Characterization of Triosephosphate Isomerase Mutants With Reduced Enzyme Activity in *Mus musculus*

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ABSTRACT

Four heterozygous triosephosphate isomerase (TPI) mutants with approximately 50% reduced activity in blood compared to wild type were detected in offspring of 1-ethyl-1-nitrosourea treated male mice. Breeding experiments displayed an autosomal, dominant mode of inheritance for the mutations. All mutations were found to be homozygous lethal at an early postimplantation stage of embryonic development, probably due to a total lack of TPI activity and consequently to the inability to utilize glucose as a source of metabolic energy. Although activity alteration was also found in liver, lung, kidney, spleen, heart, brain and muscle the TPI deficiency in heterozygotes has no influence on the following physiological traits: hematological parameters, plasma glucose, glucose consumption of blood cells, body weight and organo-somatic indices of liver, spleen, heart, kidney and lung. Biochemical investigations of TPI in the four mutant lines indicated no difference of physicochemical properties compared to the wild type. Results from immunoinactivation assays indicate that the decrease of enzyme activity corresponds to a decrease in the level of an immunologically active moiety. It is suggested that the mutations have affected the *Tpi-1* structural locus and resulted in alleles which produce no detectable enzyme activity and no immunologically cross-reacting material. The study furthermore suggests one functional TPI gene per haploid genome in the erythrocyte and seven other tested organs of the mouse.

TRIOSEPHOSPHATE isomerase (TPI, D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1.) catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) and thus plays an important role in both glycolysis and gluconeogenesis as well as in glyceride synthesis. The enzyme, a dimer of identical subunits (CORRAN and WALEY 1974; YUAN *et al.* 1979) has a molecular weight of 53,000 (GRACY 1975; KRIETSCH *et al.* 1970) and is distributed in all tissues of a wide variety of species (SCOPES 1968; GRACY 1974; SNAPKA *et al.* 1974).

In humans TPI deficiency has been described as an autosomal recessive disorder (SCHNEIDER *et al.* 1965a; review of cases by EBER *et al.* 1979; CLAY, SHOR and LANDING 1982; ROSA *et al.* 1985). Homozygous or double-heterozygous individuals have a residual TPI activity of 5–20% of normal in erythrocytes, and suffer from chronic nonspherocytic hemolytic anemia associated with severe neurologic and muscular disorders, retarded growth, and increased susceptibility to bacterial infections (SCHNEIDER *et al.* 1965a; VALENTINE *et al.* 1966; SKALA *et al.* 1977; VIVES-CORRONS *et al.* 1978). However, the incidence of homozygous TPI deficiency in human populations is quite rare when the frequency of heterozygous individuals is considered (EBER *et al.* 1979; MOHRENWEISER 1981; MOHRENWEISER and FIELEK 1982; SATOH *et al.* 1983; EBER *et al.* 1984). Data obtained by these authors indicate that heterozygotes for putative null alleles associated with an approximate 50% reduction of TPI activity are quite common occurring at a frequency of 2–48 per 1000 births.

In contrast to the situation in humans the findings in other mammals are rather scarce. Only one mouse mutant, which when homozygous expresses 42% activity of the wild type in erythrocytes, has been described (BULFIELD, HALL and TSAKAS 1984). By screening the activity of glycolytic enzymes in blood of mice CHARLES and PRETSCH (1987) identified four mutants with approximately 50% TPI deficiency in mutagenicity experiments after paternal treatment with 1-ethyl-1-nitrosourea (ENU). The present study reports the genetical, physiological and biochemical characterization of the TPI deficient mutants. The results suggest that the mutational events affected the *Tpi-1* structural locus generating null alleles resulting in neither detectable enzyme activity nor in immunologically cross-reacting material. All mutations were indicated to be homozygous lethal at an early postimplantation stage of embryonic development, probably due to a total lack of TPI activity and the resulting

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inability to utilize glucose as a source of metabolic energy.

MATERIALS AND METHODS

Animals: Four mouse mutants with approximately 50% TPI activity as compared to the wild type in blood were obtained independently in mutagenicity experiments with ENU (CHARLES and PRETSCH 1987). The heterozygous mutant offspring were selected and backcrossed at least ten generations to the C3H/El wild-type strain to transfer the mutant gene to a standard inbred genetic background. For physiological and biochemical characterization 10-week-old animals of both sexes were used. After weaning, four mice of the same sex were housed per cage and maintained under constant temperature (22 ± 2°) with a fixed 12 hr light/12 hr dark cycle. They had free access to tap water and were fed *ad libidum* with a standardized diet (Altromin 1314, Altromin International, Lage, Germany, F.R.). According to the conclusions drawn from the results of the present investigation the mutant alleles of the mutant lines TPI 2161, TPI 2312, TPI 3502 and TPI 9606 (CHARLES and PRETSCH 1987) were designated *Tpi-1*$^{m\text{-}1Neu}$, *Tpi-1*$^{m\text{-}2Neu}$, *Tpi-1*$^{m\text{-}3Neu}$, and *Tpi-1*$^{m\text{-}4Neu}$, respectively.

Genetical analysis: The transmission ratio of the mutations in heterozygotes was calculated using the percentage of mutant offspring relative to that expected for a Mendelian dominant trait in a backcross. Heterozygous mutants originating from such backcrosses with C3H/El wild types were mated *inter se* to recover possibly homozygous mutants. If no third phenotype could be observed the 50% TPI deficient offspring resulting from these intercrosses were genetically tested for homozygosity by brother-sister mating (FAVOR 1984).

Stage of homozygous lethality: Twelve-week-old homozygous wild types or heterozygous mutants, respectively, were mated *inter se* in 1:1 mode of mating. After conception, which was ascertained by the presence of a vaginal plug, the male was removed. Autopsy of the dams originating from these control crosses and intercrosses was carried out 14 days *post conceptionem*. Ovaries and uteri were examined for corpora lutea and live and dead implants following the standard protocol for the dominant lethal test on male mice (EHLING *et al.* 1978).

Sampling of tissues: For the experiments animals were weighed and blood was taken with heparinized glass capillary tubes from the retroorbital sinus. Fresh blood was used for determination of glucose consumption of blood, plasma glucose, hematocrit, red-blood-cell number and osmotic fragility. The remainder was kept on ice for biochemical determinations. Subsequently the mice were sacrificed by cervical dislocation and dissected. Organs were removed and weighed for measuring the organo-somatic indices (organ weight × 100/body weight). Tissue samples were excised and put in ice-cold 0.15 M KCl for analysis of enzyme activities. To minimize the effects of diurnal rhythm, the procedure was done between 8 and 10 a.m.

Hematological data: Hematocrit was measured by centrifugation of blood in hematocrit capillary tubes at 8000 rpm for 10 min in a Microfuge (Heraeus-Christ, Osterode, Germany, F.R.) at room temperature. Red blood cells were counted in an improved Neubauer's counting chamber (Brand, Wertheim, Germany, F.R.) using Hayem's solution. Hemoglobin (Hb) measurements were done by the cyanomethemoglobin reaction in an automatic analyzer (ACP 5040, Eppendorf, Hamburg, Germany, F.R.) according to the manufacturer's guidelines (Boehringer, Mannheim, Germany, F.R.). The osmotic fragility of erythrocytes was determined by adding 5 μl of whole blood to 1 ml of a series of different concentrations of NaCl (0–0.7%). Samples were mixed and then incubated for 2 hr at 37°. After centrifugation for 3 min the amount of hemoglobin in the supernatant was measured in the UVICON 710 spectrophotometer (Kontron Instruments, Zürich, Switzerland) at 560 nm. The mean corpuscular fragility (MCF) was determined and is defined as the NaCl concentration at which 50% of the erythrocytes lyse. Plasma glucose concentration was analyzed by the glucose oxidase-peroxidase method (WERNER, REY and WIELINGER 1970). For glucose consumption determinations, blood was preincubated at 37° for 10 min. Aliquots were taken in 15-min intervals and glucose concentration was measured. The incubated blood was shaken shortly before each sampling. The decrease of glucose concentration was linear during the chosen time.

Preparation of blood and tissue extracts: For studies of enzyme activities and physicochemical properties of TPI, lysates of whole blood and isolated erythrocytes were used. Isolation of red blood cells was done by the sulfoethylcellulose filtration technique described by NAKAO, NAKAYAMA and KANKURA 1973. Packed erythrocytes and blood (0.5 and 1 vol, respectively) were lysed in 10 vol of TRA buffer (0.1 M triethanolamine-HCl, pH 7.5, 1 mM EDTA and 5 mM DTT) containing 0.05% (w/v) saponin. After 10 min at 4° the samples were centrifuged at 8800 x *g* for 10 min at 4°. Further dilution was performed in TRA buffer. Tissues were homogenized in ice cold 0.15 M KCl (10% w/v) by sonication using a pulsed Sonifier B15 (Branson Sonic Power Company, Danbury, Connecticut). The homogenates were centrifuged at 15,000 × *g* for 20 min at 4° and the supernatant was kept on ice until used for analysis.

Enzyme specific activities in blood and tissues: The reaction mixture for determination of TPI activity contained TRA buffer, 8.4 mM glyceraldehyde-3-phosphate (Ba salt), 0.41 mM NADH and 2.8 units of L-glycerol-3-phosphate dehydrogenase per ml buffer (CHARLES and PRETSCH 1987). Substrate was prepared as described by GRACY (1975). The determination of specific activities of lactate dehydrogenase (LDH; EC 1.1.1.27), malate dehydrogenase (MDH; EC 1.1.1.37), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), 3-phosphoglycerate kinase (PGK; EC 2.7.2.3), phosphoglyceromutase (PGAM; EC 2.7.5.3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), pyruvate kinase (PK; EC 2.7.1.40) and glutathione reductase (GR; EC 1.6.4.2) in blood was as previously described (CHARLES and PRETSCH 1987). The automatic analyzer ACP 5040 was utilized for all assays. Protein concentration in tissue extracts was measured by the method of LOWRY *et al.* (1951) using crystalline bovine serum albumin as standard.

Physicochemical properties: Measurement of the Michaelis-Menten constant (K_m) for GAP was performed at 25° and pH 7.5 in the same medium as used for the TPI assay with five concentrations of GAP varying between 0.1 to 2.8 mM. The actual concentrations of GAP were detected by the method of MICHAL and BEUTLER (1974). The apparent K_m was obtained by extrapolation from the double reciprocal plot (LINEWEAVER and BURK 1934). The best linear regression fit of the data was determined by the method of least squares.

The optimum pH was determined with the TPI assay system for pH varying from 6.5 to 9.0. Thermal stability of TPI in erythrocytes was determined by incubating erythrocyte lysate at 47 and 50°. The concentration of hemoglobin in the lysates was 0.015 g/ml. At 5-min time intervals aliquots were taken and quickly mixed with ice-cold 0.1 M

TRA buffer. Residual TPI activity was assayed immediately thereafter as described above.

Polyacrylamide gel isoelectric focusing was carried out on ultrathin gels containing 2.5% ampholine (Pharmacia LKB, Freiburg, Germany, F.R.) according to the procedure of RADOLA (1980). After prefocusing at 4° and 400 V for 30 min, electrofocusing was performed at 4° following 600, 1000 and 1400 V each for 30 min on the LKB 2117 Multiphor (Pharmacia LKB, Freiburg, Germany, F.R.). The staining technique of SCOPES (1968) was used.

Immunological properties: Antiplasma raised in chickens against human TPI was kindly supplied by S. W. EBER of the Universitäts-Kinderklinik Göttingen, Germany, F.R. For immunoinactivation experiments the antiplasma was diluted 1:12 with TRA buffer, containing 154 mM/liter NaCl. Increasing amounts (0 to 0.03 ml) of antiplasma were added to 0.1 ml erythrocyte lysate containing a constant amount of enzyme activity (2 units) and the volume was brought to 0.2 ml with TRA buffer containing 154 mM/l NaCl. After 2-hr incubation at 37°, the immunoprecipitate was sedimented at 12,000 × *g* for 20 min at 4°, and the supernatant was assayed for TPI activity. The titer is defined as the number of enzyme units inactivated by 1 ml of antiplasma.

Statistical analysis: In the biochemical and physiological characterization experiments data of the same number of female and male animals were used for the mean and SEM. For statistical comparisons between the different genotypes Student's *t*-test was used. Data from the homozygous-lethality experiments were compared using a nonparametric method (Wilkoxon-rank-sum-test). Differences were stated as significant for *P* values less than 0.05.

RESULTS

Genetical characterization: Table 1 demonstrates the results of the genetical characterization studies. Crosses of heterozygotes for the four independent TPI mutant lines with wild-type C3H/El animals revealed homozygous wild type and heterozygous mutant offspring with about 50% of normal TPI activity in a ratio of approximately 1:1 with normal litter size. Intercrossing heterozygotes of each mutant line resulted in only wild types and animals with 50% reduced activity in an approximate 1:2 ratio. No third class of animals were recovered. Litter size of intercrosses was significantly reduced in each mutant line compared to the litter size of backcrosses.

The absence of a third class of animals and the decreased litter size of intercrosses suggest that homozygotes are lethal. This hypothesis was proved genetically by the absence of homozygotes among offspring of intercrosses. In each mutant line 20 randomly chosen animals resulting from intercrosses with altered TPI activity were crossed *inter se* to determine their genotype. Wild types in the progeny of each of these matings excluded homozygosity of the parents. According to FAVOR (1984) in this case the probability not to detect possible homozygotes is less than 0.001.

Stage of homozygous lethality *in utero*: To study the time of death of homozygous embryos, uterine content, and ovaries of intercrossed and control dams were examined 14 days *post conceptionem*. The results are summarized in Table 2. Data of intercrossed dams were compared with those of control dams. In all four mutant lines there was no significant difference in the number of corpora lutea, preimplantation loss or late death implants between both groups. However, the number of live implants was significantly reduced by approximately 25% and the number of early, postimplantation deaths significantly increased in dams of intercrosses. Since the mean absolute decrease in live implants is approximately as high as the mean absolute increase in early death implants these results suggest that lethality of homozygous TPI mutants occurs at an early postimplantation stage of embryonic development.

Physiological characterization: To exclude the possibility that the decreased TPI activity in blood extracts is a result of an altered red-cell population, routine hematological tests were performed. In heterozygous mutants no deviations from the wild-type values were observed (Table 3). Other physiological traits such as plasma glucose (wild-type value [WTV] = 1.41 ± 0.11 g/liter), glucose consumption of blood (WTV = 2.75 ± 0.25 mg/g Hb/hr), body weight (WTV = 21.6 ± 0.5 g) and somatic indices of liver (WTV = 5.70 ± 0.12 g/100 g body weight), lung (WTV = 0.61 ± 0.02 g/100 g body weight), kidney (WTV = 1.34 ± 0.04 g/100 g body weight), spleen (WTV = 0.36 ± 0.02 g/100 g body weight) and heart (WTV = 0.38 ± 0.01 g/100 g body weight) also indicated no difference between wild types and mutants. This finding suggests that there is no easily detectable effect of reduced TPI activity on physiological functions in heterozygotes.

Enzyme activities in blood and other tissues: Table 4 presents the level of TPI activity in blood and several tissues of wild types and heterozygous mutants. A comparable TPI reduction of approximately 50% was observed in all tissues studied. This fact is an indication for structural gene mutations in the four mutant lines or mutations affecting non-tissue-specific gene regulation.

Besides TPI the activities of nine other enzymes were routinely tested in blood lysates. Apparently the alteration involves only TPI as the activities of the nine other enzymes had normal activity in the mutants. This feature is interesting especially in the case of GAPDH. Linkage conservation of *Tpi-1*, *Gapd*, and *Ldh-2* found in 11 mammalian species (LALLEY and MCKUSICK 1985) suggests that in the mouse the structural locus for TPI maps closely to the structural locus for GAPDH. Indeed, *Tpi-1* maps 0.15 cM to *Gapd* (PRETSCH 1988). Thus, a deletion which includes both *Tpi-1* and *Gapd* could be excluded for the four independent mutations.

Physicochemical properties: Support for the hy-

TABLE 1

Genetic characterization of four TPI mutants

Cross *Tpi* genotype[a]	Litter size[b]	a/a (*n*)	a/m (*n*)	m/m (*n*)	Ratio a/a:a/m	Reduction of litter size (%)[c]
a/a × *a/a*	6.2 ± 0.3	186				
a/m-1N × *a/a*	6.4 ± 0.4	256	243		1:0.95	
a/m-1N × *a/m-1N*	5.2 ± 0.4*	92	218	0	1:2.37	19
a/m-2N × *a/a*	6.6 ± 0.4	235	246		1:1.05	
a/m-2N × *a/m-2N*	5.2 ± 0.5*	90	135	0	1:1.50	21
a/m-3N × *a/a*	6.9 ± 0.5	331	329		1:0.99	
a/m-3N × *a/m-3N*	5.3 ± 0.5*	97	143	0	1:1.47	23
a/m-4N × *a/a*	6.5 ± 0.4	245	252		1:1.03	
a/m-4N × *a/m-4N*	5.0 ± 0.5*	85	152	0	1:1.79	23

[a] a = *Tpi-1*^{a}: wild-type allele; m-1N = *Tpi-1*$^{m\text{-}1Neu}$; m-2N = *Tpi-1*$^{m\text{-}2Neu}$; m-3N = *Tpi-1*$^{m\text{-}3Neu}$; m-4N = *Tpi-1*$^{m\text{-}4Neu}$: TPI deficient alleles.

[b] Data are given as mean ± SEM of 30 litters. Significant differences ($P \leq 0.05$) between litter size of intercrosses and backcrosses are marked by *.

[c] Reduction of litter size of intercrosses compared to backcrosses is calculated as follows: (1 − litter size of intercrosses : litter size of backcrosses) × 100.

TABLE 2

***In utero* lethality of homozygotes for four TPI deficiency mutations in mice**

Cross *Tpi* genotype[a]	No. of females	Corpora lutea	Preimplantation loss[b]	Dead implants		Live implants
				Early	Late	
a/a × *a/a*	13	10.1 ± 0.4	0.8 ± 0.3	1.6 ± 0.5	0.2 ± 0.2	7.5 ± 0.5
a/m-1N × *a/m-1N*	12	10.3 ± 0.4	0.5 ± 0.3	3.7 ± 0.6*	0.5 ± 0.3	5.6 ± 0.6*
a/a × *a/a*	12	9.7 ± 0.4	0.6 ± 0.4	1.0 ± 0.4	0.3 ± 0.1	7.8 ± 0.5
a/m-2N × *a/m-2N*	9	9.6 ± 0.4	1.3 ± 0.3	2.3 ± 0.5*	0.3 ± 0.2	5.7 ± 0.5*
a/a × *a/a*	12	9.7 ± 0.5	0.8 ± 0.3	0.8 ± 0.3	0.3 ± 0.3	7.8 ± 0.5
a/m-3N × *a/m-3N*	12	9.4 ± 0.6	0.7 ± 0.2	2.3 ± 0.4*	0.3 ± 0.1	6.1 ± 0.6*
a/a × *a/a*	10	10.0 ± 0.5	1.1 ± 0.3	1.2 ± 0.3	0.2 ± 0.1	7.5 ± 0.3
a/m-4N × *a/m-4N*	15	10.3 ± 0.5	0.7 ± 0.3	3.7 ± 0.8*	0.2 ± 0.1	5.7 ± 0.6*

Data are given per female as mean ± SEM. Significant differences ($P \leq 0.05$) between females originating from control crosses and intercrosses are marked by *.

[a] Same symbols are used as in Table 1.

[b] Preimplantation loss was determined using the difference between corpora lutea and number of total implants.

TABLE 3

Hematological data of four heterozygotes for TPI deficiency mutations in mice

Tpi genotype[a]	Hematocrit (%)	Hb (g/liter)	RBC $\times 10^{12}$/liter	MCF (% NaCl)
a/a	47.3 ± 0.5	156 ± 5	8.3 ± 0.2	0.501 ± 0.015
a/m-1N	47.0 ± 0.8	153 ± 3	8.4 ± 0.2	0.506 ± 0.009
a/m-2N	47.5 ± 0.6	158 ± 5	8.6 ± 0.4	0.489 ± 0.020
a/m-3N	47.0 ± 0.4	155 ± 4	8.2 ± 0.3	0.485 ± 0.014
a/m-4N	46.5 ± 1.0	159 ± 4	8.3 ± 0.3	0.513 ± 0.025

Data are given as mean ± SEM of 10 animals. Abbreviations used: RBC, red blood cells; MCF, mean cellular fragility; Hb, hemoglobin.

[a] Same symbols are used as in Table 1.

pothesis that the mutants are true nulls comes from investigations of some physicochemical properties of the mutant erythrocyte enzyme (Table 5). With the exception of the reduced specific activity no significant alterations in the heat stability, pH optimum and K_m for GAP were detected in the mutants. TPI from normal mouse erythrocytes is resolved in three bands upon isoelectric focusing. No difference could be observed between the banding pattern of mutant and wild-type enzyme except the decreased staining intensity of the mutants.

Immunological properties: Since the above mentioned results revealed strong evidence for true null mutations the question arose whether these mutations result in TPI protein molecules not being produced, or if the mutations resulted in enzymatically inactive TPI molecules. To address this problem, immunoinactivation experiments with chicken antiplasma against human TPI were performed. TPI of mouse showed cross reactivity with the chicken antiplasma and the enzyme was inactivated by association with the chicken antibody: 1 ml antiplasma inhibited about

TABLE 4

Specific TPI activity in several tissues of wild types and heterozygotes for TPI deficiency mutations in mice

Tpi genotype[a]	Blood	Liver	Lung	Kidney	Spleen	Brain	Heart	Muscle
a/a	100 ± 2 (765)	100 ± 5 (9825)	100 ± 5 (2930)	100 ± 7 (16971)	100 ± 2 (2994)	100 ± 4 (18357)	100 ± 6 (12800)	100 ± 4 (37005)
a/m-1N	50 ± 1	50 ± 3	52 ± 5	55 ± 3	51 ± 7	48 ± 4	51 ± 7	49 ± 4
a/m-2N	50 ± 2	51 ± 3	50 ± 5	53 ± 5	52 ± 4	47 ± 6	48 ± 5	46 ± 6
a/m-3N	50 ± 1	54 ± 2	52 ± 4	52 ± 6	51 ± 3	54 ± 5	53 ± 4	52 ± 5
a/m-4N	49 ± 1	51 ± 2	52 ± 3	46 ± 3	52 ± 4	50 ± 4	48 ± 3	47 ± 4

Data are expressed as percentage of TPI wild-type activity and given as mean ± SEM of 10 animals. In parenthesis the mean specific TPI wild-type activity is given as units/g Hb in the blood and in units/g protein in other tissues. No significant differences were found in the amounts of protein extracted per gram tissue.

[a] Same symbols are used as in Table 1.

TABLE 5

Physicochemical properties of erythrocyte TPI of heterozygotes for four TPI deficiency mutations in mice

Tpi genotype[a]	Specific activity[b] (% of the wild type)	K_m (GAP)[c] (mM)	Heat stability[d] 47°	Heat stability[d] 50°	pH optimum[c]
a/a	100 ± 5	0.70 ± 0.10	75.8 ± 1.2	45.9 ± 1.2	8.0
a/m-1N	50 ± 4	0.75 ± 0.08	74.8 ± 1.0	46.5 ± 1.3	8.0
a/m-2N	47 ± 4	0.66 ± 0.10	76.6 ± 1.5	46.7 ± 2.4	8.0
a/m-3N	51 ± 2	0.81 ± 0.12	76.9 ± 1.6	46.0 ± 1.6	8.0
a/m-4N	53 ± 3	0.71 ± 0.11	75.5 ± 1.2	44.2 ± 1.4	8.0

[a] Same symbols are used as in Table 1.

[b] Data are given as mean ± SEM of 10 animals.

[c] Data are given as mean ± SEM of 4 animals (double determination).

[d] Percent remaining activity after 20-min incubation. Data are given as mean ± SEM of 4 animals (double determination).

TABLE 6

Specific activity of erythrocyte TPI and the titre determined by immunoinactivation of heterozygotes for 4 TPI deficiency mutations in mice

Tpi genotype[a]	Specific TPI activity[b] Units/g Hb	Specific TPI activity[b] % of the wild type	Titer[c] Units/ml antiplasma	Titer[c] Percent of the wild type
a/a	745 ± 38	100 ± 5	228.6 ± 8.4	100 ± 4
a/m-1N	371 ± 30	50 ± 4	225.6 ± 6.0	99 ± 3
a/m-2N	353 ± 28	47 ± 4	224.0 ± 7.2	98 ± 3
a/m-3N	380 ± 18	51 ± 2	226.8 ± 9.6	99 ± 4
a/m-4N	395 ± 25	53 ± 3	233.2 ± 4.8	102 ± 2

[a] Same symbols are used as in Table 1.

[b] Data are given as mean ± SEM of 10 animals.

[c] Data are given as mean ± SEM of 4 animals.

229 units of wild-type erythrocyte TPI (Table 6). The immunoinactivation of TPI from wild type and all mutants exhibited approximately the same level of TPI inhibition per ml of antiplasma. However, their TPI activity expressed in units/g Hb was only about 50% of the normal. Accordingly, it is suggested that in the mutants a normal enzyme is synthesized from the wild-type autosome which accounts for the 50% activity, and that the product of the second is immunologically undetectable and enzymatically inactive.

DISCUSSION

The characterization of the four TPI mouse mutants addressed two main questions: (1) the site and mode of the induced mutations leading to the reduced enzyme activity and (2) the impact of the altered enzyme activity on physiological functions.

Study of the enzyme characteristics: In general a reduction of enzyme activity in a heterozygous mutant to 50% of the wild-type level strongly indicates a null allele. In such heterozygotes the enzyme activity is derived from the intact wild-type allele. Consequently null mutants should not differ from the wild type in physicochemical properties of the enzyme under consideration. However, since null alleles may result from a variety of mutational events ranging from amino acid substitution, chain termination mutations, deletions to frame shift mutations, the immunological properties of the mutant enzyme may give clues as to the type of induced mutation.

In the case of the four TPI mutants studied the physicochemical and immunological analysis suggests mutations which (1) have affected the structural locus for TPI and (2) generate null alleles producing neither detectable enzyme activity nor immunologically cross-reacting material. Indeed, linkage studies using flanking chromosome markers indicated that the four TPI mutations reside at or near the *Tpi-1* structural locus on mouse chromosome *6* (W. PRETSCH, unpublished data). Moreover, the first suggestion is supported by the examination of a variety of tissues. In all organs studied the TPI deficiency is expressed equally. This result, is not only strong evidence for structural gene mutations, but also supports the hypothesis of only one functional TPI gene per haploid genome postulated by several authors (BROWN *et al.* 1985), at least in the tissues studied. At this time it is not possible to define further the molecular basis of the TPI deficien-

cies reported here. However, since the mutants were induced by treatment with ENU, a compound which is known to induce mainly point mutations (SIMMON *et al.* 1979; SINGER 1979; JOHNSON and LEWIS 1981; EHLING *et al.* 1982) it seems probable that the enzyme deficiencies are a result of base-pair substitutions. First results of DNA analysis of the mutant alleles revealed no restriction fragment length polymorphism (RFLP) for a series of enzymes arguing against a total deletion of the alleles being responsible for the loss of enzyme activity. This suggests the classification of the four null mutations as point mutations (H. MOHRENWEISER, personal communication).

It is noteworthy that, in contrast to other erythrocyte enzymes, *i.e.*, LDH, GPI, or MDH, in our own mutagenicity experiments with ENU (CHARLES and PRETSCH 1987) TPI mutations have only been detected leading to 50% enzyme deficiency in heterozygotes *i.e.*, leading to a null allele producing no detectable enzyme activity or immunologically cross-reacting material. Furthermore no TPI electrophoretic variants have been found in inbred strains of mice (GREEN 1981). Thus, a parallel may be drawn to the situation in humans. Population studies exhibited a high frequency of TPI null alleles and a low frequency of electrophoretic variants (PETERS, HOPKINSON and HARRIS 1973; NEEL 1978; NEEL, MOHRENWEISER and MEISLER 1980; NEEL *et al.* 1980). Moreover, mutations producing no detectable enzyme activity or immunologically cross-reacting material are by far the most prevalent abnormalities affecting the human *TPI-1* structural locus (EBER *et al.* 1979; MOHRENWEISER 1981; MOHRENWEISER and FIELEK 1982; MOHRENWEISER, FIELEK and WURZINGER 1981). These findings may be explained by the fact that for TPI many mutational events result in a null. They are also reflected in a highly conserved sequence homology of TPI isolated from various species (KRIETSCH *et al.* 1970; BANNER *et al.* 1975; ARTAVANIS-TSAKONAS and HARRIS 1980; YUAN, TALENT and GRACY 1981) indicating a high likelihood that an amino acid substitution would result in a functional effect (DAYHOFF 1978).

Genetical analysis and physiological effects of the mutations: Taken together, the results of the genetic studies of the four TPI mutants are in accordance with Mendelian expectations and clearly indicate lethality of homozygous mutants. In fact this result is not surprising: in vertebrates many tissues such as erythrocytes, platelets, leucocytes and the renal medulla nearly exclusively, and brain and muscle mainly, utilize glucose as the metabolic source of energy via glycolysis. Furthermore, in the mouse anaerobic glycolysis appears to be the predominant metabolic energy delivering pathway during the early postimplantation period (CLOUGH and WHITTINGHAM 1983). In the glycolytic pathway glucose or glycogen is converted in a number of steps to fructose-1,6-diphosphate which is then cleaved into three-carbon units, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). Only GAP is further catabolized to lactate or pyruvate. It is clear that a total depletion of TPI prevents the interconversion of DHAP and GAP and results in an accumulation of DHAP in the cell. DHAP has been suggested to be toxic for tissues and responsible for the clinical manifestations of TPI deficiencies (SCHNEIDER *et al.* 1965b; CLAY, SHOR and LANDING 1982). Furthermore a loss of net energy production would be expected in exclusively glycolytic tissues.

In consequence, a total absence of TPI activity as expected in homozygous nulls is incompatible with normal life and normal embryonal development. Indeed, there was an increased number of early postimplantation deaths in the uterus of intercrossed gravid dams in the range expected in the case of homozygous lethality. However, the development of the preimplantation embryo until its implantation appears not to be affected since there was no significant difference of preimplantation loss between dams originating from intercrosses and those originating from control crosses. Thus, the experimental data are in accordance with effects expected from a total block of glycolytic energy supply in consideration of the predominant energy supplying metabolic pathways in pre- and postimplantation embryos. In the mouse and other mammals in contrast to the early postimplantation stages the Krebs' cycle appears to be an important energy supplying pathway throughout the preimplantation period. Lactate and pyruvate are thought to be main substrates for energy metabolism during this period (BRINSTER 1970). The glycolytic capability reflected, *e.g.*, in glucose utilization (BRINSTER 1967) and activities of glycolytic enzymes such as hexokinase (BRINSTER 1968), PGK or TPI (KOZAK and QUINN 1975) is low during the first one or two days of the embryo's life and increase sharply after blastocyst formation (BRINSTER 1970). The deleterious consequences of a lack of TPI should first take effect between the implanting blastocyst and egg cylinder stages when (1) metabolic activity has strongly increased compared to the early preimplantation embryo, (2) energy supply depends mainly on glucose via anaerobic glycolysis, and (3) residual enzymes or messenger RNA synthesized during oocyte development have disappeared.

The above described situation in mice regarding TPI null alleles and homozygous lethality seems to be reflected in humans: surveys of human populations indicate a striking discrepancy between the high frequency of heterozygous carriers of TPI deficiency and the small number of detected homozygous or

double-heterozygous deficient and clinically afflicted individuals (EBER *et al.* 1979, 1984; MOHRENWEISER 1981; MOHRENWEISER and FIELEK 1982). These authors suggested that the homozygous state may result in prenatal lethality. However, since the death of the embryos should occur at a very early stage of postimplantation development the conception and subsequent early death of the embryo would not be detected.

In contrast to the fate of the homozygous TPI deficient mouse mutants with expected 0% TPI activity, the heterozygotes with about 50% activity seem to be physiologically unaffected. Hematological and other physiological traits studied showed normal values. This is in accordance with the observations of BULFIELD, BALL and PETERS (1987) who did not find signs of anemia or any significant alterations in hematological parameters in a homozygous mouse variant having 42% of the erythrocytic TPI activity of C57BL/10 mice. Also in humans 50% of normal TPI activity does not result in any obvious clinical manifestations (review of cases by EBER *et al.* 1979; ROSA *et al.* 1985). Half the normal enzyme activity is in general sufficient to maintain function under normal circumstances. This is especially true for TPI which does not represent a metabolically rate-limiting step in glycolysis.

The present investigation of the four TPI mutants thus give clear indications for the mode in which chemically induced null mutations of a structural gene coding for enzymes important in essential pathways can lead to lethal effects in homozygous carriers. It furthermore suggests the TPI mouse mutants as good animal models for the human defect.

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