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Hominoid triosephosphate isomerase: Characterization of the major cell proliferation specific isozyme

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Summary

Proliferating cells derived from hominoid species contain electrophoretically separable forms of triosephosphate isomerase (TPI), including a constitutive isozyme and major and minor cell proliferation specific isozymes. Genetic studies have shown that the constitutive and inducible isozymes are products of the same structural gene. A procedure has been developed for the rapid isolation of the constitutive and major proliferation specific TPI isozymes from human lymphoblastoid B cells. [^{35}S]methionine labeled isozymes were purified through several steps of polyacrylamide gel electrophoresis in sufficient quantities for turnover studies and preliminary structural analysis. The intact isozymes were subjected to 23 steps of automated Edman degradation; both preparations yield a [^{35}S] PTH-methionine only at cycle 14, as expected if the protein is TPI. Neither isozyme contains an blocked NH_2-terminus and length heterogenity at the amino terminal does not exist. A comparison of the two purified isozymes on 2-D PAGE confirms that the constitutive isozyme consists of only type 1 subunits while the major proliferation specific isozyme is composed of a type 1 subunit and a unique type 2 subunit. The type 1 and type 2 subunits differ by at least four charge units under native, nondenaturing conditions of electrophoresis but do not differ in molecular mass. The difference between the type 1 and type 2 subunits is covalent, as the difference in isoelectric point between the two subunits is stable to both 2% SDS and 8 M urea. The expression of TPI-2 does not correlate with the existence of the labile asparagine residues. Turnover studies indicate that the level of each subunit is regulated by differences in rates of synthesis rather than degradation but a precursor-product relationship between the subunits was not observed. Thus the mechanism for synthesis of TPI-2 must operate either during mRNA processing or nascent peptide synthesis and then only in cells from hominoid species.

Introduction

The growth of normal cells is a highly regulated process which is accompanied by a series of cellular responses (1–3). Most of the cellular changes are quantitative rather than qualitative as only 2–3% of the mRNA's and/or proteins exhibit present/absent differences between quiescent and dividing or transformed cells (5–6). Recent data suggest that, in addition to expression of many oncogenes (7, 8) and cellular growth factors (9, 10) an increased expression of several glycolytic enzymes, including triosephosphate isomerase (TPI: EC 5.3.1.1) is a relatively early response to mitogenic or growth factor stimulation of quiescent cells (11). TPI activity is increased 2–3 fold following mitogen stimulation of peripheral lymphocytes (12, 13) and is also increased in proliferating established cells, relative to quiescent cells (13,14).

The increased level of TPI observed in mitogen stimulated human lymphocytes is associated with the appearance of unique isozymes which subse-

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quently account for approximately 50% of the total TPI activity (15, 16). The induced isozymes are distinguished by an increased electrophoretic migration and thermolability, relative to the constitutive isozyme. The appearance of the induced isozymes in mitogen stimulated, human peripheral lymphocytes requires RNA synthesis and *de novo* protein synthesis (17). Isozymes analogous to the mitogen induced isozyme are observed in other actively proliferating human cells of both normal and transformed phenotypes (12–14, 16). The expression of the mitogen induced, proliferation specific isozyme is a unique feature of cells from hominoid species (18); that is, the unique induced isozymes are not detected in dividing cells from lower primates or rabbits (14, 18). The modification event is presumably not associated with a unique cytoplasmic factor as the proliferation specific human isozyme is observed in hamster cells (which express only a single isozyme) when they contain human chromosome 12 (19).

Genetic studies have conclusively demonstrated that, in humans, the constitutive and proliferation specific isozymes are products of the single structural gene, *TPI-1* (14, 16, 20). Thus, a regulated mechanism for generation of the induced isozymes must exist in hominoids. Several mechanisms for and many examples of multiple gene products being derived from a single structural locus are known (21–29) although most examples of regulated expression involve tissue and/or developmental stage specific expression (26–29). Unique aspects of the TPI system include (1) a process giving rise to a modified gene product which is apparently cell proliferation specific and, (2) a process (or event) expressed only in hominoid species, thus reflecting a recent evolutionary event.

The present communication describes a procedure for the rapid isolation of the TPI isozymes from proliferating human lymphoblastoid cells. The results confirm that the prominent proliferation specific isozyme is composed of two different subunits, a constitutive type 1 subunit and an induced type 2 subunit which is in contrast to some earlier suggestions of a homodimeric structure for this isozyme (16, 30). The modification responsible for the formation of the type 2 subunit is covalent and results in an increase in net charge of -4 charge units/subunit without a detectable change in molecular mass. Lack of a demonstratable precursor-product relationship between the type 1 and type 2 subunits suggests that the modification event occurs prior to association of the individual subunits. Several potential modifications have been excluded as the basis for the charge difference between the subunits.

Materials and methods

Cell culture

The long term human B cell lymphoblastoid line, UM-61, was originally established and described by Choi and Bloom (31). UM-61 cells were grown as stationary suspension cultures in RPMI 1640 media (GIBCO, Grand Islands, NY) supplemented with 20% heat-inactivated horse serum (GIBCO), penicillin G (100U/ml), streptomycin (0.01% w/v), and 0.2 mM L-glutamine at 37 °C, 5% CO_2. Grown under these conditions, the UM-61 cells had a doubling time of 30 hrs and reached a final density of 1.6–1.8 $\times$ 10^6 cells/ml.

Radioactive precursors.

L-[^{35}S]methionine (met, 1000–1300 Ci/mmole, Amersham, Arlington Heights, IL) was used to label cellular protein. Cells were harvested while in exponential growth, washed (4 °C) twice in methionine-free RPMI 1640 supplemented as described above and resuspended in this same media (37 °C) at 10^6 cells/ml. [^{35}S]met was added to a final concentration of 40 uCi/ml. The cultures were incubated in the presence of [^{35}S]met for the indicated times at 37 °C. Cells were harvested by transfer to pre-chilled tubes (4 °C), diluted with medium containing excess methionine (0.2 M) and pelleted. The cells were washed twice by resuspension in Ringer's solution (4 °C), the cell pellets drained and stored at -70 °C. The cultures for the pulse labeling experiments were treated similarily except that a single 50 ml culture was labeled for 8.0 hrs. Following the pulse, the cultures were chilled to 4 °C, the cells were pelleted, resuspended in the same volume of excess methionine media (37 °C) and then distributed to duplicate, 7 ml cultures.

Cell lysates

The cell pellets were resuspended in hypotonic buffer (50 mM triethanolamine-HCl (TEA), 1 mM

EDTA, pH 8.0, 1 mM dithiothreitol (DTT)) at a concentration of 5 × 10^7 cells/100 ul. $MgCl_2$ was also added to a final concentration of 10 mM. Cell lysis was completed by five cycles of freezing in liquid nitrogen. DNase I (type DN-CL, Sigma, St. Louis, MO) was added to a final concentration of 10000 U/ml of lysate and the lysate was clarified at 35000 xg for 30 minutes at 4 °C. The supernatants, excluding an upper layer of lipid, were stored undiluted at -70 °C; the TPI activity was stable for at least six months. No changes in the TPI isozyme patterns were observed after more than a year of storage at -70 °C.

TPI activity was measured as described by Fielek and Mohrenweiser (32). The TPI activity in the UM-61 cell lysates was routinely 500 ± 50 U/ml (Units are defined as umoles of NADH oxidized/min at 30 °C.) Densitometry of the isozyme activity staining patterns were performed on a Joyce-Lobel Recording Microdensitometer, MK IIIC. A 35 mM negative (HC-35, Kodak) was made by photographing the stained cellulose acetate overlay; the negative was scanned using a 3.0 O.D. bar.

Total soluble cellular protein concentration was determined by the method of Sedmak and Grossberg (33). The assay was modified for use with the centrifugal analyzer (32), with absorbance readings at 465 and 620 nm. The assay was linear over a range of 2.5 and 50.0 ug/ml using bovine serum albumin (Sigma, A–4378) as a standard and Coomassie G–250 from Eastman Kodak Chemical Co. (Rochester, NY).

Isozyme purification

Previous work by O'Farrell (34) and Walsh *et al.* (35) suggested that multiple steps of gel electrophoresis should yield a protein which would be sufficiently pure for structural characterization. The [^{35}S]methionine labeled cell lysates were prepared for electrophoresis by the addition of a one-tenth volume of 40% sucrose, 0.025% bromophenol blue (BPB). The lysates were electrophoresed at 4 °C under nondissociating conditions and stained for TPI activity (15). The individual isozymes were excised as 1.0 cm × 0.5 cm gel slices and stored at -70 °C.

The frozen gel slices were thawed and equilibrated at room temperature immediately prior to a second step of electrophoresis. Each slice was soaked for 2 hrs in 5 ml of 62.5 mM TRIS-HCl (pH 6.8), 10 mM DTT, 2 percent sodium dodecyl sulfate (SDS), 4 percent glycerol and for an additional hour in 1 ml of fresh equilibration solution plus 0.1 percent BPB. The equilibration solution was prepared fresh daily. The individual isozymes were then electrophoresed by a modification of the system described by Laemmeli (36). The resolving gel was 10 percent acrylamide (30: 0.8, acrylamide: bisacrylamide) and measured 6.5 × 10.0 × 0.3 cm. The stacking gel was 1.5 cm. The equilibrated gel slices were placed directly on top of the stacking gel, at right angles to the direction of the first electrophoresis. The slices were held in place with 1.0 percent agarose (Sigma, St. Louis, MO), in 125 mM TRIS-HCl (pH 6.8), 2 percent SDS, 5 mM DTT. Five percent (w/v) rabbit TPI (Calbiochem, La Jolla, CA) was included in the buffered agarose and served as a marker for locating TPI following this and the next step (below) of electrophoresis. The electrode buffer was 62 mM TRIS, 200 mM glycine (pH 8.8–9.0), and 0.1 percent SDS. Electrophoresis was at room temperature for 1 hr at 50 volts and 2.5 hrs at 75 volts. The TPI protein was stained by soaking the gel in 0.1 M KCl for 20 min (37). The TPI, [^{35}S]isozymes plus rabbit carrier, was excised as 1.0 cm × 0.5 cm gel slices and stored at -70 °C.

The final electrophoresis step was as described for the previous step, but a 13 percent resolving gel which measured 14.0 × 13.0 × 0.18 cm was used. The gel slices from the second electrophoresis step were equilibrated and placed at right angles directly on a 2.5 cm stacking gel. Carrier rabbit TPI was omitted from the agarose for this step. The samples were electrophoresed for 1400 volt hrs. The TPI were located by KCl–SDS precipitation (25), excised, and stored at -70 °C for subsequent analysis. In some cases, the gel was equilibrated in 10 percent glacial acetic acid, 25 percent methanol and stained with 0.2 percent Coomassie R–250 (Sigma); after destaining, the gel was treated with ENHANCE (New England Nuclear, Boston, MA) and exposed to X-ray film (38).

Edman degradation

Gel slices from the final step of electrophoresis were pooled for each isozyme. The gels were fragmented and then soaked in several changes of elution buffer (24 mM TEA (pH 8.0), 1.0 mM DTT,

0.1 percent SDS). An additional 3 mg of rabbit TPI was added to the final buffer change. The samples were chromatographed on a Sephadex G–25 column equilibrated in 50 mM ammonium bicarbonate before the volume of each isozyme sample was reduced to 1.0 ml under a N_2 stream. The sample was again desalted on the Sephadex column and the volume reduced to 0.6 ml under N_2. The sample contained, in addition to the TPI protein, 15 mM TEA and residual protein bound SDS. The recovery of radioactive material was monitered at each step of elution and desalting; recoveries from the Sephadex columns were >90 percent.

Automated Edman degradation was done in the liquid phase with a Beckmann 890B Sequenator as described by Tanis and Tashian (39). One mg of NH_2-terminus acetylated carbonic anhydrase I was added to each sample (40). Each sample was washed for two sequenator cycles in which phenyl isothiocynate (PITC) was omitted and a third cycle in which n-heptafluorobutyric acid (HFBA) was omitted. A portion of the thiazoline products were directly assayed by liquid scintillation and the remainder of the sample was converted to phenylthiohydantoin (PTH) derivatives. The PTH-amino acids were identified by reverse phase liquid chromatography (RPLC) (41). For the thiazoline steps containing radioactivity, the PTH-methionine RPLC fractions were assayed by liquid scintillation.

In order to ascertain the effects of any protein bound SDS or buffer solutes on the liquid phase Edman degradation, initial sequence analysis was performed on unlabeled rabbit muscle TPI. The rabbit enzyme (2.5 mg) was made 0.1% SDS in the gel elution buffer (Methods and Materials), desalted on a Sephadex column, concentrated and then analyzed in an automated sequenator run of 25 cycles. The first 23 residues were clearly identified and were in complete agreement with the sequence for rabbit TPI. Thus, SDS present in the protein preparation does not have an adverse effect on the automated degradation procedure (35). The rabbit TPI was included in the sequenator analyses of the human isozymes and was used to compare the efficiencies of individual sequenator runs.

Two dimensional page

The [^{35}S] labeled TPI isozymes were purified and eluted from the gel slices as described above for the Edman degradation. The samples were subjected to two dimensional PAGE (42). After electrophoresis, the gels were stained with Coomassie dye, photographed and prepared for fluorography. For quantitation of individual radioactive polypeptides, the spots were excised, rehydrated, solubilized with NCS tissue solubilizer and assayed by liquid scintillation counting.

Results

TPI levels in lymphoblast cultures

TPI isozymes with characteristics identical to those observed in mitogen stimulated human peripherial lymphocytes are observed in long term proliferating human cell lines (13, 14). Fig. 1 shows the TPI isozyme pattern of UM-61 cells. The UM-61 cells express the constitutive isozyme, a second isozyme referred to as the major mitogen induced or proliferation specific isozyme, and trace levels of the first deamidated form of the constitutive isozyme. The deamidated forms are more clearly seen in the hemolysate sample shown in Fig. 1. Each of the sequential deamidation forms of the constitutive isozyme differ by a net charge of one per dimer (43, 44). Based upon the similar mobility of the major induced isozyme and the fourth deamidation form, the constitutive and major mitogen induced/proliferation specific isozymes should differ by a net charge of approximately four per dimer, given the subsequent data that the subunits do not differ in molecular mass. Although not observable in this figure, an additional proliferation specific, more anodally migrating band of TPI activity is observed in lysates from UM-61 cells following prolonged staining and in more rapidly dividing cells where the TPI-2 subunit is more prevalent (14).

Suspension cultures of the human B cell lymphoblastoid line, UM-61, contained 120 units of TPI activity per 10^8 cells, an amount equivalent to 12 ug of TPI protein, assuming a specific enzyme activity of 10000 units/mg (30). The TPI protein is thus estimated to be 0.8% of the soluble cellular protein. The activity of the major proliferation specific isozyme was quantitated by heat inactivation (16). Upon heating the cell lysates at 56 °C, 20% of the TPI activity is rapidly lost while the re-

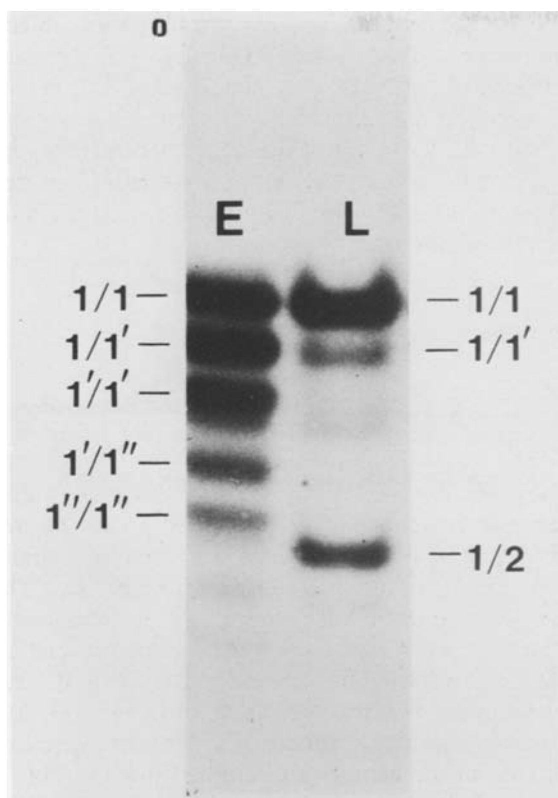


Fig. 1. Native polyacrylamide gel electrophoresis of TPI isozymes. The sample at the left is from human erythrocytes (E). The sample at the right is UM-61 lymphoblastoid cell lysate (L). The erythrocyte isozymes are designated on the left; the lymphoblastoid isozymes on the right. 1 and 2 refer to the subunits while ′ indicates the subunits derived by deamidation of asparagines resudues. The origin is at the top (O). The preparation of the lysate, the composition of the gel and buffers and the methods for staining for TPI activity are described in Methods and also Ref. 14 and 16.

maining 80% activity is stable for at least 45 minutes. Electrophoresis of the lysates after heating at 56 °C for 30 minutes revealed a specific loss of the induced isozyme; the staining intensity of both the constitutive isozyme and the first deamidation form remained unchanged (data not shown). The ratios of the individual isozymes were also measured by densitometry of the staining pattern on gels similar to that shown in Fig. 1. The induced isozyme accounted for 24% of the total staining activity, a level of activity which is in good agreement with the results from the heat inactivation assay. The quantitative relationship between the isozymes was independent of total activity applied to the gel and also the staining time, suggesting that the relative staining intensity is a reasonable measure of enzyme activity in each isozyme band. The major induced isozyme accounts for approximately 20% of the enzyme activity but because this isozyme is a heterodimer, the proliferation specific subunit, TPI−2, is 10% of the TPI protein in UM-61 cells. The TPI−2 subunit accounts for up to 30% of the TPI protein in more rapidly dividing human cell lines (Landa, unpublished observations).

Isozyme purification

UM-61 cultures of 10^8 cells were labeled with [^{35}S]methionine for 8 hours, harvested and lysates prepared. The cell lysates were electrophoresed under native conditions and the polyacrylamide gels were stained for TPI activity as shown in Fig. 1. The individual isozymes were then excised and electrophoresed under denaturing conditions in a second dimension, perpendicular to the first direction of electrophoresis. This second electrophoresis step served to quickly separate the bulk of the contaminating [^{35}S] labeled proteins from the TPI isorymes. The TPI containing gel slices from the second electrophoresis step were then subjected to a third and final cycle of electrophoresis with increased resolving power, also under denaturing conditions. The final polyacrylamide gel is shown in Fig. 2; the carrier rabbit TPI is readily visualized by protein staining. A fluorograph of the final SDS gel reveals a single radioactive spot for the constitutive isozyme in a position which corresponds precisely with the densest staining area of the rabbit TPI. A fluorograph of the gel of the induced isozyme reveals two radioactive spots; the uppermost and denser of the two spots comigrated precisely with the densest area of the rabbit TPI; the second spot represents a contaminating protein carried through from the second cycle of electrophoresis and migrated outside of the Coomassie staining area of rabbit TPI. Thus, the final round of SDS electrophoresis was necessary for isolation of the TPI 1/2 isozyme. The individual isozymes from the 2nd SDS electrophoresis step were eluted from the gel and used for sequence analysis in order to confirm that the isolated protein was TPI and also free of contaminating protein. This purification scheme

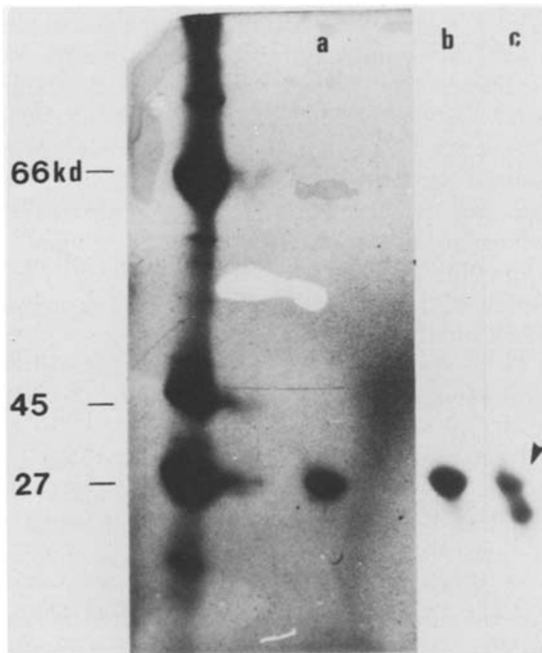
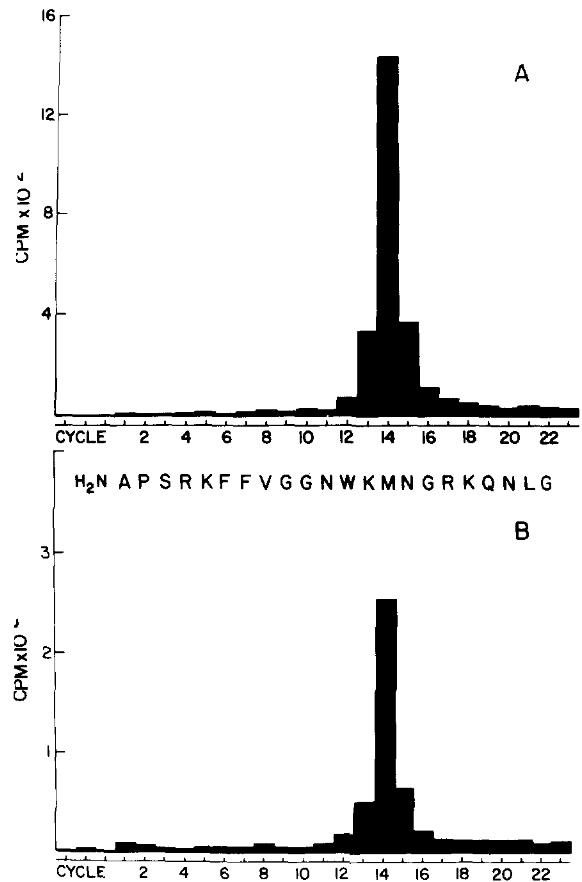


Fig. 2. SDS gel electrophoresis of isolated TPI isozymes. The left figure is stained for protein with Coomassie dye and the right figure (lanes b and c) is a fluorograph of a separate but similar gel. Molecular weight markers on the left are bovine albumin (66000), ovalbumin (45000) and rabbit TPI (27000). Lane a is rabbit TPI, lane b is TPI 1/1 and lane c is TPI 1/2. The small arrow on the right marks the position of the rabbit carrier TPI.

was also used for the isozyme turnover studies.

The purification of TPI from 10^8 cells yielded 2.6×10^5 dpm of total TPI; total lysate protein from the same number of cells was 3.4×10^7 dpm of [^{35}S] labeled soluble protein. The lymphoblastoid TPI is thus 0.8% of the total soluble protein



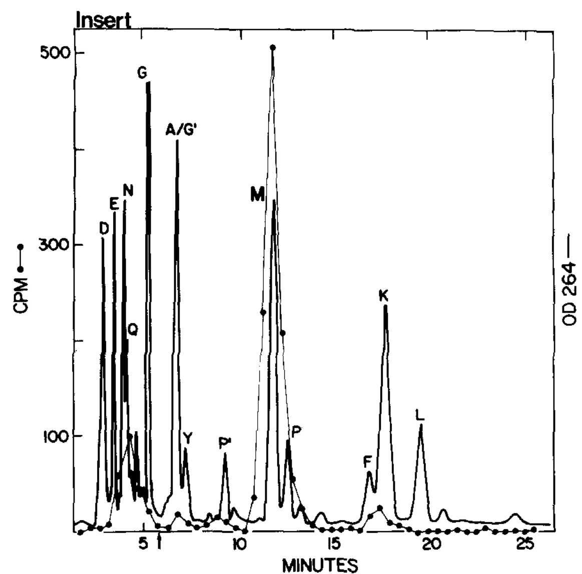


Fig. 3. Analysis of thiazolinone amino acids from [^{35}S] methionine labeled TPI isozymes. TPI was isolated from UM-61 cells by the electrophoretic methods described in the text, subjected to automated Edman degradation. An aliquot of the product was assayed for radioactivity prior to acid hydrolysis. The radioactivity released from TPI 1/1 is in Panel A and TPI 1/2 is in Panel B. Results of the three initial wash cycles are plotted before the first cycle of degradation. The amino acid sequence of the human enzyme is displayed between the panels. The results of analysis of the products released at sequenator step #14 by reverse phase HPLC chromatography is in the insert.

as assayed by recovery of radiolabeled enzyme which is in agreement with the value as determined by enzyme activity measurements of the crude cell lysate. The values for the individual [^{35}S]methionine labeled isozymes were 18–22% for the induced isozyme and 78% for the constitutive isozyme. The initial deamidated isozyme was also isolated in some preparations and was found to contain 4–5% of the total TPI radioactivity. The value of 18–22% for the pure, [^{35}S] labeled, proliferation specific isozyme is in excellent agreement with the values of 20% as determined by thermoinactivation and densitometric scanning.

Identification of the isolated [^{35}S]proteins as TPI

The sequence of the 23 amino terminus residues of human TPI is shown in the center of Fig. 3. The sequence is taken from both direct sequencing of the erythrocyte protein (45) and derived from the cDNA isolated from cultured cells (46). The NH_2-terminus sequence of the human enzyme is identical to that of the rabbit skeletal muscle TPI (47). We reasoned that if we had indeed isolated pure isozymes of TPI, direct sequencing of the intact protein should yield a peak of radioactivity coincident with the 14th cycle of Edman degradation. Furthermore, as TPI contains only two methionine residues, 50% of the original radioactivity should be recovered at this step.

The isozymes were labeled with [^{35}S]methionine and prepared as described above. The final SDS gel was soaked in 0.1 m KCl rather than Coomassie stain and the rabbit carrier TPI was visible as a distinct spot of opaque precipitate. Multiple samples of each isozyme were eluted from the gel and pooled, unlabeled rabbit TPI was added, and then each isozyme sample was sequenced. Both isozymes yielded a single major peak of radioactivity in the first 23 cycles; this single major peak of radioactivity was in the 14th cycle which corresponds with the position of the first methionine residue in both the human and rabbit sequences (Fig. 3).

Small amounts of radioactivity were also recovered in sequenator cycles 13 and 15. The radioactivity in cycle 15 is attributed to incomplete hydrolysis of peptide bonds during each sequencing cycle. Carryover of a small fraction of residues into subsequent cycles was routinely observed during the analysis of the rabbit TPI. The radioactivity in cycle 13 is the result of reagent contamination during the initial wash cycles. A control experiment using the alpha chain of human hemoglobin was performed in which the reagents present in the wash cycles were systemically altered. It was found that the HFBA present in the first wash (+HFBA, -PITC) was not completely removed prior to the second wash (-HFBA, +PITC) and that these trace amounts of acid were initiating a small fraction of premature peptide bond cleavage during the subsequent second and third blank cycles (D. Hewett-Emmett and R. S. Decker, unpub. obs.).

The quantitation of [^{35}S] radiolabel which was recovered during the sequence analysis of each isozyme is presented in Table 1. The total radioactivity for each isozyme is assumed to be represented equally between the two methionine residues (#14 and #82) in the enzyme. An expected overall yield of 36% for the 14th cycle was derived by assuming a repetitive yield of 93% for each degradation cycle. The 93% value is considered conservative as compared to prior studies on this particular sequenator (40). The observed yields were identical for both isozymes at step 14, and 50% of the expected radiolabel was recovered in cycle 14 alone. If the radiolabel which was recovered in the preview (steps #12–13) and carryover (steps #15–16) cycles are taken into consideration, the observed yields approach 80% of the theoretical values for each of the isozymes. These data are taken to indicate that both isozyme preparations are pure, at least with respect to other [^{35}S]methionine labeled protein. Any significant contamination would be expected

Table 1. Analysis of results from Edman degradation of [^{35}S]-methionine labeled isozymes.

	TPI-1/1	TPI-1/2
Initial CPM[a]	55133	9126
Theoretical yield #14[b]	9979	1652
Observed yield #14	4907	861
Observed yield #12–19	7880	1345
% Theoretical yield #12–19[c]	79	81

UM-61 cells were labeled and the isozymes isolated as outlined in Methods.

[a] Total radioactivity (^{35}S) loaded onto sequenator.

[b] Expected yield at step 14 assumes 100% initial yield and 93% repetitive yield at each step of hydrolysis thus the expected recovery at step 14 is 36%. Each subunit has only 2 methionine residues per subunit.

to alter the observed recoveries relative to the expected values for either isozyme.

The identity of the radioactive product in cycle 14 as a hydantoin derivative of methionine was confirmed by reverse phase HPLC analysis. Identical profiles (insert, Fig. 3) were obtained for both of the isozymes. Seventy-two percent of the radioactivity from the constitutive isozyme eluted in the position of PTH-met, whereas for the induced isozyme 68% of the material was recovered as PTH-met. In both profiles, a small peak (15%) of radiolabel was also seen at 4.7 minutes. This material may represent a secondary methionine derivative formed during conversion of the unstable PITC products.

Subunit structure of the major proliferation specific isozyme

The subunit composition of the constitutive and major proliferation specific isozymes was compared on two dimensional polyacrylamide gels (2–D PAGE; 8 M urea isoelectric focusing in the first dimension, followed by electrophoresis in the presence of SDS). The constitutive and major induced isozymes differ by a net charge of at least 4 charge units (Fig. 1) under native conditions, and are easily separated by either native electrophoresis or isoelectric focusing (16, 30).

In preliminary experiments, unlabeled rabbit TPI was denatured in SDS and subjected to standard 2–D gel electrophoresis. As seen in Fig. 4, the rabbit TPI is resolved as a series of distinct polypeptides separated in the IEF dimension. The multiple rabbit TPI forms can be attributed to the posttranslational deamidation of asparagines 15 and 71 (43, 44).

The human isozymes were labeled with [^{35}S]methionine and isolated as described above. Equal amounts of each radioactive isozyme plus an additional 170 ug of rabbit TPI were loaded onto separate IEF gels, focused to equilibrium, and then electrophoresed in a second dimension in the presence of 0.1% SDS. The rabbit TPI was resolved into

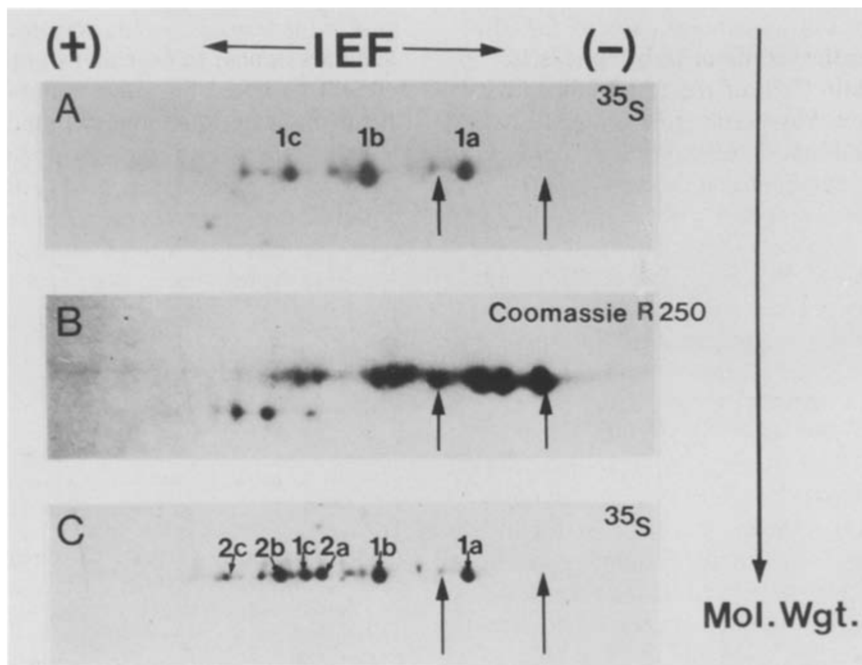


Fig. 4. Urea-SDS two dimensional PAGE of purified [^{35}S] methionine labeled TPI. The Coomassie dye stained rabbit carrier is shown in (B). The autoradiograph of the constitutive isozyme is in Panel A and the autoradiograph of the major induced isozyme is in Panel C. The arrows mark the positions of two carrier rabbit TPI subforms which were located by protein staining (see panel B) before radioautography of the human isozymes and which were subsequently used to align the gels ((A) and (C)) for comparison; the ampholine range was pH 3.5 to 10.0. The 1 and 2 designations are described in the text.

a pattern of polypeptides which was identical in both gels. This pattern was used to align the gels containing the individual radiolabeled isozymes.

The constitutive isozyme profile consists of three major isoelectric forms which we have designated *1a, 1b,* and *1c* in order of decreasing pI (Fig. 4). The major induced enzyme contains the same set of constitutive *1a–c* polypeptides. The positions of these radiolabeled peptides were in perfect register when the gels (and films) were superimposed using the Coomassie stained rabbit peptides for orientation. In addition, the proliferation specific isozyme also contains three unique polypeptides which we have designated *2a, 2b* and *2c.* One of the major characteristics of the native mitogen induced isozyme is its relatively large increase in negative charge and a lower isoelectric point (pI = 5.2) relative to the constitutive isozyme (pI = 5.6) (30). Consistent with this, the unique *2a–c* polypeptides of the induced isozyme profile focus with a lower pI range than the constitutive *1a–c* polypeptides.

Table 2 presents the relative radioactive content of the individual polypeptides from the gels shown in Fig. 4. The three major polypeptides, (*1a–c*) of the constitutive isozyme contain 83% of the total radioactivity and the six major polypeptides (*1a–c* plus *2a–c*) contain 77% of the total radioactivity of the major induced isozyme, relative to the radioactivity in the original samples applied to the IEF gels. The *1a–c* polypeptides in the induced isozyme profile contain 55% of the radioactivity of the same polypeptides in the constitutive isozyme profile. Further, within the induced isozyme profile, the *2a* and *2b* species are present in amounts approximately equal to the *1a* and *1b* species respectively. The *a–c* heterogeneity in both the type 1 and type 2 polypeptides is presumed to result from deamidation of asparagines 15 and 71 during the isolation procedure. The *a–c* heterogeneity is reflected to an equal extent in both of the isozyme profiles suggesting that the *a–c* heterogeneity is unrelated to the mechanism which is responsible for the appearance of the type 2 subunit. The major mitogen induced, proliferation specific isozyme is composed of 2 electrophoretically distinct subunits (and associated deamidated subforms) which would be consistent with it being a 1/2 heterodimer as previously suggested (14, 48). The constitutive isozyme is a 1/1 homodimer (discounting the deamidated asparagines) and the minor induced isozyme (described in ref. 14, 48) would be the 2/2 homodimer. No significant difference in migration rate in the SDS direction of electrophoresis appears between any of the individual radiolabeled polypeptide species. Thus, as previously noted, the TPI subunits appear to be equal in molecular mass. It should be noted that the results from this experiment indicate that the difference between the subunits involves a covalent modification in that the difference in charge is still detected after two

Table 2. Comparison of polypeptide compositions of TPI-1/1 and TPI-1/2.

	TPI-1/1			TPI-1/2					
	1a	1b	1c	1a	1b	1c	2a	2b	2c
% cpm of individual polypeptide subunits within each isozyme	30.5	49.1	20.1	18.9	27.3	12.2	16.5	20.1	4.8
Ratio of type 1 subunits in TPI-1/2 relative to type 1 subunits of type-1/1				0.61[a]	0.56	0.60			
Proportion of type 2 subunits relative to type 1 subunits of TPI-1/2							0.88[b]	0.74	0.39

The initial amounts of radioactive TPI-1/1 and TPI-1/2 were 16730 and 17280 cpm, respectively. The three polypeptide spots of TPI-1/1 and the six spots in TPI-1/2 represent >83% and 77% of the total material applied to each gel, respectively.

[a] Expected values are 0.50.

[b] Expected values are 1.00.

steps of SDS gel electrophoresis and also IEF in the presence of 8 M urea.

Steady state conditions

The relative rates of synthesis and degradation of TPI-1 and TPI-2 were studied to examine a possible precursor product relationship between the type 1 and type 2 subunits. An initial lag of 20–24 hrs in growth was observed following resuspension of exponentially growing cells into fresh medium. Following this lag period, the cells enter an exponential growth phase with a mean doubling time of 30 hrs. During the lag period (7 time points), a constant cell density of 8.5 ± 1.0 × 10^5 cells/ml was maintained, the soluble cellular protein concentration was 4.8 ± 0.3 mg/culture, the total TPI activity was 87 ± 5 units/mg soluble protein and the induced isozyme was 21% of the total TPI activity. Thus, it appears that the initial lag phase exhibited by these cells approximates steady state conditions as necessary for isozyme turnover studies.

Apparent rates of degradation of TPI-1 and TPI-2

The kinetics of radiolabel loss from the TPI-1 and TPI-2 subunits were determined by pulse-labeling cultures with [^{35}S]-methionine, followed by incubating the cells in nonradioactive medium for appropriate time intervals. Both subunits follow similar first-order kinetics of radiolabel loss (Fig. 5). The rate of loss or degradation (k_d) was 0.021 hr^{-1} for TPI-1 and 0.0235 hr^{-1} for TPI-2 in these experiments (Table 3). The radiolabel in total protein was lost at a rate of 0.028 ± 0.009 hr^{-1}. TPI-1 has an apparent half-life of 34 hrs and TPI-2 an apparent half-life of 32 hrs while total protein has an apparent half-life of 25 hrs. Thus, TPI-1

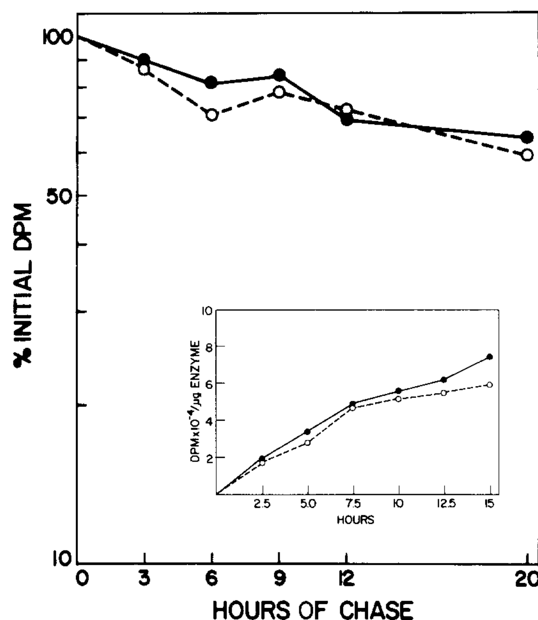


Fig. 5. Relative rates of synthesis and degradation of TPI-1 and TPI-2. UM-61 cells were labelled for 8 hrs before initiation of the chase. At indicated times, cells were washed, the TPI isozymes isolated and the isotope remaining determined. The results of a [^{35}S] methionine incorporation experiment are in the insert. Cells were harvested at the times indicated, following isotope addition, the TPI isozymes isolated and the specific radioactivity determined. (○) is TPI-1 and (●) is TPI-2.

Table 3. Turnover of TPI subunits in human lymphoblastoid cells (UM-61).

	Relative K_s^a	APP $K_d(\cdot Hr^{-1})$		$T_{1/2}$ (hrs)	Net DPM[b] incorporated
		Exp 1	Exp 2		
TPI-1	0.415 ± 0.083	0.020	0.022	34	7.20 × 10^3
TPI-2	0.040 ± 0.004	0.023	0.024	32	0.77 × 10^3
Total soluble protein	100.0	0.028 ± 0.009[c]		25	1.75 × 10^6

[a] The level of incorporation into each subunit is expressed relative to the rate of incorporation into total soluble protein (Mean ± SD (N = 4)).
[b] Mean incorporation into each subunit (3 experiments) at initiation of chase period. The labeling period was 7.5 hrs as described in Methods.
[c] Mean ± SD (N = 3). The rate of isotope incorporation (or loss) into the TPI 1/2 heterodimer was assumed to be the average of the rate for the TPI-1 subunit and the TPI-2 subunit. Since data for the TPI-1 subunit could be obtained independently (from the TPI 1/1 dimer), the data for the TPI-2 subunit contribution to the 1/2 heterodimer were calculated.

and TPI-2 appear to have rates of decay which do not differ significantly from each other.

Kinetic relationship of TPI-1 and TPI-2

The pulse-chase experiments outlined above also provide results which bear on the possible precursor-product relationship of the TPI-1 and TPI-2 subunits. Given the approximately 30 hr half-lifes for the subunits, a pulse of 7.5 hours would allow for sufficient incorporation of radiolabel for adequate detection of a precursor-product relationship. As seen in Fig. 5, there is no increase in specific radioactivity for either of the isozymes during the initial chase period, with both TPI-1 and TPI-2 showing an immediate loss of radiolabel and following essentially identical exponential decay kinetics. A precursor-product relationship between the two forms of the enzyme would require an immediate increase in specific radiolabel of the product and concurrent loss of radiolabel from the precursor. A precursor-product relationship would also be reflected in a slower apparent rate of degradation for the product relative to the precursor; this was not observed.

In a preliminary experiment, a precursor-product relationship could be observed between the constitutive isozyme and the first deamidation product of this isozyme (TPI 1/1'; see Fig. 1). The peak radioactivity in the deamidation product was at 3 hrs after initiation of the chase phase of the experiment; the specific radioactivity at zero time was 82% of that observed at the 3 hr time point. The specific radioactivity of the TPI 1/1' isozyme was approximately 10 percentage points above that of the TPI 1/1 isozyme at 3 hrs and all subsequent time points. At the 3 hr time point in this experiment, both TPI-1 and TPI-2 have lost 10% of the radiolabel present at zero time.

Relative rates of synthesis of TPI-1 and TPI-2

The equivalent half-lifes of TPI-1 and TPI-2 indicate that differential rates of degradation do not account for the differences in relative isozyme levels. From the relationship describing protein turnover, it is known that the differences in steady state levels between two proteins with identical rates of degradation is directly proportional to their relative rates of synthesis.

The results of monitoring the continuous incorporation of radiolabel into the TPI subunits are seen in Fig. 5. The relative rate of TPI-1 subunit synthesis was nine-fold greater than the rate of TPI-2 subunit synthesis in these experiments (Table III), consistent with the nine-fold differences in quantity of subunits.

Discussion

TPI is an essential and highly efficient enzyme which is central to glycolysis, as well as to gluconegenesis, the pentose shunt, and glycerol/lipid biosynthesis (49, 50). The constitutive enzyme is a dimer composed of identical 26–27000 dalton subunits in a range of organisms from bacteria to man (45, 47, 51, 52). The amino acid sequence has been highly conserved through evolutionary time, with 98% sequence homology between the human and rabbit enzymes (46, 47) and 90% between the human and chicken enzymes (53). The constitutive form of TPI undergoes a sequential deamidation of asparagine residues 15 and 71 in mammalian species (43, 44). In other species (e.g. chicken) where the asparagine 71 is replaced with a lysine residue, this spontaneous deamidation process is not observed (51). The stepwise deamidation of the asparagine residues generates enzymatically active subforms, each differing by a net negative charge. The TPI-1/2 heterodimer has a mobility which is similar but detectably different from that of the fourth deamidation subform of the constitutive isozyme (13). More importantly, the absence of expression of the TPI-2 subunit in mitogen stimulated and/or proliferating cells from several species where the TPI retains the labile asparagines (14, 18), argues against the involvement of deamidation as a mechanism for generation of the hominoid specific induced isozymes.

The results from the denaturing IEF experiment argue against the TPI-2 isozyme being the result of noncovalent ligand binding or being a conformational isomer of the constitutive isozyme. The more acidic pI is retained by the type 2 isozyme following denaturation in the presence of 2.0% SDS and subsequent electrophoresis in the presence of 8 M urea. The modification is probably not mediated through proteolysis as both isozymes exhibit similar migration in the SDS dimension of the 2–D

PAGE. This same gel is capable of resolving molecular weight differences resulting from the loss of the two amino terminal residues from the rabbit TPI. A similar difference in the molecular weight of Dipteran glycerolphosphate dehydrogenase isozymes was detectable by electrophoresis on similar SDS gels (54). It is noted, however, that conformation effects may qualify conclusions based upon migration rates on SDS PAGE (55).

Many mechanisms for covalent modification of protein structure are known (21, 22). NH_2-terminus acetylation is one example of a cotranslational protein modification (23). However, the Edman degradation experiments demonstrate that neither TPI isozyme is blocked at the NH_2-terminus. The Edman degradation experiments also indicate no heterogenity in length at the amino terminus which is consistent with an absence of size differences.

The data presented indicate that the nine-fold difference in levels of the TPI-subunits in the UM-61 cells, under steady state conditions, is reflected in similar differences in the level of isotope incorporation. The specific radioactivity of each of the purified isozymes is virtually identical. Furthermore, pulse chase experiments indicate nearly identical rates of degradation for each subunit with half lifes of 34 and 32 hrs for TPI-1 and -2, respectively, and the maximum specific activity of both subunits was at the zero time point (initiation of the chase component of the experiments). These observations are consistent in arguing against a posttranslation mechanism of modification between the isozymes. Such a relationship is observed for the deamidation product of TPI-1, where the maximum specific activity of TPI 1/1' is observed 3 hr following initiation of the isotope chase. The absence of a precursor-product relationship between TPI-1 and TPI-2 is consistent with the observations of Kester *et al.* (17) who observed that the appearance of TPI-2 required RNA synthesis and *de novo* protein synthesis. The results are inconsistent with a modification via a posttranslational pathway, thus the modification must occur during protein synthesis and involve only nascent peptides or possibly be due to differential mRNA processing, given that both subunits are products of a single structural locus (14, 16, 20).

In summary, the results indicate that the electrophoretic difference of 4 charge units between the TPI-1 and TPI-2 subunits results from a covalent modification. The modification is not associated with NH_2 terminus modification or deamidation of the labile asparagines. Heterogeneity in length at the amino terminus of the molecule and detectable differences in molecular weight have also been excluded. The subunits do not exhibit a precursor-product relationship, at least at the level of posttranslational modification. The exact nature of the type 2 modification of hominoid TPI relative to the constitutive subunit is still unknown at this time, however.

Many examples of developmentally regulated or tissue specific expression of closely related proteins are known. In several cases, these multiple proteins are known to be products of a single structural locus (26, 29). At least two mechanisms, including (1) co- or posttranslational modification and (2) differential mRNA processing, could be responsible for generating multiple isozymes. The present data exclude several possible posttranslational modifications. Given the increasing prominence of differential mRNA processing, this mechanism is an intriging possibility.

The regulation of TPI-2 expression is uniquely different from many other examples of differential expression in that it is restricted to rapidly dividing cells (e.g. mitogen stimulated lymphocytes, transformed lymphoblasts or fibroblasts, placental tissue). Furthermore, TPI-2 expression is a very recent evolutionary event, as it is seen only in hominoids, even though the amino acid sequence of this isozyme is highly conserved. Given the uniquely restricted expression of this isozyme, it will be of interest to define the mechanism responsible for generation of the TPI-2 subunit. The mitogen induced form of TPI presents a unique opportunity to study the regulation of gene expression in the general context of cellular proliferation.

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