To remove 2-mercaptoethanol protein was precipitated by addition of solid ammonium sulfate to 60% saturation, and the precipitates were redissolved in 45 ml of 50 ml Hepes' buffer at pH 7.5.

**Dithionitrobenzoate: Thiopropyl-Sepharose Column Connected to Phosphocellulose Column**—A thiopropyl-Sepharose 6B (Pharmacia) column (1 x 6 cm) was first exchanged with 5 mg/ml of 5,5'-dithio-bis(2-nitrobenzoic acid) in 50 mM sodium phosphate at pH 7.4 and 1 mM EDTA, then equilibrated with 0.2 M ammonium sulfate in 50 mM Hepes buffer at pH 7.5. The concentrated Sepharose 6B pool was loaded onto the dithionitrobenzoate:thiopropyl Sepharose column. The column was washed with 50 ml of 0.2 M ammonium sulfate column as one major peak at about 0.3 M NaCl. The active fractions were pooled (340 ml) and protein in this fraction was salted out by addition of solid ammonium sulfate to 60% saturation. The protein precipitate was collected by centrifugation and redissolved in 10 ml of 0.5 M NaCl in 25 mM potassium phosphate at pH 7.4, 10 mM 2-mercaptoethanol, and 10% glycerol.

**Renaturation of Calf Thymus Terminal Transferase after Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate**—The detection of terminal transferase activity after polyacrylamide gel electrophoresis was carried out by a modification of the procedure described by Spanos et al. (10). The enzyme fractions were separated on a 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (13) and 0.15 mg/ml of activated calf thymus DNA. After electrophoresis, the gel was washed with 3 changes of 2 liters of 50 mM sodium phosphate at pH 7.4 in 10 mM 2-mercaptoethanol for a total of 2 h at room temperature to remove sodium dodecyl sulfate. The gel was then equilibrated with 2 changes of 500 ml of 6 M guanidine HCl in 50 mM sodium phosphate at pH 7.4 and 10 mM 2-mercaptoethanol at room temperature for a total of 1 h. Guanidine HCl was removed by washing with 50 mM sodium phosphate at pH 7.4 in 10 mM 2-mercaptoethanol, and the gel was equilibrated with 0.2 M potassium cacodylate buffer at pH 7.2, 1 mM 2-mercaptoethanol, and 8 mM MgCl₂. The gel was then transferred into a 30-ml reaction mixture containing 0.2 M potassium cacodylate buffer at pH 7.2, 1 mM 2-mercaptoethanol, 8 mM MgCl₂, and 0.05 mM [α-³²P]dATP at 0.25 μCi/nmol. Incubation was at 35 °C for 17 h. After the enzyme reaction, the gel was detected on the washed gel by exposing it to Kodak X-O-X AR5 film for 14 h at 4 °C. The gel was then stained with Coomassie blue to locate protein bands present in the enzyme fractions and the protein standards. The protein standards used were bovine serum albumin, rabbit IgG, ovalbumin, and whale myoglobin.

**Isoelectric Focusing of Terminal Transferase on Glycerol Gradients**—Isoelectric focusing of terminal transferase was carried out in 10 to 40% (v/v) glycerol gradients containing 2% Ampholine in the pH range of 6 to 10 in a glass tube. A 0.75 ml 15% polyacrylamide plug containing 2% Ampholine (pH range 6 to 8) was focused for 1.5 h in the bottom of the tube and 0.5 ml of 2% Ampholine (pH range 5 to 10) in 40, 50, 35, 30, 25, 20, 15, and 10% glycerol was added sequentially into the tube. An aliquot of terminal transferase and 25 μg of horse heart cytochrome c were mixed in the 25% glycerol layer of the gradient. The density gradient was formed by diffusion for 16 h at 4 °C. Isoelectric focusing was carried out in a Buchler electrophoresis apparatus for 3.5 h at 4 °C at 800 V with 0.2 M NaOH and 0.2 M acetic acid as cathode and anode solutions, respectively. After focusing, the cathode solution was removed from the top of the tube and the gradients were fractionated from the top into 40 equal volume fractions using a Buchler Densi-Flow apparatus. Immediately after fractionation of the gradient, an aliquot of each fraction was diluted with 9 volumes of H₂O for pH determination. Terminal transferase activity in each fraction was assayed as previously described (9).

**RESULTS**

**Purification of High Molecular Weight Terminal Transferase from Calf Thymus Gland**—During purification of the high molecular weight terminal transferase from calf thymus glands, both enzyme activity and the presence of high molecular weight immunoreactive peptides of terminal transferase were monitored. When enzyme pools were made, the fractions having terminal transferase activity but enriched in low molecular weight (e.g. M, = 26,000 or lower) terminal transferase peptides were discarded. A summary of the purification scheme is shown in Table I.

In this purification procedure we have expressed enzyme activity in dGTP units, which are about 4 times as great as dATP activity used in our earlier study (1). Thus, we are describing the same amount of total terminal transferase activity present in higher molecular weight forms. Three steps included in the new purification scheme are devised to remove low molecular weight terminal transferase from the enzyme. Separate analytical experiments showed that: 1) purified low molecular weight terminal transferase cannot be precipitated from solution at 55% saturation of ammonium sulfate.

²The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.
ious molecular weight forms of terminal transferase from calf thymus glands. Since the var-
eteolysis occurs during purification until the enzyme has been
extracts made from fresh tissue. In any case, continued pro-

These multiple species representing minor differences in mo-
activity peak while the lower molecular weight peptides
of the column shows that higher molecular weight peptides
in the low molecular weight preparation shows renaturable
duce in situ on a polyacrylamide gel, both the 58,000- and the 44,000-
dalton peptides produce acid-insoluble deoxynucleotide prod-
ons are not enzymatically active, the procedure de-

TABLE I

<table>
<thead>
<tr>
<th>Purification of high molecular weight terminal transferase from calf thymus glands</th>
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<tr>
<td>Enzyme fraction</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>I. Crude extract</td>
</tr>
<tr>
<td>II. Protamine sulfate supernatant</td>
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<tr>
<td>III. 30–55% (NH₄)₂SO₄</td>
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| IV. Isoleucine-Sepha-
rose | 5,400              | 354                       | 1.91 × 10⁶               |
| V. Phosphocellulose | 367               | 4,930                     | 1.81 × 10⁶               |
| VI. Sepharose 6B | 90                 | 16,700                    | 1.52 × 10⁶               |
| VII. Dithionitroben-
zoate-thiopropyl-
Sepharose-phos-
phocellulose | 39.3              | 22,700                    | 0.89 × 10⁶               |
| VIII. Hydroxylapatite | 9.04              | 98,500                    | 0.89 × 10⁶               |

Purified terminal transferase protein (5 μg) was separated on two separate 15% polyacrylamide gels in the presence of sodium dodecyl sulfate. After electrophoresis, one gel was stained with Coomassie blue and the other gel was used for immunoblotting as described under “Experimental Procedures.” Lane A is the stained gel, and lane B shows the immunoblot. The markers used are bovine serum albumin, rabbit IgG, ovalbumin, and whale myoglobin.

Fig. 1. Polyacrylamide gel and immunoblot analysis of high molecular weight terminal transferase. Purified terminal transferase protein (5 μg) was separated on two separate 15% polyacrylamide gels in the presence of sodium dodecyl sulfate. After electrophoresis, one gel was stained with Coomassie blue and the other gel was used for immunoblotting as described under “Experimental Procedures.” Lane A is the stained gel, and lane B shows the immunoblot. The markers used are bovine serum albumin, rabbit IgG, ovalbumin, and whale myoglobin.

The purification procedure described starts with frozen calf thymus glands. Low molecular weight immunoreactive peptides can be detected in the crude extract, but it is not known at the present whether these forms of the enzyme have any biological significance. While it is possible that these forms arise during storage of the glands, they are also found in extracts made from fresh tissue. In any case, continued proteolysis occurs during purification until the enzyme has been eluted from the isoleucine-Sepharose column. Since the various molecular weight forms of terminal transferase from calf thymus glands are enzymatically active, the procedure described in this report produces an enzyme fraction enriched in the high molecular weight (Mᵣ = 58,000 and 44,000) terminal transferase peptides, but not a pure enzyme. The enzyme protein is estimated to be about 50% of the total protein present in the final preparation.

Demonstration that the 58,000- and 44,000-dalton Immunoreactive Peptides Have Terminal Transferase Activity—Since the antibody used for detection of immunoreactive peptides was prepared using homogeneous calf thymus enzyme (32,000 daltons), it is reasonable to conclude that the 58,000- and 44,000-dalton immunoreactive peptides are precursors of the low molecular weight species. Direct demonstration that these high molecular weight peptides contain terminal transferase activity was accomplished by demonstration of enzyme activity in situ in polyacrylamide gels after electrophoresis in the presence of sodium dodecyl sulfate. When the calf thymus terminal transferase preparation containing high molecular weight, immunoreactive peptides (Fig. 1) was analyzed for terminal transferase activity after renaturation in situ on a polyacrylamide gel, both the 58,000- and the 44,000-dalton peptides produce acid-insoluble deoxynucleotide products with the 58,000- and 56,000-dalton peptides (also the 44,000- and 42,000-dalton peptides) showing up as a doublet (Fig. 2, lane A). The 26,000-dalton peptide of the low molecular weight enzyme, on the other hand, cannot be renatured to active enzyme by the technique used (Fig. 2, lanes B and B'), suggesting that both the 26,000-dalton (β-peptide) and the 10,000-dalton (α-peptide) peptides of the low molecular weight enzyme are required for enzyme activity. A minor peptide contaminant with Mᵣ = 44,000 (Fig. 2, lanes B and B') in the low molecular weight preparation shows renaturable activity. The purified terminal transferase preparations used produce no radioactive products from [α-³²P]dATP in this procedure if DNA is omitted from the gel.
weight terminal transferase is analyzed, the isoelectric pH of the bulk of the enzyme is 7.4 (Fig. 3A). This preparation of low molecular weight terminal transferase contains about 10% of the 44,000-dalton form having an isoelectric pH of 8.2 (Fig. 2, lane B and Fig. 3A). When the purified high molecular weight calf thymus terminal transferase is focused on the gradient, the bulk of the enzyme activity is found at pH 8.6 with a shoulder of activity at pH 8.2 (Fig. 3B). These results suggest that the peptides removed by proteolysis are somewhat enriched in basic amino acids.

The starting materials for the isoelectric focusing experiment are the high molecular weight enzyme preparation described in this communication and a low molecular weight enzyme prepared essentially as previously described (1). When this low molecular weight terminal transferase preparation is analyzed by polyacrylamide gel electrophoresis, the predominant stained bands are at 26,000 and 10,000 daltons with a minor band at 44,000 daltons (Fig. 2, lane B'). When the same preparation is analyzed by the immunoblot procedure, an additional band at 24,000 daltons is detected, and the 10,000-dalton band appears quite broad (Fig. 4, lane A). The immunoreactive bands found in the enzyme after isoelectric focusing

Fig. 2. Renaturation of terminal transferase on polyacrylamide gel after electrophoresis in the presence of sodium dodecyl sulfate. Purified high molecular weight (500 units) and low molecular weight (1500 units) terminal transferase were separated on a 12% polyacrylamide gel containing activated calf thymus DNA in the presence of sodium dodecyl sulfate. The renaturation procedure and the terminal transferase reaction are described under “Experimental Procedures.” Lane A shows the activity of the high molecular weight terminal transferase (Fig. 1) after renaturation on the gel. Lane B shows the activity of the low molecular weight enzyme after renaturation on the gel. Lane B' shows the Coomassie blue stain of the gel shown in lane B.

Although the published procedure for renaturing DNA polymerase activity after electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (10) works well for DNA polymerase-β (14), renaturation of terminal transferase required some changes of procedure. No terminal transferase activity can be detected on the gel unless it is treated with 6 M guanidine HCl after the removal of sodium dodecyl sulfate. It is possible that the high molecular weight terminal transferase peptides are not completely unfolded during electrophoresis and the treatment with guanidine HCl results in complete unfolding prior to proper refolding of the enzyme. Even with the guanidine HCl treatment, the efficiencies of renaturation of the 58,000-, 56,000-, and 44,000-dalton peptides are not the same. There is more 58,000-dalton peptide than 44,000-dalton peptide in the enzyme preparation and the 56,000-dalton peptide is scarcely detectable on the stained gel (Fig. 1), yet the amount of terminal transferase product formed by the 44,000-dalton peptide is much greater than that formed by the 58,000-dalton peptide, and the 56,000-dalton peptide appears to be as active as the 58,000-dalton peptide after the renaturation process. Because of the difference in efficiency of renaturation of terminal transferase peptides, it is conceivable that the 26,000-dalton peptide does contain the entire sequence of the active site of the enzyme but is not properly refolded in the renaturation process.

Isoelectric pH of Various Species of Calf Thymus Terminal Transferase—From the behavior of the high molecular weight species of terminal transferase during purification, we believe that the portion of enzyme lost due to proteolysis is hydrophobic and contains accessible sulfhydryl groups. When purified high molecular weight and low molecular weight enzymes are analyzed on isoelectric focusing gradients, a major difference in pH is observed (Fig. 3). When the low molecular weight terminal transferase is analyzed, the isoelectric pH of the bulk of the enzyme is 7.4 (Fig. 3A). This preparation of low molecular weight terminal transferase contains about 10% of the 44,000-dalton form having an isoelectric pH of 8.2 (Fig. 2, lane B and Fig. 3A). When the purified high molecular weight calf thymus terminal transferase is focused on the gradient, the bulk of the enzyme activity is found at pH 8.6 with a shoulder of activity at pH 8.2 (Fig. 3B). These results suggest that the peptides removed by proteolysis are somewhat enriched in basic amino acids.

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Fig. 3. Isoelectric forms of the low molecular weight and the high molecular weight terminal transferases. Purified terminal transferases (1250 units each) were separated by isoelectric focusing on glycerol gradients as described under “Experimental Procedures.” After the gradients have been fractionated, 5 μl of each fraction was assayed in a 55-μl terminal transferase reaction using [β-3H]dGTP at 20 counts/pmol (9). Incubation was for 30 min at 35 °C. At the end of the reaction, 40 μl of the reaction mixture was processed for acid-insoluble radioactive material. The pH of each fraction was measured after diluting 10 μl of each fraction with 90 μl of H2O immediately after the fractionation of the gradient. A shows the isoelectric focusing gradient profile of the low molecular terminal transferase; B shows the profile of the high molecular weight terminal transferase.
are at 26,000, 24,000, and 10,000 daltons (Fig. 4, lane C). The presence of both 26,000- and 24,000-dalton species may account for the broad peak observed in the pH gradient activity profile (Fig. 3A).

When the peak fraction of the focused high molecular weight terminal transferase (Fig. 4, lane B) was analyzed on immunoblots only the 58,000-dalton peptide was found (Fig. 4, lane D), suggesting that the native calf thymus terminal transferase is a rather basic protein with an isoelectric pH at 8.6. The 44,000-dalton species appears to have an isoelectric pH at about 8.2 as judged by the minor activity peak seen when the low molecular weight enzyme is focused (Fig. 3A). Immunoblot analysis of the shoulder of the activity peak in the focusing gradient of the high molecular weight calf thymus terminal transferase (Fig. 3B) also shows the 44,000-dalton peptide (Fig. 4, lane E) as well as overlapping 58,000- and 56,000-dalton peptides.

**Generation of Active Low Molecular Weight Terminal Transferase by Controlled Proteolysis**—Calf thymus terminal transferase activity is relatively resistant to trypsin degradation. When the high molecular weight terminal transferase at 200 μg/ml is incubated with 35 μg/ml of trypsin no activity is lost during the first 20 min of incubation. After 60 min of incubation with trypsin, about 60% of the enzyme activity still remains (data not shown). Immunoblot analysis of the trypsin-degraded samples shows essentially complete conversion of the high molecular species to the 26,000- and the 10,000-dalton peptides prior to any loss in enzyme activity (Fig. 5, lane D). Kinetic analysis using the immunoblot procedure shows that both the 58,000- and the 44,000-dalton species are extremely sensitive to degradation by trypsin (Fig. 5, lane B), and the loss of these two peptides gives rise to the appearance of transient peptides of 56, 42, 32, and 14,000 daltons as well as the 26,000- and the 10,000-dalton peptides (Fig. 5, lanes B and C). Enzyme activity loss is observed only when the 26,000- and 10,000-dalton peptides are further degraded (data not shown).

Examination of the immunoreactive peptides during trypsin degradation suggests that trypsin mimics the action of endogenous proteases in the extracts of calf thymus glands. Results obtained from isoelectric focusing of the trypsin-degraded high molecular terminal transferase support this hypothesis (Fig. 6). The isoelectric focusing profile of the high molecular weight enzyme is illustrated in Fig. 6A. Fig. 6B shows the profile of activity after a brief treatment with trypsin and Fig. 6C shows the profile after prolonged degradation with trypsin. During early stages of trypsin degradation, small amounts of the 44,000- and 42,000-dalton species still remain with an isoelectric pH at about 8.2, while the bulk of the enzyme activity is focused at pH 7.4 (Fig. 6B). With prolonged degradation, net loss in enzyme activity is observed but the isoelectric pH of the residual enzyme activity remains at 7.4.

Kinetic analysis of the trypsin degradation of terminal transferase using the immunoblot procedure (Fig. 5, lanes A and B) and the procedure to renature terminal transferase in polyacrylamide gel (Fig. 7) both show that the 38,000- and 44,000-dalton peptides are exquisitely sensitive to trypsin conversion to 56,000- and 42,000-dalton peptides. In the experiment shown in Fig. 7, no net loss of enzyme activity was observed during trypsin degradation prior to denaturation for gel electrophoresis. The only terminal transferase peptide that can be renatured following polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate migrates as 58-, 56-, 44-, and 42,000-dalton peptides (Fig. 7). From the results shown in Fig. 5, it is obvious that other immunoreactive peptides are produced during the course of trypsin degradation shown in Fig. 7. Peptides produced by trypsin degradation having molecular weights less than 42,000 daltons do not exhibit renaturation in the gels. These results confirm the previous experiment, shown in Fig. 2, lanes B and B', which indicated that the two-chain structure of terminal transferase cannot be renatured after separation of the peptides on polyacrylamide gel. This observation is expected if both chains are...
Fig. 7. Renaturation of trypsin-generated terminal transferase peptides. High molecular weight terminal transferase at 200 pg/ml was incubated with 20 μg/ml of trypsin at 35 °C in 50 mM Hepes buffer at pH 7.5. Samples were removed at 0 min (lane A), 30 s (lane B), 1 min (lane C), 2 min (lane D), and 30 min (lane E), and trypsin activity was terminated with soybean trypsin inhibitor. The terminal transferase peptides were separated by polyacrylamide gel electrophoresis and the enzyme was renatured and assayed on the gel as described under "Experimental Procedures" except that 1 μg/ml of soybean trypsin inhibitor was included in all wash buffer solutions and 10 μg/ml of soybean trypsin inhibitor was added to the terminal transferase reaction mixture.

required for activity. The results shown in Fig. 7 further suggest that the α-peptide of the molecular weight enzyme is cleaved from the high molecular weight terminal transferase at the 44- and 42,000-dalton stage during proteolysis.

DISCUSSION

The demonstration of the 58,000-dalton terminal transferase peptide in extracts of calf thymus gland using the immunoblot technique with an antibody against homogeneous low molecular weight calf thymus terminal transferase (3) raises the possibility that the low molecular weight form of the enzyme is derived from the 58,000-dalton form by proteolysis. Whether proteolytic degradation of terminal transferase occurs as a natural biological process within cells is not known at this point. Calf thymus terminal transferase does appear to be rather susceptible to proteolysis during fractionation of extracts and the degradation does not appear to be random. By comparing the immunoreactive peptides in immunoblots of fractions in early stages of purification and in purified enzyme fractions, it is possible to order the appearance of various immunoreactive peptides. The first peptide derived from 58,000-dalton terminal transferase is the 56,000-dalton peptide, followed by the doublet at 44,000 and 42,000 daltons. In other preparations of purified high molecular weight terminal transferase we have seen another doublet at 34,000 and 32,000 daltons, but we have not been successful in demonstrating renaturation of these species. Purified (essentially homogeneous) low molecular weight terminal transferase preparations frequently contain a minor 24,000-dalton peptide as well as the 26,000-dalton β-peptide. The β-peptide in extensively degraded terminal transferase preparations is at 24,000 daltons. The pattern of doublets suggests the presence of a sensitive bond about 20 amino acids from one terminus of the 58,000-dalton terminal transferase peptide, and this is consistent with the recurring doublet pattern seen upon trypsin degradation. One possible scheme for proteolytic degradation of terminal transferase in calf thymus extract is shown in Fig. 8 for discussion purposes.

The generation of the 26,000-dalton β-peptide and the 8,000- to 10,000-dalton α-peptide by trypsin suggests that an analogous mechanism of proteolysis is occurring in calf thymus extract, that is, predominately produced by serine proteases. The identity of the degraded peptides in the two procedures is not clearly established, but the transient peptides observed during trypsin degradation of the high molecular weight species support this hypothesis. The exact nature of the α-peptide is not easy to define since steps 1, 2, and 3 (Fig. 8) can produce
a set of peptides in the same molecular weight range as the \( \alpha \)-peptide. It is possible that continued proteolysis results in total or partial loss of two of the peptides generated by these three steps since \( \text{NH}_2 \)-terminal analysis of the \( \alpha \)-peptide of the homogeneous enzyme shows it to be cysteine. We cannot exclude the possibility that the \( \alpha \)-peptide of the homogeneous terminal transferase is composed of a mixture of peptides averaging 10,000 daltons.

The chemical properties of the high molecular weight and low molecular weight form of the enzyme are quite different, although both forms retain full enzyme activity. From the differences in their binding properties to the hydrophobic column and the thiopropyl-Sepharose column, it appears that the inactive portion of terminal transferase peptide lost by the proteolytic cleavage is hydrophobic and has accessible sulfhydryl groups. The difference in isoelectric pH values of the 58,000-dalton enzyme (pI = 8.6) and the 32,000-dalton enzyme (pI = 7.4) suggests that the portion lost is also quite basic. The conversion of 58,000-dalton peptide to the 44,000-dalton peptide resulted in a decrease of 0.4 pH unit in pI of the enzyme, and the conversion of 44,000-dalton peptide to the \( \alpha \)- and \( \beta \)-peptides of the low molecular weight enzyme resulted in a further decrease of 0.8 pH unit. These observations suggest that most and perhaps all of the small peptides generated in the degradation are basic peptides.

Renaturation of peptides after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that both 58,000- and 56,000-dalton, and 44,000- and 42,000-dalton peptides are enzymatically active. This rules out the possibilities that the \( \alpha \)-peptide arose from the 14,000-dalton fragment and that part of the active site of the enzyme is on the 2,000-dalton peptide. Because the 32,000-dalton immunoreactive peptide generated during trypsin degradation does not appear to contain the entire active site of the enzyme, it seems reasonable to propose that the low molecular weight \( \alpha \)-peptide is formed by proteolytic cleavage of the 44,000-dalton (or 42,000-dalton) peptide to the 34,000-dalton (or 32,000-dalton) peptide, which is step 2 of the proposed degradation scheme. Further removal of basic residues from the 10,000-dalton peptide and the 34,000-dalton (32,000-dalton) peptide generated by step 2 of the degradation scheme would result in the decrease in pI observed when terminal transferase is converted from 44,000 daltons to the low molecular weight terminal transferase (\( \alpha \)- and \( \beta \)-peptides).

The proposed scheme indicates how sequential degradation of the 58,000-dalton terminal transferase peptide might lead to the \( \alpha \)- and \( \beta \)-peptides of the homogeneous low molecular weight terminal transferase. It is likely that the degradation occurs also by other pathways such as the direct generation of \( \beta \)-peptide from the 58,000-, 56,000-, and 44,000-, and 42,000-dalton peptides. The \( \alpha \)-peptide would have to be derived from other portions of the cleaved peptides.

When terminal transferase activity from crude calf thymus extract is analyzed directly by chromatography on a phosphocellulose column, enzyme activity elutes as a sharp peak and the immunoreactive peptide is found to be predominately at 58,000 daltons (8). After the more extensive manipulation in the large scale purification presented in this communication, the activity elutes as a broad peak when rechromatographed on phosphocellulose. The heterogeneity induced in calf thymus terminal transferase during purification is probably due to the different active peptide species present and is quite different from the chromatographic heterogeneity observed with terminal transferase activity present in human lymphoblastoid cells (8). The different chromatographic forms of terminal transferase found in human lymphoblastoid cells all have the same 58,000-dalton peptide, and the heterogeneity here is most likely due to other kinds of charge modification, such as glycosylation, phosphorylation, etc.

Experiments on autolysis (utilizing endogeneous proteases) of terminal transferase peptides in the crude unfractionated calf thymus extracts produced no clear pattern of degradation. The lack of significant proteolysis by self-digestion of these extracts could indicate the presence of protease inhibitors. Proteolytic activity is readily demonstrated after addition of sodium dodecyl sulfate to the extract. The peptides formed during autolysis in detergent have different and much lower molecular weights than those observed during purification. Early steps in the purification of terminal transferase appear to remove these protease inhibitors and result in gradual, rather specific, degradation of the native terminal transferase peptide. The degradation of terminal transferase peptides can be easily monitored with the immunoblot technique during initial stages of purification. Examination of other peptides present in the fractions by Coomassie blue staining of polyacrylamide gels shows that proteolysis is not general since major peptides present in the calf thymus extract are not degraded. This rather specific proteolysis of terminal transferase protein may be related in the in vivo function of this enzyme which is present only in progenitor lymphoid cells.

We believe that our current studies resolve a major question about the various molecular weight species of terminal transferase that have been described in purified form (1, 4–6) by suggesting that all forms arise by proteolysis of the 58,000-dalton peptide. The immunoblot procedure and renaturation gels are most useful in establishing this point. Charge heterogeneity in specimens exhibiting only the 58,000-dalton species (3, 8) still requires further explanation.

REFERENCES
Proteolytic degradation of calf thymus terminal deoxynucleotidyl transferase.
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