Cathepsin T (Convertase) Generates the Multiple Forms of Tyrosine Aminotransferase by Limited Proteolysis†

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ABSTRACT: A lysosomal enzyme first termed "convertase", and now cathepsin T [Gohda, E., & Pitot, H. C. (1981b) *J. Biol. Chem. 256*, 2567], that generates the multiple forms of tyrosine aminotransferase has been characterized with respect to its action on various proteins. When cathepsin T was incubated with highly purified tyrosine aminotransferase I, tyrosine aminotransferase III and a peptide of about 4500 daltons were generated, and no amino acids were released. Incubation of $^{32}P_i$-labeled tyrosine aminotransferase I with cathepsin T generated $^{32}P_i$-labeled tyrosine aminotransferase III; no ^{32}P was found in the region of the peptide after electrophoresis on 15% polyacrylamide gels. Cathepsin T did not act on the cytosolic or mitochondrial forms of aspartate aminotransferase, nor on β-actin. When incubated with a heat-stable fraction of ^{35}S-labeled proteins from hepatoma cell cytosol, cathepsin T caused complete conversion of the 53 000-dalton monomer of tyrosine aminotransferase to the 49 000-dalton monomer as detected by two-dimensional electrophoresis. A number of other proteins were also converted to smaller entities. The peptide from tyrosine aminotransferase was purified by passage over a Sephadex column, and its amino acid composition was determined. It appeared to be generated from the carboxyl terminus, and its acidic nature accounted for the different isoelectric points of the 53 000- and 49 000-dalton subunits of tyrosine aminotransferase (5.6 and 5.9, respectively). This work confirms that cathepsin T is an endopeptidase and that the multiple forms of tyrosine aminotransferase are products of proteolysis.

Three forms of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) are found in extracts of rat liver and are separable by chromatography on hydroxylapatite or CM-Sephadex C-50 (Kenney, 1962; Iwasaki et al., 1973; Johnson et al., 1973). These forms differ in abundance when isolated under differing extraction conditions (Johnson & Grossman, 1974; Rodriguez & Pitot, 1975). The origin of the multiple forms has been attributed to carbamylation (Johnson et al., 1973), phosphorylation (Smith et al., 1976), and proteolysis (Hargrove et al., 1980; Gohda & Pitot, 1980), and a difference in titratable sulfhydryl groups has been noted (Rodriguez & Pitot, 1976). Recently, we have purified the three forms separately and demonstrated that they differ in molecular weight. The forms are designated I, II, and III in order of their elution from hydroxylapatite; form I is comprised of two 53 000-dalton monomers, form III has two 49 000-dalton monomers, and form II contains one of each sized monomer (Hargrove et al., 1980). Gohda & Pitot (1980) have purified the factor that causes the conversion and demonstrated the generation of forms II and III in vitro from a highly purified preparation of form I. Their further studies demonstrated that the factor has the properties of a novel thiol proteinase for which they have proposed the name cathepsin T (Gohda & Pitot, 1981a).

The proteolytic characteristics of cathepsin T and the difference in molecular weight of multiple forms of tyrosine aminotransferase indicate that the forms probably originate through limited proteolysis, although this has not been demonstrated directly. Since tyrosine aminotransferase is degraded with a half-time of 2 h in vivo, the multiple forms may be part of the degradation pathway of this enzyme, or their formation may only indicate a general susceptibility of this enzyme to proteases. The results of the present study demonstrate that cathepsin T released a low molecular weight peptide from tyrosine aminotransferase during the conversion of form I to form III. The cathepsin T catalyzed conversion of the 53 000-dalton subunit of tyrosine aminotransferase to the 49 000-dalton subunit did not alter the $^{32}P_i$ content of radiolabeled enzyme nor was a $^{32}P_i$-labeled peptide found. These studies demonstrate that the multiple forms of tyrosine aminotransferase arise through the endoproteolytic activity of cathepsin T.

Materials and Methods

Materials. Tyrosine aminotransferase I (EC 2.6.1.5) was purified from rat livers as previously described (Hargrove & Granner, 1980) or by the modified procedure described below. Cathepsin T was obtained from rat kidney as described by Gohda & Pitot (1981b). 9α-Fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16α,21-diacetate (triamcinolone)[1] was obtained from Lederle, DEAE-cellulose was purchased from Whatman, and CM-Sephadex C-50 and Sephadex G-75 were from Sigma. Reagents for polyacrylamide gel electrophoresis, including protein standards for M_r determination, were from Bio-Rad, and ampholytes were from LKB. Protein standards used to estimate molecular weights were as follows: insulin B chain, M_r 3600; lysozyme, M_r

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[1] Abbreviations: triamcinolone, 9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16α,21-diacetate; dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione; HTC cells, hepatoma tissue culture cells; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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14 300; soybean trypsin inhibitor, M_r 21 000; carbonic anhydrase, M_r 30 000; ovalbumin, M_r 43 000; bovine serum albumin, M_r 68 000; phosphorylase B, M_r 94 000. HTC cells were originally obtained from Dr. Brad Thompson and have been maintained in continuous culture in this laboratory for 13 years. [^{35}S]Methionine (>700 Ci/mmol) was from New England Nuclear, and [^{32}P]orthophosphate (carrier free) was from Amersham.

Purification of Tyrosine Aminotransferase I. The following procedure was adopted to produce tyrosine aminotransferase I without generating the other forms of the enzyme: rats were injected intraperitoneally with 10 mg of triamcinolone per 100 g body weight at 10:00–11:00 p.m. and killed by decapitation 8 h later. The livers were excised, rinsed briefly in ice-cold 0.15 M KCl, and placed ten at a time in a Waring blender at room temperature. The livers were homogenized with two 30-s bursts at a rheostat setting of 70 V in 500 mL of extraction buffer (50 mM Hepes buffer, pH 8.0, containing 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 2.5 mM α-ketoglutaric acid, and 0.1 mM each of pyridoxal 5′-phosphate and phenylmethanesulfonyl fluoride). This homogenate was immediately decanted into a 1-L beaker, placed in a 70 °C water bath, and stirred until the temperatured reached 60 °C. The heating continued for 5 min, whereupon the extracts were cooled in a −8 °C brine and purified over DEAE-cellulose and CM-Sephadex C-50 as previously described (Hargrove & Granner, 1980).

Reaction of Cathepsin T with Tyrosine Aminotransferase. Tyrosine aminotransferase I (0.1–7 mg) or another substrate was dialyzed against 50 mM Mops buffer, pH 7.0, containing 2 mM dithiothreitol. To this solution was added 0.2–14 μg of cathepsin T; the solution was mixed and allowed to incubate on ice for periods of up to 2 h, and samples were withdrawn periodically. For examination of the conversion of tyrosine aminotransferase I to III, 5–10-μg samples were analyzed on 10% polyacrylamide gels (Laemmli, 1970); for attempts to detect peptides released during the reaction, samples of up to 100 μg were applied to 15% or 20% polyacrylamide gels, as stated in the text. Samples containing 5 nmol of tyrosine aminotransferase were reacted with cathepsin T for an hour and then heated to 100 °C for 5 min to examine whether amino acids were released. Denatured protein was removed by centrifugation. The supernatant was lyophilized, taken up in 20 mM, pH 2.2 sodium citrate buffer, and analyzed for amino acid content on a Beckman 121 MB amino acid analyzer.

Characterization of the Peptide Released from Tyrosine Aminotransferase. The reaction products obtained as described in the preceding section were applied to a 1.6 × 90 cm column of Sephadex G-75 equilibrated with 0.1 M ammonium acetate, pH 4.0, and eluted with the same buffer while monitoring the optical density of the effluent at 278 nm; peaks of light-absorbing material were pooled, lyophilized, and resuspended in water. Samples of pooled fractions from the column were subjected to electrophoresis on 20% polyacrylamide slab gels (Laemmli, 1970), stained in 50% trichloroacetic acid containing 0.2% Coomassie blue R250, and destained in 10% (v/v) acetic acid. Other aliquots containing about 5 nmol of peptide were lyophilized in ignition tubes, taken up in 0.25 mL of 6 M HCl, degassed, and hydrolyzed in vacuo for 24 h. One aliquot was oxidized with excess performic acid by dissolving the dried sample in 25 μL of 99% formic acid, adding 5 μL of methanol, and cooling the sample in −7 °C brine. Performic acid was prepared by mixing 1 mL of 30% (v/v) H_2O_2 with 9 parts of 99% formic acid and allowing the mixture to remain at room temperature for 1 h. A 25-μL aliquot of this solution was mixed with the solution containing the peptide; the reaction was stopped after 15 min by adding 450 μL of water, diluting and lyophilizing the sample, and preparing it for amino acid analysis as described above. Amino acid compositions were determined at the University of Iowa Protein Structure Facility with a Beckman 121 MB amino acid analyzer.

Purification of ^{32}P-Labeled Tyrosine Aminotransferase I. Reuber H35 cells were grown to confluence in 75-cm^2 flasks in Swim's S-77 medium containing 2.5% (v/v) fetal bovine serum and 2.5% calf serum. The cells were incubated for 18 h in fresh medium containing 1 μM dexamethasone phosphate. N^6,$O^{2'}$-Dibutyryladenosine 3′,5′-monophosphate (1 mM final concentration) and insulin (1 μM) were added to the medium for the final 2 h to further increase tyrosine aminotransferase concentration. This medium was decanted, and 6 mL of phosphate-free Swim's S-77 medium containing the same concentrations of hormones was added to the flasks. Carrier-free [^{32}P]orthophosphate (Amersham) was added to yield 200 μCi/mL, and the incubation was continued for 2 h. The medium was aspirated, the cells were scraped from the flasks and washed twice with ice-cold, pH 7.6 buffer containing 8 g/L NaCl, 0.2 g/L KH_2PO_4, 1.1 g/L Na_2HPO_4, and 0.2 g/L KCl, and the cells were lysed in extraction buffer by the addition of Nonidet P40 (0.1% v/v; Particle Data Laboratories) with agitation. The extract was heated to 70 °C for 5 min, cooled in a −7 °C brine, and centrifuged at 15000*g* for 20 min. The supernatant was pooled with a liver extract containing 500 units of enzyme activity and purified by chromatography on DEAE-cellulose and CM-Sephadex C-50 as described above. The product had a specific activity of 410 units of enzyme activity per mg of protein and 170 000 dpm/mg protein; 0.4 mg was recovered.

Reaction of Cathepsin T with ^{35}S-Labeled Proteins from HTC Cells. HTC cells were grown to confluence in monolayer culture in 60-cm^2 Petri dishes with Swim's S-77 medium containing 2.5% bovine serum, 2.5% fetal bovine serum, 2.4 mM $CaCl_2$, and 0.1 μM dexamethasone phosphate. This medium was decanted, and the cells were washed once in medium lacking L-methionine. Fresh medium (2 mL) containing 250 μCi/mL [^{35}S]methionine was added to each petri dish, and the cells were incubated in this medium for 30 min. The medium was decanted, and the cells were washed 3 times in ice-cold Swim's 77 containing 1 mM L-methionine. Cells were dislodged with a rubber policeman, taken up in 1 mL of extraction buffer, and sonicated at 20 W for 30 s. The extract was heated to 45 °C for 10 min, centrifuged at 30000*g* at 4 °C for 20 min, and equilibrated with 50 mM Mops, pH 7.0, containing 2 mM dithiothreitol and 1 mM EDTA by using a Sephadex PD-10 column (Pharmacia). The resulting solution had an A_{280} of 0.912 and contained 6450 cpm/μL trichloroacetic acid insoluble [^{35}S]methionine. Two aliquots of 150 μL each were incubated for 1 h at 4 °C with or without 1 μg of cathepsin T. Aliquots (40 μL each) of the samples were lyophilized, taken up in 40 μL of lysis buffer [9.5 M urea, 1.6% (v/v) pH 5–7 ampholytes, 0.4% pH 3.5–10 ampholytes, 2% Nonidet P40, and 5% β-mercaptoethanol] containing 5 μg each of tyrosine aminotransferases I and III, and subjected to two-dimensional electrophoresis as described by O'Farrell (1975). The resulting gels were stained in 50% trichloroacetic acid containing 0.2% Coomassie blue R250, destained in 10% acetic acid, and subjected to fluorography (Chamberlain, 1979). The dried gels were marked on the edge with ^{14}C-containing ink and exposed to Kodak X-Omat R film at −70

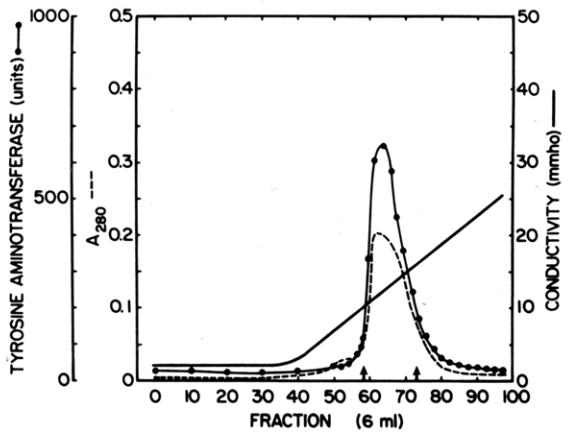

FIGURE 1: Elution profile of tyrosine aminotransferase from CM-Sephadex C-50 after rat livers were homogenized in pH 8.0 Hepes buffer and rapidly heated to 65 °C, as described under Materials and Methods. Following purification over a DEAE-cellulose column, the extract was applied to a CM-Sephadex C-50 column. Note that a single peak of tyrosine aminotransferase catalytic activity and a corresponding peak of material absorbing light at 280 nm were eluted, corresponding entirely to form I. No peaks corresponding to forms II or III were found.

°C for 4 days prior to being developed. The gels were aligned with the fluorograms by means of the ^{14}C-containing ink spots in order to determine which radiographic images corresponded with authentic monomers of tyrosine aminotransferases I and III.

Results

Form I of Tyrosine Aminotransferase Exists in Vivo. The presence of three immunologically and catalytically similar forms of tyrosine aminotransferase in rat liver has represented an enigma with respect to how they might function in metabolism. Once the properties of cathepsin T were disclosed (Gohda & Pitot, 1980, 1981a,b), it became possible to test whether the three forms coexisted in liver cytosol by inhibiting the activity of this protease during the purification procedure. To test these alternatives, livers from triamcinolone-treated rats were homogenized at room temperature and immediately heated to 65 °C and purified further as described under Materials and Methods. Activity of cathepsin T was minimized by employing 0.1 M Hepes, pH 8.0, containing 0.1 M KCl; at this pH, cathepsin T has very little activity, and the heating denatures the protease (Gohda & Pitot, 1980). The profile of this preparation during elution from CM-Sephadex C-50 is shown in Figure 1. Only one peak of tyrosine aminotransferase (form I) eluted from this column, indicating that form I is the principal if not the only form of the enzyme present in the soluble fraction of liver cells. This is the first reported extraction procedure that yields only the antive enzyme at this stage; all others yield two or more peaks or only the degraded form (Hargrove & Granner, 1980; Belarbi et al., 1977; Iwasaki et al., 1973).

Cathepsin T Excises a Peptide from Tyrosine Aminotransferase. For characterization of the action of cathepsin T on tyrosine aminotransferase, the native enzyme of Figure 1 was dialyzed against pH 7.0, 50 mM Mops buffer containing 2 mM dithiothreitol. The two enzymes were incubated together at a 200:1 molar ratio (aminotransferase to protease) as described under Materials and Methods. Analysis of the products on 15% polyacrylamide gels showed that a fragment of low M_r was released during the reaction (arrow in Figure 2). A sample of 100 μg of tyrosine aminotransferase in the absence of convertase showed several contaminating bands but

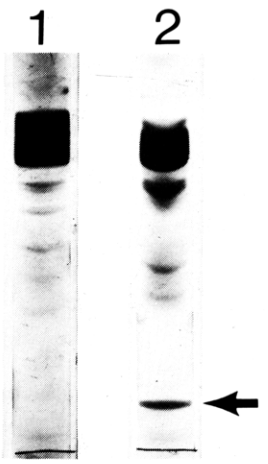

FIGURE 2: Release of a peptide from tyrosine aminotransferase I by cathepsin T. Tyrosine aminotransferase I was incubated in pH 7.0, 50 mM Mops buffer with or without cathepsin T at a 200:1 molar ratio for 1 h at 4 °C. Gel 1, electrophoretic pattern of 100 μg of tyrosine aminotransferase I incubated in the absence of cathepsin T and analyzed on a 15% polyacrylamide gel. Gel 2, pattern shown after incubation of tyrosine aminotransferase I with cathepsin T. Note the appearance of low molecular weight material that stains with Coomassie blue R250 (arrow).

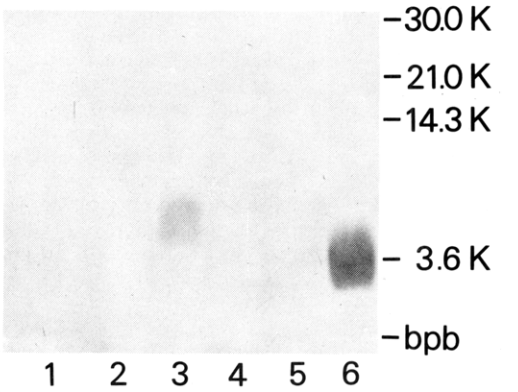

FIGURE 3: Analysis on 20% polyacrylamide gels of the eluate from a Sephadex G-75 column to which products of the reaction between cathepsin T and tyrosine aminotransferase had been applied. An aliquot of pooled fractions 20–22 is shown on lane 1, 23 and 24 on lane 2, 25–27 on lane 3, 28–30 on lane 4, and 31–33 on lane 5. A 10-μg sample of insulin B chain (M_r 3600) was applied to lane 6 as a standard. Note that only fractions 25–27 contained material that stained with Coomassie blue R250; these corresponded to the only peak of material absorbing light at 278 nm that eluted between the excluded and included volumes. Molecular weights were estimated by using the protein standards described under Materials and Methods and are indicated on the right side of the figure.

none of this size. Analysis of the supernatant showed that no amino acids were released during the incubation (data not shown). A preparation containing 7 mg of tyrosine aminotransferase was then purified to homogeneity as previously described (Hargrove & Granner, 1980), incubated with cathepsin T, and applied to a column of Sephadex G-75. The elution profile showed that one peak of material absorbing light at 278 nm eluted between the included and excluded volumes (data not shown). Form III of tyrosine aminotransferase eluted in the excluded volume. Samples of these fractions showed that all the tyrosine aminotransferase was converted to form III and that only one peptide could be seen on 20% slab gels of the fractions tested (Figure 3, lane 3). This peptide was soluble in destaining solution (10% acetic acid) but could be detected by fixing the gel in 50% (w/v) trichloroacetic acid containing 0.2% (w/v) Coomassie blue R250 for 15 min and destaining rapidly in 10% acetic acid. About 86 nmol of

Table I: Amino Acid Composition of the Peptide Derived from Tyrosine Aminotransferase I[a]

residue	residues/4500 g: calcd value	nearest integer
Asx	5.82	6
Glx	3.04	3
Thr	1.57	2
Ser[b]	5.00	6
Pro	1.71	2
Gly	3.67	4
Ala	1.76	2
half-cystine	0.30	0
Val	2.97	3
Met	1.21	1
Ile	1.01	1
Leu	2.08	2
Tyr	0.83	1
Phe	0.57	1
Lys	2.76	3
His	2.05	2
Trp[c]	(+)	
Arg	2.96	3
total		42

[a] An aliquot of fractions 25–27 from the G-75 column was oxidized with performic acid and another was lyophilized without oxidation. Samples were taken up in 250 μL of 6 N HCl containing 0.3% (v/v) each of 88% phenol and 2-mercaptoethanol and hydrolyzed at 110 °C for 24 h, dried, taken up in 20 mM sodium citrate, pH 2.2, and subjected to amino acid analysis. Values shown were calculated on the assumption that the peptide's molecular weight was 4500, as estimated by polyacrylamide gel electrophoresis. Methionine and half-cystine were quantitated from the amount of methionine sulfone and cysteic acid found in the performic acid oxidized sample. [b] The integral value for serine was rounded to six on the assumption that 20% of this amino acid was destroyed during the 24-h hydrolysis. [c] Tryptophan content was not estimated; a peak was noted during the analysis however.

peptide was recovered from the Sephadex G-75 column; this represented 65% of the amount that would be generated by complete conversion of 7 mg of tyrosine aminotransferase I to III.

Characterization of the Peptide. The molecular weight of the peptide was estimated by electrophoresis in 20% gels by using the M_r standards listed under Materials and Methods. A M_r value of 4500 was found, in good agreement with estimates that the subunits of tyrosine aminotransferases I and III differ in mass by 4000 daltons (Hargrove et al., 1980; Gohda & Pitot, 1980). The amino acid composition of the peptide was determined by hydrolyzing it in 6 N HCl with or without performic acid oxidation as described under Materials and Methods. The amino acid composition, calculated by assuming an M_r of 4500, is given in Table I. Although tryptophan was not quantitated, a small amount of tryptophan was found when the peptide was hydrolyzed in the presence of phenol and 2-mercaptoethanol. Cysteic acid was not present in the performic acid oxidized sample. Acidic amino acids predominated over basic ones (nine Asx plus Glx vs. eight Lys, His, and Arg). This feature may explain why subunits of tyrosine aminotransferase III have a more basic isoelectric point than subunits to form I when electrofocused in urea (Hargrove & Granner, 1981).

Tyrosine Aminotransferases I and III Are Phosphorylated. Tyrosine aminotransferase is phosphorylated (Lee & Nickol, 1974), and the three forms of tyrosine aminotransferase have been reported to contain different amounts of phosphate, with form I having much more than form III (Kenney et al., 1975; Belarbi et al., 1980). These data indicate that the peptide

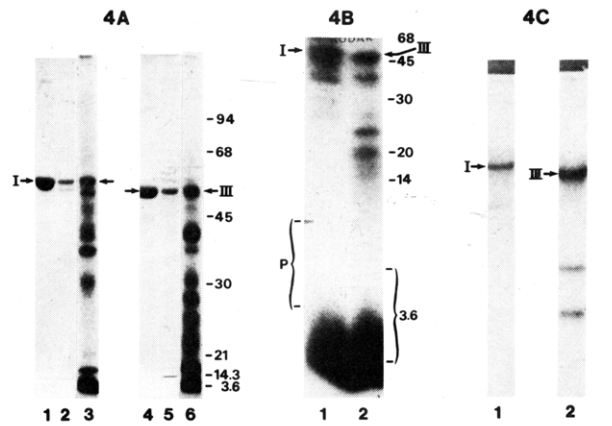

FIGURE 4: Analysis of $^{32}P_i$-labeled tyrosine aminotransferase before and after reaction with cathepsin T. (A) Lanes 1 and 4, 10% polyacrylamide gels (Laemmli, 1970) containing purified tyrosine aminotransferases I and III, respectively; lanes 2 and 5, gels of total reaction products of purified $^{32}P_i$-labeled tyrosine aminotransferase incubated without and with cathepsin T, respectively, and stained with Coomassie blue R250; lanes 3 and 6, autoradiograms of lanes 2 and 5, respectively. Positions corresponding to forms I and III are shown by arrows. (B) Lanes 1 and 2, autoradiograms of 20% polyacrylamide gels of the products shown in (A). Positions of M_r markers and region to which the 4500-dalton peptide migrated are indicated. (C) Autoradiograms of 10% polyacrylamide gels containing $^{32}P_i$-labeled tyrosine aminotransferase immunoprecipitated after incubation without (lane 1) or with (lane 2) cathepsin T. Note the appearance of $^{32}P_i$-labeled subunit III and lower M_r fragments of tyrosine aminotransferase after treatment with cathepsin T.

cleaved from tyrosine aminotransferase may also be phosphorylated. To study this possibility in vitro, $^{32}P_i$-labeled tyrosine aminotransferase was prepared as described under Materials and Methods and stored frozen at −20 °C in pH 6.5, 50 mM sodium phosphate containing 0.15 M KCl. Dithiothreitol was added to thawed aliquots containing 10 μg of $^{32}P_i$-labeled enzyme at a final concentration of 2 mM, and 2 μg of cathepsin T was added to an aliquot to generate form III of the enzyme. These samples were denatured, analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate, and subjected to fluorography. Although only one band of Coomassie blue staining material could be seen in each lane, corresponding to the subunits of tyrosine aminotransferases I and III, many other bands of radioactivity were observed, and treatment with cathepsin T caused many of these bands to migrate more rapidly during electrophoresis, indicating that they had been fragmented (Figure 4A; compare lanes 2 with 5 and 3 with 6, respectively). Both the higher and lower M_r subunits of tyrosine aminotransferase corresponded to bands containing $^{32}P_i$. In contrast, $^{32}P_i$ was not evident in the region corresponding to the 4500–dalton peptide, as seen in a fluorogram of the reaction products separated on a 20% polyacrylamide gel (Figure 4B). Reaction products were immunorecipitated to ascertain that tyrosine aminotransferase III is phosphorylated; the majority of the contaminating, $^{32}P_i$-labeled bands seen in Figure 4A did not precipitate and were assumed not to be related to tyrosine aminotransferase. Immunoreactive bands containing $^{32}P_i$ were found that comigrated with subunits I and III (Figure 4C). The 49 000-dalton phosphoprotein also comigrated with tyrosine aminotransferase III subunits on two-dimensional gels (data not shown). Note that cathepsin T also released two smaller phosphorylated, immunoreactive fragments from tyrosine aminotransferase (Figure 4C, lane 2). The molecular weights of these fragments were about 30 000 and 25 000, respectively. These bands were also seen in 10% gels of the reaction product stained with Coomassie blue R250 but were

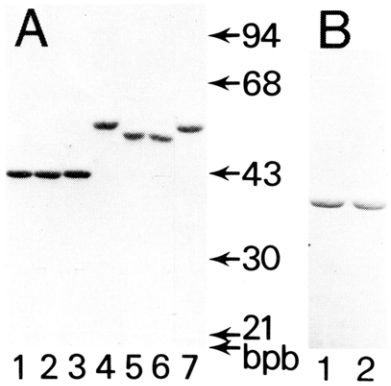

FIGURE 5: Comparison of cathepsin T action on tyrosine aminotransferase I and cytosolic and mitochondrial aspartate aminotransferases. Protease (1 μg) was present per 100 μg of the other enzymes. (A) Lane 1, cytosolic aspartate aminotransferase before incubation with cathepsin T; lanes 2 and 3, the same enzyme after 1 and 2 h of incubation, respectively; lanes 4–6, tyrosine aminotransferase under the same conditions; lane 7, tyrosine aminotransferase incubated for 2 h in the absence of cathepsin T. (B) Lanes 1 and 2, mitochondrial aspartate aminotransferase before and after 2 h of incubation with cathepsin T. Note that no change in migration is noted for the isoenzymes of aspartate aminotransferase, whereas the tyrosine aminotransferase I monomer is completely converted to form III. Molecular weights, determined with standards listed under Materials and Methods are given on right of panel A.

of low intensity (not shown, but see Figure 2, gel 2). These data suggest that cathepsin T does not cleave a phosphopeptide from tyrosine aminotransferase but does release larger, phosphorylated fragments.

Action of Cathepsin T on Other Proteins. Although cathepsin T degrades performic acid oxidized ribonuclease and oxidized hemoglobin (Gohda & Pitot, 1981a), the only native protein that it is known to act upon is tyrosine aminotransferase. Since mitochondrial aspartate aminotransferase also transaminates tyrosine (Miller & Litwack, 1971) and shows 50% homology in amino acid sequence to the cytosolic isoenzyme of aspartate aminotransferase (Kagamiyama et al., 1980), we tested the ability of cathepsin T to act on these proteins. In neither case was a change in molecular weight apparent, whereas tyrosine aminotransferase was readily cleaved under these circumstances (Figure 5). Cathepsin T also failed to alter β-actin (data not shown).

Since the conversion of tyrosine aminotransferase I to III occurs readily in unpurified supernatants of liver cells (Johnson et al., 1973), we next examined the action of cathepsin T on the proteins found in cell extracts. HTC cells were incubated with [^{35}S]methionine, and a postheat supernatant fraction was obtained as described under Materials and Methods (the heating is necessary to degrade endogenous cathepsin T). This fraction was passed over a Sephadex G-25 column to equilibrate it with 50 mM Mops buffer, pH 7.0, containing 2 mM dithiothreitol, and 1 μg of cathepsin T was added to an aliquot containing 150 μg of protein. A second aliquot was incubated in the absence of protease for 1 h at 4 °C, and the aliquots were subjected to two-dimensional electrophoresis along with carrier tyrosine aminotransferases I and III. Figure 6A shows a fluorogram of the untreated aliquot; the circled spot corresponds to tyrosine aminotransferase I as located by staining the gel with Coomassie blue R250. In Figure 6B, a fluorogram of the aliquot treated with cathepsin T is shown. Note that the spot circled in Figure 6A has disappeared, and a new spot that comigrates with tyrosine aminotransferase III has appeared (circled spot in Figure 6B). The positions of a number of other proteins were plainly altered by cathepsin T treatment, since several new spots appeared (arrows in Figure 6B) and

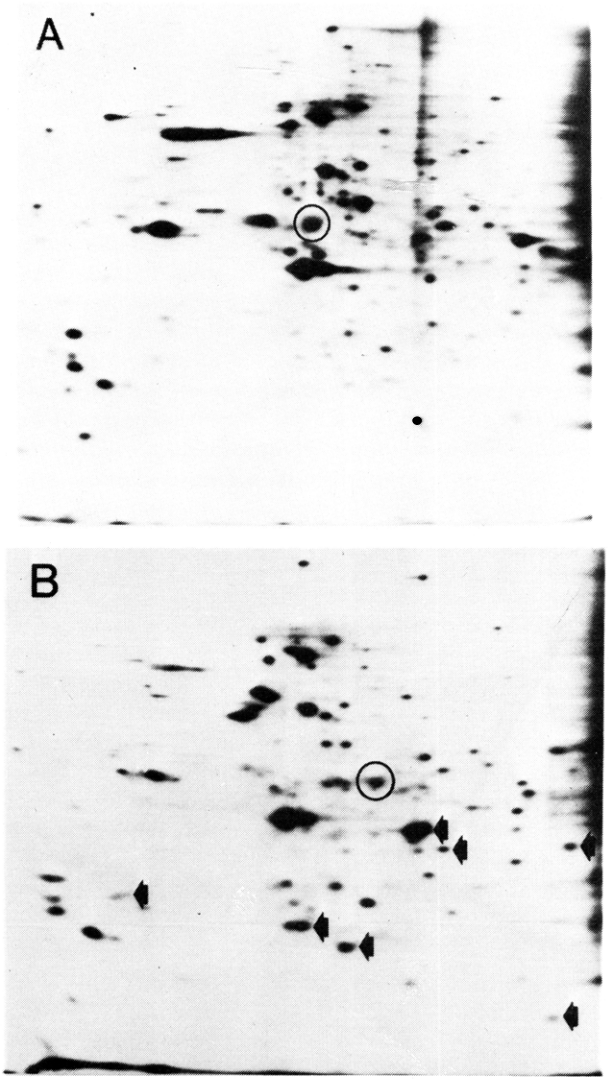

FIGURE 6: Analysis of two-dimensional gels of ^{35}S-containing proteins from HTC cells before and after reaction with cathepsin T. Sample preparation and electrophoresis were performed as described by O'Farrell (1975). (A) Fluorogram of proteins after 1 h of incubation at 4 °C in the absence of cathepsin T. The circled protein is tyrosine aminotransferase I, detected by adding 5 μg of carrier to the extract and staining the gel with Coomassie blue R250 prior to fluorography. (B) Fluorogram of proteins after incubation with cathepsin T; the circled protein is tyrosine aminotransferase III monomer, detected by adding 5 μg of carrier enzyme to the reaction products and staining the gel prior to fluorography. The acidic side of the electrofocusing gel is on the left, and the sodium dodecyl sulfate–polyacrylamide gel is in the vertical dimension. Note that conversion of form I to III was complete and that a number of new spots appeared (arrows) while other disappeared. In addition, no form III was apparent in gel A.

the intensity of other spots was altered. Cathepsin T therefore degrades a number of proteins from hepatoma cells that remain soluble after a heat treamtent; at least 5% of the proteins visible on the fluorograms were altered.

Discussion

The data presented in this paper confirm that cathepsin T, the lysosomal "converting factor" that generates the multiple forms of tyrosine aminotransferase, is an endopeptidase. A peptide is released when the protease is incubated with tyrosine aminotransferase; no evidence was found for a change in the phosphoryl content of the latter enzyme during the conversion of the 53 000-dalton subunit to the 49 000-dalton subunit. No evidence that the released peptide was phosphorylated could be obtained. These results clarify the mode by which the

multiple forms are generated, which has been obscure since they were discovered by Kenney (1962) during his investigation of suitable purification procedures for this enzyme. Originally, form III was thought to be the unmodified translational product since it could be found on polysomes; addition of cyanate to the homogenization buffer caused most of the enzyme to elute as form I, and the modification was suggested to involve carbamylation (Johnson et al., 1973). This effect was later shown to be due to inhibition by cyanate of the converting enzyme (now termed cathepsin T; Rodriguez & Pitot, 1976). With the subsequent discovery that tyrosine aminotransferase is phosphorylated (Lee & Nickol, 1974), it was proposed that different degrees of phosphorylation might generate the multiple forms (Kenney et al., 1975; Belarbi et al., 1980). Other data suggested that the interconversion could be prevented by inhibition of phosphatase activity (Beneking et al., 1978). Differences in titratable sulfhydryl groups among the three forms were also found, leading to the hypothesis that sulfhydryl modification might be involved in this process Rodriguez & Pitot, 1976). Purification of the three forms of tyrosine aminotransferase and analysis by polyacrylamide gel electrophoresis revealed that they differed in apparent molecular weight (Hargrove et al., 1980). Simultaneously, the lysosomal converting factor (cathepsin T) was purified to homogeneity and shown to alter the molecular weight of tyrosine aminotransferase subunits with no requirement for cofactors (Gohda & Pitot, 1980). The latter fact, and the ability of the converting factor to hydrolyze azocasein, a synthetic substrate for proteases, strongly implied that the modification entailed limited proteolysis with no change in tyrosine aminotransferase's catalytic activity.

The peptide released from tyrosine aminotransferase is of approximately 4500 daltons (Figure 3), which accounts for the difference in size observed between form I and III monomers on polyacrylamide gels (Hargrove et al., 1980; Gohda & Pitot, 1980). Its amino acid composition indicates that it is an acidic peptide and explains the fact that the subunit of form III has a more basic isoelectric point than the subunit of form I (Hargrove & Granner, 1981). The peptide contains both serine and threonine, but we have been unable to demonstrate loss of phosphoryl content on conversion of form I to III, as has been suggested to occur (Belarbi et al., 1980). Other recent evidence suggests that both forms I and III have blocked amino-terminal residues, implying that the peptide is cleaved from the carboxyl terminus (Hargrove & Granner, 1981). We have not detected cysteinyl residues in the peptide cleaved from tyrosine aminotransferase.

Cathepsin T releases a peptide from tyrosine aminotransferase I (Figures 2 and 3) but not from the cytosolic or mitochondrial isoenzyme of aspartate aminotransferase. We tested the action of cathepsin T on these enzymes because an altered form of aspartate aminotransferase is generated in crude homogenates of liver (Smith et al., 1976) and because mitochondrial aspartate aminotransferase transminates tyrosine, and may therefore be structurally similar to tyrosine aminotransferase (Miller & Litwack, 1971). The similarity may not be extensive, however, since mitochondrial aspartate aminotransferase differs in molecular weight from tyrosine aminotransferase by at least 6000 (Figure 5), and its isoelectric point is 9.0 as compared to 5.6 for tyrosine aminotransferase (Ohisalo & Pispa, 1976). The only other known substrates for cathepsin T action are performic acid oxidized ribonuclease A and oxidized hemoglobin; no exopeptidase activity has been detected (Gohda & Pitot, 1981a,b; this work). Analysis on two-dimensional gels, however, shows that a number of proteins from HTC cells are susceptible to cathepsin T (Figure 6). Proteins such as tyrosine aminotransferase that are degraded rapidly in vivo are generally more susceptible to proteolysis than those that are degraded slowly (Bond, 1975). The half-time of degradation for tyrosine aminotransferase is about 2 h (Kenney, 1967), whereas the turnover time for cytosolic aspartate aminotransferase is about 3 days (Rosen et al., 1958). The ability of cathepsin T to hydrolyze tyrosine aminotransferase but not the aspartate aminotransferase isoenzymes might indicate a selective role for cathepsin T in degradation. If so, the data accrued so far suggest that the process must occur within the lysosomes, since very little or no altered tyrosine aminotransferase is found among soluble, heat-stable proteins when cathepsin T activity is destroyed rapidly by heating (Figure 1). The "group-specific protease" cleaves a peptide from several pyridoxal-dependent enzymes (not including tyrosine aminotransferase) and has been posited to limit the rate of the degradative process (Katunuma et al., 1976). This enzyme, however, originates in the mast cells and is not now thought to participate in degradation of intracellular, pyridoxal 5′-phosphate dependent enzymes (Holzer & Heinrich, 1980). Although lysosomal proteases certainly participate in turnover of proteins, the processes that cause various proteins to be degraded at differing rates are complex and appear to involve several factors, not just proteolytic susceptibility (Segal et al., 1978; Ciechanover et al., 1980; Francis & Ballard, 1980). Our failure to find altered forms of tyrosine aminotransferase in liver cytosol may indicate that once the enzyme enters the lysosome and is partially hydrolyzed it does not escape further degradation and reenter the cytoplasm. This is an important consideration that deserves further testing (Segal et al., 1978).

The possibility that specific proteases could control gluconeogenesis by altering the activity of fructose 1,6-bisphosphatase has been raised, since the activity of a converting enzyme from rabbit liver increases severalfold during fasting (Melloni et al., 1981). Tyrosine aminotransferase is potentially gluconeogenic, but a comparison of the properties of cathepsin T with those of fructose 1,6-bisphosphatase converting enzyme from rat liver shows several differences. In particular, cathepsin T is highly active at pH 7.0 and is inhibited by leupeptin but not phenylmethanesulfonyl fluoride (Gohda & Pitot, 1981a,b), whereas the other converting enzyme is inactive at pH 7.0, is inhibited by phenylmethanesulfonyl fluoride, and is not sensitive to leupeptin (Crivellaro et al., 1978). Cathepsin T, therefore, is a novel lysosomal protease that cleaves an acidic peptide from tyrosine aminotransferase, thereby generating the multiple forms. This represents one of the few instances in which a rapidly turning over enzyme and a protease that acts on that enzyme with some specificity have been shown to coexist in a tissue. Since both enzymes have now been purified to homogeneity, further characterization of their interaction can be obtained in vitro.

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Characterization of a Human Cryoglobulin Complex: A Crystalline Adduct of a Monoclonal Immunoglobulin G and Albumin†

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ABSTRACT: An unusual human cryoglobulin complex was characterized as a two-component system containing monoclonal immunoglobulin G (IgG) and serum albumin in a 1:2 mole ratio. This complex appeared to be an antibody–antigen complex, since the mole ratio was appropriate and the isolated Fab of the IgG associated with the albumin. The cryoglobulin apparently arose as a result of specific association and/or aggregation of the IgG albumin adduct, since the cryoglobulin complex formed a crystalline precipitate. The IgG and albumin were separated and characterized with respect to immunological cross-reactivities, sedimentation velocities, isoelectric properties, and amino acid composition. The extent of precipitation of the cryoglobulin complex was maximal at pH 7.8, was decreased by added ions including citrate, ethylenediaminetetraacetic acid, NaCl, and $CaCl_2$, and was decreased by increasing temperature. Both the nature of the cold-precipitable complex and the solubility properties differed from those described for other cryoglobulins.

Cryoglobulins comprise those immunoglobulins which precipitate reversibly in the cold. In humans, the following three classes or types have been recognized: type I, monoclonal immunoglobulins with reduced cold solubility; type II, mixed immunoglobulins with a monoclonal component possessing antibody activity toward polyclonal immunoglobulins; and type III, mixed polyclonal immunoglobulins which may also contain nonimmunoglobulin molecules (Brouet et al., 1974). This paper describes the Tu cryoglobulin complex, a cryoglobulin which does not readily fit any of the above categories. The characterization of the Tu cryoglobulin as a cold-insoluble, crystalline 1:2 complex of monoclonal immunoglobulin G (IgG) and serum albumin is described in this report.

The mechanism of cryoprecipitation has been investigated for a number of human cryoglobulins of all three types. No unifying mechanism has been reported (Middaugh et al., 1978); rather, a variety of different mechanisms have been

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