Differential Tissue and Flight Developmental Expression of Glycerol-3-Phosphate Dehydrogenase Isozymes in the Chagas Disease Vector Triatoma infestans

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Abstract. Glycerol-3-phosphate dehydrogenase (GPDH) isozymes are differentially expressed among tissues and during flight development. GPDH-1 is involved in the flight-muscle metabolism and GPDH-2 provides precursors for lipid biosynthesis in many tissues. We have isolated and characterized from Triatoma infestans, a Chagas disease vector, two cDNAs encoding for GPDH-1 and GPDH-2 isozymes. The inferred amino acid sequences showed high identity with other GPDH sequences from flying insects. A GPDH-2 transcript was found in fifth instar nymphs, thoracic muscles, adult gonads, and fat bodies. Both isozymes are present in 30-day-old adult thoracic muscle transcripts, and the pattern of expression differs between sexes. The expression of GPDH-1 begins earlier in females, and GPDH-2 is expressed more abundantly in female adult thoracic muscles than in those from males. This finding is consistent with those of other investigators who showed a higher flight initiation probability in T. infestans females than in males.

INTRODUCTION

American trypanosomiasis or Chagas disease is well recognized as the most serious human parasitic disease of Latin America in terms of its social and economic impact,1 with approximately 12 million persons infected and approximately 90 million living in disease-endemic areas.2 Triatoma infestans (Hemiptera; Reduviidae; Triatominae), an hematophagous insect, is the main vector in the Southern Cone of Latin America in terms of its social and economic impact.1,2 With approximately 12 million persons infected and approximately 90 million living in disease-endemic areas.2 Triatoma infestans (Hemiptera; Reduviidae; Triatominae), an hematophagous insect, is the main vector in the Southern Cone of Latin America in terms of its social and economic impact.1,2 It is estimated that 500,000 to 1,000,000 cases of this disease occur every year in the Americas, with approximately 12 million persons infected and 90 million living in disease-endemic areas.2

MATERIALS AND METHODS

Insects. Triatoma infestans was reared at 28 ± 1°C at a relative humidity of 60–70% with a 6-hour light/18-hour dark cycle and fed once every two weeks after molting on restrained chickens. The tissues were dissected under aseptic conditions and stored in liquid air. Each sample was a pool of tissue from 20 adults or 50 fifth instar nymphs specimens. Adult, 1–5 day-old thoracic muscles, fat bodies, and gonads were collected from both sexes. For the flight developmental study, females and males thoracic muscles were stored separately from adults and fifth instar nymphs 1–5 days of age and 30 days of age of each stage. Fifth instar nymphs were sexed by the differences described by Espinola.10 Only adult thoracic muscles were used for the reverse transcription–polymerase chain reaction (RT-PCR) with degenerate oligonucleotides and rapid amplification of cDNA end by PCR (RACE) assays.

Apis mellifera worker bees were obtained from a farm beehive. Drosophila melanogaster were obtained from the Departamento de Ecología, Genética y Evolución, Universidad de Buenos Aires (Buenos Aires, Argentina).

Isolation of total RNA. Total RNAs were isolated from pools of insect tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Samples used were T. infestans adult and fifth instar nympha thoracic muscles, adult gonads, and fat bodies; A. mellifera adults thoracic muscles; and D. melanogaster whole bodies. Extracts were diluted 1:100 with nuclease-free water (0.1% diethylpyrocarbonate) and RNA concentration determined by absorption at 260 nm.

Degenerated primer selection, retrotranscription, PCR, and cloning. First-strand cDNA synthesis was performed with 1 μL of Oligo-dT20 (50 μM) (Invitrogen), 3 μg of total RNA

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FIGURE 1. A, Nucleotide sequences and deduced amino acid sequences of glycerol-3-phosphate dehydrogenase (GPDH) from Triatoma infestans adult thoracic muscles EU139315 (A) and n gonads EU139316 (B). B, CLUSTAL W version 1.83 protein multiple sequence alignment. T. infestans adult thoracic muscle GPDH isozyme GPDH-1 and T. infestans adult gonad GPDH isozyme GPDH-2.
from three-day-old adult *T. infestans*, and 300 U of SuperScript III-RT (Invitrogen) in a 20-µL reaction volume that was incubated at 55°C for 1 hour. For subsequent PCR, 1 µL of first-strand cDNA (template), 4 µM of each degenerate primer, 0.2 µM of each specific primer, 0.5 U of Taq Platinum DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl₂, and 2.5 µL of 10× PCR buffer minus M (Invitrogen) were added to a 25-µL reaction volume. Degenerate oligonucleotide primers for PCR were derived from the conserved region of the sequence of GPDH from the flying insects *D. melanogaster* (gi|16197915|gb|AAL13721.1), *A. mellifera* (gi|3064138|gb|AAC14552.1), *Locusta migratoria* (gi|4163991|gb|AAD03500.1), *Anopheles gambiae* (gi|55245320|gb|EAA03917.3), and *Aedes aegypti* (gi|99031624|pdb|1X0V). The α-helices are shown as black bars, β-sheets as black arrows, ligand interaction residues are shaded in gray, and active center residues are shaded in black.

**FIGURE 2.** Amino acid sequence alignment and secondary structure assignment of glycerol-3-phosphate dehydrogenase (GPDH) using the SwissModel workspace program. Hs = *Homo sapiens* GPDH-1 (gi|99031624|pdb|1X0V); Ti = *Triatoma infestans* EU139315-isoform-1; Am = *Apis mellifera* gi|3064138|gb|AAC14552.1; Dm = *Drosophila melanogaster* gi|295746|emb|CAA32381.1; Lm = *Locusta migratoria* gi|4163995|gb|AAD03502.1; Mf = *Methuselah* gi|15984772|gb|CAD36856.1; Ne = *Neurospora crassa* gi|3510469|gb|CAB43778.1; Os = *Oryza sativa* gi|24145364|gb|CAD36857.1; Sa = *Saccharomyces cerevisiae* gi|8052|gb|CAD36858.1; Sc = *Saccharomyces cerevisiae* gi|8053|gb|CAD36859.1; Sm = *Saccharomyces cerevisiae* gi|8054|gb|CAD36860.1. The α-helices are shown as black bars, β-sheets as black arrows, ligand interaction residues are shaded in gray, and active center residues are shaded in black.

**FIGURE 1.** Continued
GLYCEROL-3-PHOSPHATE DEHYDROGENASE IN TRIATOMA INFESTANS

The cDNA product was amplified by conventional PCR using degenerate primers F4: 5'-TTTGYGARACNACNATYYGYGC-3' and R2: 5'-ATNACNGCNGCYYTNGRATT-3', and corresponding specific primers for A. mellifera F5: 5'-TGGGAAACTAC-TATTGGGTC-3' and R3: 5'-ATTTACTGAGCTTTTGATATT-3' and D. melanogaster F6: 5'-TGGCAGGACA-CATCCGGCTGC-3' and R4: 5'-ATGACAGGCCGTCTTG- GTTT-3'. The PCR was performed with a Thermocycler (Mycycler; Bio-Rad, Hercules, CA) with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 1.5 minute, and finally an incubation at 72°C for 7 minutes. Amplification was confirmed and fragment sizes was estimated by electrophoresis of 10 µL of the PCR product on a 10-cm 1.5% agarose gel (Tris-acetate EDTA buffer, pH 8) containing 0.5 µg/mL of ethidium bromide.

The PCR products with a 200-bp predicted size were obtained for T. infestans and positive control samples A. mellifera and D. melanogaster. The T. infestans PCR product was purified from the agarose gel using the QIAEX II kit (Invitrogen) and was cloned into pCR4-TOPO TA cloning vector (Invitrogen) following the manufacturer's instructions. Analysis of the sequence was performed for several clones using the BLAST program and comparing it with the SWISSPROT database. The T. infestans fragment was shown to be part of the GPDH gene and cDNA.

**RACE-PCR.** Four gene-specific primers were designed based on the T. infestans sequence. Two primers had the same sequence as the coding strand: FGSP: 5'-GGGAGAGCC-CAACCTCTGGAAGATA-3' and FNGSP: 5'-GGTGTCCAGCAGTTAACCCCCGTAGA-3'. The resulting products of the RT-PCR with each primer as template were cloned into the pCR4-TOPO TA cloning vector and DNAs of several clones obtained by this procedure were purified and sequenced.

For amplification of 5' ends of GPDH isoforms, 5 µg total RNA were treated with calf intestine phosphatase to remove 5' phosphates and eliminate truncated mRNA and non-mRNA. Dethiophorylated RNAs were treated with tobacco acid pyrophosphatase to remove the 5' cap structure from intact, full-length mRNA. Using T4 RNA ligase, the RNA oligonucleotide was ligated to the 5' region of the RNAs. The GeneRacer RNA oligonucleotide provides a known priming region of the RNAs. The GeneRacer RNA oligonucleotide provides a known priming region of the RNAs. Reverse transcription was conducted using the RGSP primer and Superscript III RT. To amplify the first-strand cDNA and to obtain the 5' end, GSRP and the GeneRacer 5' Primer (homologous to the GeneRacer RNA oligo) were used. It was necessary to perform nested PCR with RNGP and Gene Racer 5' nested primer. The resulting cDNA fragment was cloned into the pCR4-TOPO TA cloning vector and DNAs of clones obtained by this procedure were purified and sequenced.

For the amplification of 3' end, 5 µg of total RNA were reverse transcribed with Gene Racer Oligo dT and Superscript III RT. To amplify the first-strand cDNA and to obtain the 3' cDNA encoding for GPDH isoforms, an FGSP and a GeneRacer 3' primer (homologous to GeneRacer Oligo dT Primer) were used. Only mRNA with a poly A tail was reverse transcribed and amplified using PCR. It was necessary to perform additional PCR with nested primers FNGSP and GeneRacer 3' nested primer. The resulting products were cloned into the pCR4-TOPO TA cloning vector and DNAs of clones obtained were purified and sequenced.

**Sequencing.** The resulting products of the RT-PCR with degenerate oligonucleotide primers and RACE-PCR were cloned into the pCR4-TOPO TA cloning vector, and DNAs of 20 clones from each experiment obtained by this procedure were purified and sequenced by the ATGen Molecular System (Faculty of Sciences, Montevideo, Uruguay) using primers M13 forward and M13.

**Sequence analysis and homology modeling.** Sequence analysis was carried out with the DNA SeqEq version 1.03 program (Applied Biosystems, Darstard, Germany). The GenBank database was searched using the BLAST utility program. Analysis of the T. infestans GPDH isozyme sequences was performed using the BLAST-x. version 2.1.14 program and comparing it with the SWISSPROT database. To calculate its identity with other GPDHs, complete sequences were obtained from the SWISSPROT database available at the NCBI website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was carried out using CLUSTALW version 3.2. The deduced protein was analyzed using ExPASy Proteomics Tools (http://www.expasy.org/tools/).

The amino acid sequence of Homo sapiens GPDH-1 (gi|99031624|pdb|11x0|/A/99031624|) was obtained from Swiss Prot protein sequence database (http://www.expasy.org/sprot). A homology model was generated using the Swiss-Model Repository (http://swissmodel.expasy.org/repository/) and an automated protein modeling server (GlaxoSmithKline, Research Triangle Park, NC).

**Expression of GPDH isoforms.** Isolation of total RNA from tissue pools and first-strand cDNA synthesis was performed and subjected to RT-PCR analysis. Subsequent PCRs used 1 µL of first-strand cDNA as template, 0.2 µM of each specific primer and β-actin primers as positive controls, 0.5 U of Taq Platinum DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl2, and 2.5 µL of 10× PCR Buffer minus M (Invitrogen) in a 25-µL reaction. The PCR was performed using a thermocycler (Mycycler; Bio-Rad) with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles 94°C for 30 seconds, 60°C for 40 seconds, and a final incubation at 72°C for 7 minutes. Specific primers for GPDH were F: 5'-GGCTGGTTTCCATTGATGGCCATTAGG-3' and different reverse primer corresponding to GPDH-1 Rm: 5'-TTTCTTCATTGATGGCCATTAGG-3' and different reverse primer corresponding to GPDH-2 Rm: 5'-GTCCACGATTTAACCCTGCCCATAGA-3' and different reverse primer corresponding to GPDH-3 Rm: 5'-TCTTCACTGGTGTCTCTC-3' and GPDH-2 Rg: 5'-GTCACAGATACTTGATTTT-3'. The β-actin-specific primers AF1: 5'-AAGGGCTACTCTTTTACA-3', AF2: 5'-ATTGCCACCCAGCATTTT-3', AR1: 5'-CCATCCAGCAATTATCAGA-3', and AR2: 5'-AGCGTATCAGCCTTCTTACA-3' were designed based on a sequenced 300-bp PCR product amplified with the β-actin universal primer pair. The RT-PCR products (100 µL) were separated by electrophoresis on a 10-cm 1.5% agarose gel (Tris-acetate EDTA buffer, pH 8) containing 0.5 µg/mL of ethidium bromide. Digital images were obtained under ultraviolet illumination with a Chemi Doc System (Bio-Rad). Semi-quantification of PCR bands was performed with the ImageJ program through a graphical method that measures peak areas.
RT-PCR amplification using degenerate primers. A 200-bp fragment was obtained from *T. infestans* thoracic muscles with the degenerate primer pair F4 and R2 using a cDNA pool as a template. Upon cloning, sequencing, and database alignment, the fragment was shown to be part of GPDH gene. On the basis of the cDNA sequence obtained, specific primers (GSPF, GSNPF, GSPR, and GSPNR) were designed for RACE analysis.

RACE analysis of *T. infestans* GPDH transcripts. We carried out the RACE experiment using RNA extracted from adult *T. infestans* thoracic muscles and gonads. We amplified full-length cDNAs sequences of the GPDH gene using gene specific primers. It was necessary to perform nested PCR to obtain visible product bands.

Sequence analysis, conserved domains, and homology modeling. The RACE 3' and 5' products were obtained from thoracic muscles and gonads. Full-length cDNA fragments encode polypeptides with amino acid sequences similar to GPDH found in other flying insects. Figure 1 shows the complete cDNA sequences and the inferred amino acid sequences for *T. infestans* GPDH isoforms. The cDNA from adult muscles contained 1,253 nucleotides with an open reading frame (ORF) of 1,065 (EU139315) nucleotides that encoded 355 amino acids. The cDNA sequence includes the start codon ATG and the stop codon TAA. Adult gonad cDNAs contained 1,262 nucleotides with an ORF of 1,074 (EU139316) nucleotides that encoded 358 amino acids. These sequences have the start codon ATG and the stop codon TAA. The amino acid sequences of GPDHs that are inferred from the nucleotide sequence were aligned with those of four of the same enzymes from *G. pallida* (gi|3064138|gb|AAC14552.1|), *D. melanogaster* (gi|2957546|emb|CA32381.1|), and *L. migratoria* (gi|4163995|gb|AAD05302.1|isoform 3b). The nucleotide sequences of coding regions showed 61%, 65%, 67%, and 64% identity, respectively, and the amino acid sequences showed 73%, 80%, 81%, and 81% identity, respectively.

The GPDH amino acid sequence can be divided into two functional domains: NAD+–binding domain (pfam01210), a member of FAD/NAD(P) binding Rossmann Fold superfamily clan, and catalytic domain (GOG0240), a member of 6-phosphogluconate dehydrogenase C-terminal–like super family clan. The NAD+–binding domain in crystallized *H. sapiens* GPDH-1 consists of N-terminal residues 1–189 arranged into eight β sheets and seven α helices. All β sheets are flanked by two α helices structures. The first αβ unit contains the highly conserved GxGxxG NAD+–binding motif. The catalytic domain, which extends from residue 196 to residue 336 for *H. sapiens* GPDH-1, is organized into several α helices whose number varies with the species. Figure 2 shows GPDH aligned amino acid sequences of *H. sapiens*, *T. infestans* (EU139315), and three other insect species and illustrates secondary structure assignment using the Swiss-Model workspace. The amino acid positions are referred to in the *H. sapiens* sequence.

Catalytic residues K204–T264 and residues involved in substrate specificity (K120–D260) are fully conserved. Residues involved in ligand interaction in *H. sapiens* GPDH-1 structures G12, N13, W14, G15, S16, K20, M38, H67, K68, P94, R187, N205, G267, G268, R269, N270, R271, Q298, E305, and P346 are conserved among insect species. Residues K240D and S248K are replaced in all insect species except in *L. migratoria* (K240D) and in *A. mellifera* (S248K). Residue Q182E is replaced in *T. infestans* and *A. mellifera* sequences. Residues K178H and S249V are replaced only in *T. infestans*.

Expression of GPDH isozymes. GPDH-1 and GPDH-2 exhibit a unique temporal and tissue-specific pattern of expression in *T. infestans*. The RT-PCR with specific primers for isozymes was carried out. The positive control was a fragment of β-actin with two pairs of specific primers for *T. infestans* (AF1-AR1 and AF2-AR2). Figure 3 shows that the GPDH-1 transcript is predominant in pools from both sexes of 1–5-day-old adult thoracic muscles, and the GPDH-2 transcript is present in pools of both sexes of fifth instar nymph thoracic muscles, adult gonads, and fat bodies. Both transcripts are present in 30-day-old adult thoracic muscles. Figure 4 shows that the pattern of expression during flight muscle development shows differences between sexes. Semi-quantification in Figure 5 shows that GPDH-1 transcript is expressed later in males and GPDH-2 is highly expressed in female after the last molt to adults. These results are consistent with the metabolic role described for GPDH isozymes in flying insects.

DISCUSSION

Identity and similarity comparison of the amino acid sequences of GPDH among different species demonstrates that sequences have changed slowly over time. Transcripts coding for GPDH isozymes isolated using RNA extracted from adult thoracic muscles and gonads differs only at the 3' cDNA end and the C-terminal of the deduced amino acid sequence. The GPDH from thoracic muscles differs from the gonad isozyme that the same sequence is extended by an ORF.

![Figure 3. Polymerase chain reaction products of Triatoma infestans.](image-url)

M1, M2, M3, M4, G1, G2, G3, G4, F1, F2, F3, F4
that encodes three and nine amino acids, respectively. The predicted molecular weights are 38,964.32 daltons and 39,369.00 daltons (DNAStar-Protean; http://www.dnastar.com/). The expected electrophoretic mobility of GPDH from gonads is slower than that from thoracic muscles; their estimated pI values are 8.04 and 6.26, respectively. This finding is consistent with previous electrophoretic studies of *T. infestans* GPDH isoforms. According to molecular weights, identity, and homology, the transcript from adult thoracic muscles corresponds to GPDH-1 and the transcript from gonads corresponds to GPDH-2.

The GPDH locus in *D. melanogaster* and other insects expresses three classes of transcripts corresponding to GPDH-1, GPDH-2 and GPDH-3 by alternative splicing. In *A. mellifera*, loss of introns that are present in *D. melanogaster* or mouse genes gives rise to only one GPDH transcript. Whether the isoforms are coded by the same locus and expressed by alternative splicing or by different loci and gene organization to express two mature transcripts in *T. infestans* remains to be elucidated.

The NAD+-binding domain shows especially high conservation throughout the NAD+-binding motifs and binding residues S16, E38M, K120, and Q298. The amino acid substitutions observed do not substantially alter the predicted positions of the α and β-elements in the model predicted for *T. infestans* deduced amino acid sequence.

The catalytic domain is less conserved, and the region of the protein linking the NAD+-binding site with the catalytic domain seems particularly divergent. Overall, the amino acid replacements observed in domains do not suggest any substantial alterations to the secondary structure assignments on the basis of predictions.

The GPDH isoforms are not distributed equally in space or time. Expression is related to insect tissue or development stage; it tends to favor lipid accumulation in gonads, fat bodies, and larva or nymph thoracic muscles (GPDH-2 and GPDH-3) or to provide NAD+ for flight muscles (GPDH-1). The glycolytic enzymes hexokinase and fructose-6-phosphate dehydrogenase increase their activities in *T. infestans* adult thoracic muscles. A 30-fold increase in GPDH activity in adult flight muscles was also described for Triatomine insects and can be related to a metabolic adaptation to support flight requirements. Adult muscles should have higher glycolytic and respiratory activity and probably have higher
levels of GPDH-1 expression and activity to transfer reducing equivalents to mitochondria for ATP synthesis. Modification of the activity of an enzyme during development can be brought about through several mechanisms. These include changes in the concentration of messenger RNA for that enzyme, varying rates of transcription or mRNA processing, or mRNA degradation. The level of enzyme activity may also fluctuate because of changes in translation rates or changes in rates of enzyme degradation. Enzyme activity may be controlled by altering the catalytic efficiency of a given number of enzymes. In T. infestans, the increment may be produced by GPDH-1 expression.

GPDH-2 is the predominant isozyme in T. infestans gonads, fat bodies, and nymph thoracic muscles. The presence of GPDH-2 is probably related to the synthesis of triacylglycerols, which requires production of glycerol-3-phosphate from dihydroxyacetone phosphate. It was demonstrated that flight initiation probability in T. infestans is higher in females than in males. Accordingly, expression of GPDH-1 begins earlier in females and expression of GPDH-2 is higher in adult thoracic muscles from females than in those from males. It is known that insects use lipids as fuel for flight and reproduction. Electron microscope observations of T. infestans thoracic muscles showed large inclusions of partially extracted lipids. In addition, fatty acid binding proteins partially characterized from Dipetalogaster maximus (Triatome) flight muscles showed high identity (approximately 71% in N-terminal residues) with other flying insects. The different 3' ends of GPDH isoforms inferred for C-terminal amino acid sequences ETSEE for GPDH-1 and FFTKKSLKP for GPDH-2 could condition the enzymatic activity and the subcellular localization needed to accomplish their metabolic role.

Chagas disease is recognized as the most serious human parasitic disease of Latin America in terms of its social and economic impact. Because of their biology and close association with humans, T. infestans is one of the most important vectors of Trypanosoma cruzi. There is no vaccine or effective treatment for infection with T. cruzi. Studies on enzymes involved in flight and reproduction of the vector may contribute to understand the basic processes that give T. infestans the capacity to invade new habitats and colonize human dwellings. This knowledge of the properties of vector enzymes could provide new data for designing control campaigns.

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