Human triose-phosphate isomerase deficiency: A single amino acid substitution results in a thermolabile enzyme

(hereditary hemolytic anemia/glycolytic enzyme/missense mutation/thermolabile protein/x-ray crystallography)

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ABSTRACT Triose-phosphate isomerase (TPI; 6-glycer-aldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) deficiency is a recessive disorder that results in hereditary anemia and neuromuscular dysfunction. To determine the molecular basis of this disorder, a TPI allele from two unrelated patients homozygous for TPI deficiency was compared with an allele from a normal individual. Each disease-associated sequence harbors a G-C → C-G transversion in the codon for amino acid-104 and specifies a structurally altered protein in which a glutamate residue is replaced by an aspartate residue. The importance of glutamate-104 to enzyme structure and function is implicated by its conservation in the TPI protein of all species that have been characterized to date. The glutamate-to-aspartate substitution results in a thermolabile enzyme as demonstrated by assays of TPI activity in cultured fibroblasts of each patient and cultured Chinese hamster ovary (CHO) cells that were stably transformed with the mutant alleles. Although this substitution conserves the overall charge of amino acid-104, the x-ray crystal structure of chicken TPI indicates that the loss of a side-chain methylene group (-CH2CH2COO– → -CH2COO–) is sufficient to disrupt the counterbalancing of charges that normally exists within a hydrophobic pocket of the native enzyme.

Triose-phosphate isomerase (TPI; 6-glycer-aldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) catalyzes the movement of a single proton to interconvert dihydroxyacetone phosphate and glyceraldheyde 3-phosphate in glycolysis and gluconeogenesis (1). The enzyme, a dimer of identical subunits that are 248 amino acids in humans (2, 3), has no cofactors, required metal ions, or cooperativity between subunits. Both prokaryotic and eukaryotic TPI are very similar in sequence and structure and are characterized by a high catalytic efficiency. The second-order rate constant for the formation of dihydroxyacetone phosphate, the thermodynamically favored reaction, is close to the rate constant for a diffusion-limited encounter of substrate and enzyme (4, 5).

Site-directed mutagenesis combined with crystallographic and kinetic studies have provided a means of defining the contribution of specific TPI amino acids to enzyme activity (6, 7). This method has focused on amino acids implicated by high-resolution structural data and chemical-modification experiments to be important to the catalytic mechanism of the enzyme. We have chosen a complementary approach to understand TPI structure-function relationships in studying natural TPI gene mutations that exist within the human population. Hereditary TPI deficiency is an autosomal recessive disease that has severe clinical manifestations including chronic hemolytic anemia and neuromuscular disorders (8). Homozygous-deficient individuals usually have 3–20% of normal TPI activity; all of this activity is heat labile, suggesting that at least one allele encodes a structurally altered protein (3, 9, 10). The other allele is presumably null, since mutations producing no detectable enzyme activity or immunologically cross-reacting material are by far the most prevalent abnormality affecting the human TPI locus (11–15).

In the present study, we have determined the nucleotide sequence of two TPI alleles, each of which was isolated from unrelated individuals homozygous for TPI deficiency. Relative to a normal allele (16), each allele harbors a single base pair change. This change consists of a G-C → C-G transversion in the codon for amino acid-104 and results in a thermolabile protein having an aspartate residue in the place of a glutamate residue. The significance of glutamate-104 to enzyme structure and function has been deduced from the crystal structure of chicken TPI.

MATERIALS AND METHODS

Cell Cultures. Fibroblast and Chinese hamster ovary (CHO) cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (DMEM). CHO cells transformed with pHyg (17), a plasmid DNA encoding Escherichia coli hygromycin B phosphotransferase, were maintained under selective pressure in DMEM supplemented with 700 µg of hygromycin B per ml.

Construction and Analysis of Genomic Libraries. DNA was purified from TPI-deficient fibroblast cell lines (18) and partially digested with Sau3A I. Fragments in the range of 15–25 kilobase pairs were isolated (19), treated with calf intestinal alkaline phosphatase, ligated to phage λ EMBL3B DNA (20) that had been digested to completion with BamHI and EcoRI, and packaged into bacteriophage particles (19, 21). Phage were screened (22) with a 32P-labeled intron 4-specific sequence of the expressed TPI gene that does not cross-react with TPI processed pseudogenes (16). DNA from TPI recombinant phage was isolated for subcloning into phage M13 or plasmid vectors (19) and sequenced (23–25).

Hamster Cell Transformation. Calcium phosphate-mediated DNA transformation of CHO cells was essentially as described (26). Coprecipitates consisted of 1 µg of pHyg, a 10-fold molar excess (relative to pHyg) of plasmid DNA harboring either a normal TPI allele or a TPI-deficient allele, and carrier CHO DNA to bring the total amount of transforming DNA to 20 µg/ml per 100-mm dish. After 2–3 weeks, colonies resistant to increasing concentrations of hygromycin B (300–700 µg/ml) were either pooled and assayed for TPI enzyme or isolated, expanded, and then assayed for enzyme.

TPI Enzyme Assay. Cells were lysed by two cycles of freezing and thawing, and centrifuged at 8000 × g and 4°C for 30 min. Vertical starch gel electrophoresis of 150 µg of

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Abbreviations: TPI, triose-phosphate isomerase; CHO, Chinese hamster ovary.
protein was in Tris/EDTA (pH 9.3) at 8 V/cm for 18 hr (27).
To test for thermolabile enzyme, the extracts were incubated for 36 min at room temperature or 52°C prior to electrophoresis. TPI enzyme activity was determined by using D-glyceraldehyde 3-phosphate as a substrate for TPI in an α-glyceroephosphate dehydrogenase/NADH-coupled assay (27). TPI isozymes were photographed under short-wave ultraviolet light.

**Computer Analysis.** The crystal structure of chicken TPI at 2.5-Å resolution (28) has been refined by using a restrained-structure-factor least-squares program (ref. 29; P.J.A., W. Taylor, and D.C.P., unpublished data). The standard crystallographic R factor was 0.20, with good geometry, using all 17,042 terms to 2.5-Å resolution. Interatomic distances were calculated as the means of equivalent distances in the two subunits. The average discrepancy between these distances was ±0.25 Å. Amino acid substitution studies were done on an Evans and Sutherland PS300 graphics system with the FRODO program (30).

For computer graphic studies of the glutamate-to-aspartate substitution, the Glu-104 side chain was replaced by an aspartate side chain in the chicken TPI coordinates. By assuming that the rest of the TPI structure was unchanged, coordinate sets for two torsion angles of the aspartate side chain, $\chi_1$ (N-Cr-Cr-Cr) and $\chi_2$ (C-Cr-Cr-Cr), were generated at 10-degree intervals. These sets were evaluated by using a simple hard-sphere repulsion potential to determine which side-chain conformations had unfavorable contacts with other amino acid residues. Only two favorable minima were found: one at $\chi_1 = -75$ to $-55$ degrees, $\chi_2 = -25$ to $+25$ degrees; and the other at $\chi_1 = -75$ to $-55$ degrees, $\chi_2 = -155$ to $+155$ degrees. These two minima are equivalent, since the 180-degree rotation of $\chi_2$ corresponds to an interchanging of the equivalent O2 and O4 atoms. This small range of possible conformations was examined on the graphics for potential associations with neighboring amino acids.

**RESULTS**

**Isolation and Characterization of the TPI-Deficient Alleles.** Patient 1 was an infant of unknown race whose cultured fibroblasts have 19.5% of normal TPI activity; patient 2 was a male Caucasian of Northern European extraction whose cultured fibroblasts have 8% of normal TPI activity (3). Genomic libraries in λ phage EMBL3B were constructed with DNA of the two patients. A minimum of 660,000 recombinant phage were screened from each library to yield nine plaques that hybridized to intron 4-specific sequences of a normal, expressed TPI gene (16): eight for patient 1 and one for patient 2. One TPI gene from each library that conformed in structure to the full-length expressed gene was sequenced in entirety. Relative to the normal gene (ref. 16; I.O.D. and L.E.M., unpublished intron sequences), a single base pair change was present in each TPI-deficient allele (Fig. 1). This change, a $C\rightarrow G$ transversion in the codon for amino acid-104, results in a protein having an aspartate residue in the place of a glutamate residue (Fig. 1). Therefore, the mutant proteins, designated TPI[104 Glu→Asp], differ from the normal protein by lacking a single methylene group in the side chain of residue-104.

**Enzymatic Properties of TPI[104 Glu→Asp].** TPI enzyme in extracts prepared from cultured fibroblasts of patient 1 is thermolabile as demonstrated by a total disappearance of activity after incubation for 40 min at 52°C (3). In contrast, enzyme activity in extracts of normal fibroblasts decreases by only 17% after an equivalent treatment (3). This decrease is due to inactivation of the least abundant and more acidic protein product of the TPI gene (see below). If the glutamate-to-aspartate change at position 104 confers susceptibility to heat inactivation, then fibroblasts from patient 2 should also contain thermolabile TPI.

To test this hypothesis, lysates from the normal fibroblast cell line L153 and cultured fibroblasts of each patient were electrophoresed in a starch gel with or without prior incubation for 36 min at 52°C. We chose an electrophoretic approach in measuring TPI activity to resolve the several isozymes known to be present in cultured human fibroblasts (27, 31–33). Upon staining the gel for TPI activity, TPI-1/1 and TPI-1/2 (32), also referred to as isozymes $a$ and $e$, respectively (27) were readily detected in L153 extracts (Fig. 2A). With prolonged staining, TPI-2/2 (32), also called TPI i (33), became visible. TPI-1 and TPI-2 subunits are products of the same DNA locus, as demonstrated by studies of naturally occurring variant isozyme patterns (34) and TPI cDNA and genomic sequences (3, 16). TPI-1 is constitutively expressed, whereas TPI-2 expression is restricted to dividing cells and is the more unstable of the two forms (32, 33). When L153 extracts were incubated at 52°C, the thermolabile TPI-1/2 and TPI-2/2 isozymes disappeared, while TPI-1/1 activity remained constant (Fig. 2A) (27, 31, 32). In contrast to L153 extracts, untreated extracts of the TPI-deficient fibroblasts contained detectable amounts of only the TPI-1/1 isozyme, and all enzymatic activity was lost after incubation at 52°C (Fig. 2A). These results indicate that the glutamate-to-aspartate substitution at position 104 destabilizes all three TPI isozymes. The mutant TPI-1/2 and TPI-2/2 isozymes

![Fig. 1. Comparison of normal and defective TPI nucleotide and amino acid sequences. (A) Arrows indicate the single nucleotide difference between the mRNA complementary strand of a normal TPI allele and an allele isolated from patient 2. This allele from patient 2 is identical in sequence to an allele isolated from patient 1 (data not shown). (B) Nucleotides of a normal TPI allele and the corresponding amino acids in TPI protein are shown. Arrows designate the base pair substitution of the TPI-deficient alleles and the resultant amino acid change at position 104 of the defective TPI enzyme.](image-url)
may be so unstable that either they do not constitute a detectable amount of the steady-state TPI in the TPI-deficient fibroblasts or they are inactivated during extract preparation. It has not been determined if the TPI in either individual consists exclusively of TPI<sup>104</sup> Glu<sup>-</sup>Asp. Accordingly, the unsequenced allele of either patient may encode the glutamate-aspartate substitution, encode a different heat-labile protein, or be null and produce no protein. DNA sequence analysis indicates that only one allele of patient 1 produces a protein with an abnormal amino acid-104 (I.O.D. and L.E.M., unpublished data).

To test the effect of the glutamate-to-aspartate substitution on TPI stability in the absence of any contributions from the protein product of the uncharacterized allele, a normal and the two TPI<sup>104</sup> Glu<sup>-</sup>Asp alleles were subcloned into pBR322 DNA and introduced with the plasmid pHyg, encoding the dominant selectable gene for hygromycin B phosphotransferase (17), into cultured CHO cells. CHO-cell transformants resistant to hygromycin B were either pooled or established as clonal cell lines, lysed, and assayed for TPI. All data presented below are derived from transformed cells of mixed clonal origin. Identical results were obtained with cloned transformants (data not shown).

CHO TPI is resistant to heat inactivation (Fig. 2A), electrophoretically distinct from the two most abundant human isozymes (35) (Fig. 2A), and, therefore, serves as an internal control for measurements of human TPI activity. In extracts prepared from CHO cells that had been transformed with pTPI (a plasmid containing a normal human allele), human TPI-1/1 was detected at levels comparable to the endogenous hamster enzyme (Fig. 2B). TPI-1/2, if present, comigrated with the human/hamster enzyme (see below); the minor human TPI-2/2 species comigrated with CHO TPI and, thus, was not assayable (Fig. 2B). After extract incubation at 52°C, TPI-1/1 was resistant to heat inactivation as expected (Fig. 2B). What appears to be stability of the heat-labile TPI-1/2 isozyme (Fig. 2B) is best attributed to stability of a human/hamster heterodimer that comigrates with TPI-1/2 at a position half-way between TPI-1/1 and the hamster enzyme. This heterodimer would most logically, consist of one TPI-1 subunit and one hamster TPI subunit, both of which are resistant to heat treatment. In untreated extracts prepared from CHO cells that had been transformed with pTPI<sup>104</sup> Glu<sup>-</sup>Asp (a plasmid containing the sequenced allele of either TPI-deficient patient), the pattern of human isozymes was identical to the pattern in untreated extracts prepared from cells that had been transformed with pTPI (Fig. 2B).

Upon heating, only the CHO enzyme was detected (Fig. 2B), indicating that all enzyme forms consisting of one or two of the defective human monomers were unstable. These results establish that the TPI<sup>104</sup> Glu<sup>-</sup>Asp alleles isolated from each patient encode a thermolabile enzyme.

**DISCUSSION**

We have demonstrated that two unrelated individuals homozygous for TPI deficiency are characterized by a TPI allele that differs from normal by a single base pair substitution. This substitution results in a thermolabile protein having an aspartate residue in the place of a glutamate residue at amino acid position 104.

The functional importance of Glu-104 to TPI structure and activity is implicated by its conservation in the enzymes of all species that have been characterized to date, including *Bacillus stearotherophilus* (36), *Escherichia coli* (37), *Saccharomyces cerevisiae* (38), coelacanth (39), chicken (40, 41), rabbit (42), and human (2, 3). Approximately 25% of the TPI amino acid sequence is invariant in all of these species. TPI is a dimer of subunits that lack covalent crosslinks, prostatic groups, disulfide bonds, or the need for cofactors or metal ions. While x-ray crystallographic data are not available for the human enzyme, complete three-dimensional structures of the chicken enzyme (refs. 29 and 43; P.J.A., W. Taylor, and D.C.P., unpublished data) and the yeast enzyme (43, 44), both of which are essentially free of isozymic forms, have been established. For the purposes of this paper, it is more appropriate to infer aspects of human TPI conformation from the chicken rather than the yeast data, since human and chicken TPI differ by 33 amino acids whereas human and yeast TPI differ by 116 amino acids. The electron density map of chicken TPI indicates that each subunit is roughly spherical and is folded to form an inner cylinder or barrel consisting of eight strands of parallel ß-pleated sheets (Fig. 3). These strands are joined primarily by ß-helical segments that constitute the outer surface of each subunit. As is typical of globular protein molecules, those amino acid side chains that are shielded from contact with the solvent medium are either nonpolar or are hydrogen-bonded to complementary groups of one or more neighboring amino acids. Glu-104 lies between two ß-helical regions (designated D<sub>1</sub> and D<sub>2</sub> (45)) in a short stretch of random coil near the subunit interface (Fig. 3). Most of the negatively charged side chain of Glu-104 is buried...
in a hydrophobic pocket together with the positively charged side chains of Arg-98 and Lys-112 in the same subunit. Like Glu-104, both Arg-98 and Lys-112 are conserved in the TPI of all species for which sequence data exist, suggesting that they are important to enzyme structure and function. The side chain of Glu-104 is situated between the side chains of Arg-98 and Lys-112 and functions, at least in part, to shield the positive charges on the Arg-98 and Lys-112 side chains from one another (Fig. 4). The N\(^{\text{2}}\) carboxyl oxygen of Glu-104 hydrogen bonds to the N\(^{\text{2}}\) and N\(^{\text{3}}\) guanidinium nitrogens of Arg-98, and the O\(^{\text{2}}\) carboxyl oxygen of Glu-104 hydrogen bonds to the N\(^{\text{3}}\) side-chain nitrogen of Lys-112. Of these atoms, only O\(^{\text{1}}\) of Glu-104 and N\(^{\text{2}}\) of Arg-98 of Glu-104 and N\(^{\text{2}}\) of Arg-98 forms intersubunit bonds with O\(^{\text{1}}\) of the Glu-77' side chain and the main-chain carbonyl oxygen of Thr-75' (the ' indicates residues of the second subunit). Therefore, Glu-104 may function to orient the guanidinium group of Arg-98 in such a way that optimizes these intersubunit contacts. Also noteworthy, Thr-75' hydrogen bonds to the side chains of Asn-11 (O\(^{\text{2}}\) - O\(^{\text{1}}\) of Glu-111) and Glu-97 (O\(^{\text{1}}\) - O\(^{\text{2}}\) of Glu-97). These two side chains further hydrogen bond to Lys-13 (O\(^{\text{2}}\) - O\(^{\text{1}}\) of Glu-13; O\(^{\text{1}}\) - O\(^{\text{2}}\) - O\(^{\text{1}}\) of Glu-13; O\(^{\text{2}}\) - O\(^{\text{1}}\) - O\(^{\text{2}}\) - O\(^{\text{1}}\) of Glu-13). (data not shown). Lys-13 interacts with the C\(^{\text{6}}\) oxygen of the substrates and, most likely, serves as a catalytic electrophile for the carbonyl group of dihydroxyacetone phosphate (6, 40). Therefore, through a succession of hydrogen bonds, Glu-104 establishes several indirect although potentially significant connections to the active site of the enzyme.

The side chain of Asp-104 in the mutated enzyme of each patient is identical in charge but shortened by a methylene group relative to the side chain of Glu-104 in the normal enzyme. Currently, studies of how this glutamate-to-aspartate substitution affects human TPI structure and thermal stability are not possible because of the lack of purified normal and TPI\(^{\text{104}}\) Glu->Asp isozymes. However, with the data available, a potentially informative yet speculative analysis of TPI\(^{\text{104}}\) Glu->Asp structure was undertaken by introducing an aspartate residue at position-104 in a computer graphics representation of the chicken enzyme. Two major assumptions were made in this analysis. One assumption was that the substitution does not grossly perturb protein folding. This assumption seems to be reasonable because, with the exception of an alanine-to-threonine substitution in bacteriophage T4 lysozyme (46), we have found no precedent in the literature for the induction of structural changes that propagate more than a few tenths of an Å away from a single amino acid change that confers thermostability. X-ray crystallographic comparisons of wild-type and mutant ferredoxins (47), hemoglobins (47), thermolysins (48, 49), and T4 lysozymes (47, 50, 51) suggest that differences in the thermostability of proteins are due to subtle changes in, for instance, hydrophobic interactions and hydrogen bonds rather than to more pervasive changes in secondary and tertiary structure. To minimize disturbance to the rest of the TPI structure, a second assumption was that possible conformations for the TPI\(^{\text{104}}\) Asp side chain can be determined solely on the basis of minimal repulsion potentials between Asp-104 and neighboring amino acid residues. When these two assumptions were made for the 1296 coordinate sets that were tested as possible torsion angles for the aspartate side chain, one favorable conformation encompassing a small range of torsion angles was obtained. As exemplified by model building, a χ\(^1\) (N\(^{\text{5}}\)-C\(^{\text{6}}\)-C\(^{\text{7}}\)-O\(^{\text{6}}\)) torsion angle of ~70 degrees and a χ\(^2\) (C\(^{\text{1}}\)-C\(^{\text{7}}\)-C\(^{\text{8}}\)-O\(^{\text{8}}\)) torsion angle of +20 degrees allowed formation of a 3.10-Å hydrogen bond from O\(^{\text{1}}\) of Asp-104 to N\(^{\text{2}}\) of Lys-112 without any need to modify the crystallographically observed conformation of Lys-112. However, in this conformation, O\(^{\text{4}}\) of Asp-104 was more than 4.2 Å from the positively charged guanidinium group of Arg-98. When the conformation of the Arg-98 side chain was subsequently altered to allow Arg-98 hydrogen-bonding to residues-104 and Glu-77' as in the normal enzyme, the contacts between Arg-98 and Thr-75' were strengthened or lost. From these limited evaluations, it can be hypothesized that the glutamate-to-aspartate substitution, while maintaining an overall negative charge, minimizes the distance between the hydrogen-bonded ion pairs involving the side chain of position-104 so that one or more of these pairs are reduced to, at best, a weak electrostatic interaction. Alternatively, the side chains of Arg-98 and/or Lys-112 might move to accommodate the shortened Asp-104 side chain. In either situation, an unfavorable interaction between the positive charge on N\(^{\text{2}}\) of Lys-112 and the partial positive charge on N\(^{\text{2}}\) of Arg-98 would lower the stability of the native enzyme and promote unfolding, explaining the in-
creased temperature sensitivity of the mutated enzyme. Particular to the situation to evoke a change in Arg-98
conformation, the intersubunit association of Arg-98 and Thr-75’ would be jeopardized under conditions where bonding
between Arg-98 and Glu-77’ is maintained. This would weaken the overall intersubunit interaction because 9 of the
21 intersubunit hydrogen bonds that are <3.2 Å in length involve Thr-75 or Thr-75’ and/or Arg-98 or Arg-98’. It should
be noted that a reduction in intersubunit bonding does not necessarily imply a reduction in enzymatic activity because
TPI does not exhibit cooperativity between the two subunits. An additional and more speculative outcome of the glutam-
ate-to-aspartate substitution involves effects extending to Lys-13 in the active site of TPI with effects on enzyme
catalysis.

Definitive analysis of TPI Glu→Asp properties should come from the synthesis and characterization of yeast
TPI Glu→Asp (E. Lolis and G. A. Petsko, personal communication). Yeast TPI was chosen for study because its complete three-dimensional structure has been determined (43, 44), and site-directed mutagenesis of the yeast TPI gene has been successfully applied to studies of TPI catalysis (6).

Note Added in Proof. Sequence analysis of steady-state TPI RNA by primer extension has demonstrated that the uncharacterized allele of patient 2 also harbors a G:C→C:G transversion in the codon for amino acid-104.

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