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ARTICLE *in* PROTEINS STRUCTURE FUNCTION AND BIOINFORMATICS · JUNE 2004

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PROTEINS: Structure, Function, and Bioinformatics 55:824–834 (2004)

An Unusual Triosephosphate Isomerase from the Early Divergent Eukaryote *Giardia lamblia*

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ABSTRACT **Recombinant triosephosphate isomerase from the parasite *Giardia lamblia* (GlTIM) was characterized and immunolocalized. The enzyme is distributed uniformly throughout the cytoplasm. Size exclusion chromatography of the purified enzyme showed two peaks with molecular weights of 108 and 55 kDa. Under reducing conditions, only the 55-kDa protein was detected. In denaturing gel electrophoresis without dithiothreitol, the enzyme showed two bands with molecular weights of 28 and 50 kDa; with dithiotretitol, only the 28-kDa protein was observed. These data indicate that GlTIM may exist as a tetramer or a dimer and that, in the former, the two dimers are covalently linked by disulfide bonds. The kinetics of the dimer were similar to those of other TIMs. The tetramer exhibited half of the k_{cat} of the dimer without changes in the Km. Studies on the thermal stability and the apparent association constants between monomers showed that the tetramer was slightly more stable than the dimer. This finding suggests the oligomerization is not related to enzyme thermostability as in *Thermotoga maritima*. Instead, it could be that oligomerization is related to the regulation of catalytic activity in different states of the life cycle of this mesophilic parasite. Proteins 2004;55: 824–834.** © 2004 Wiley-Liss, Inc.

Key words: glycolysis; giardiasis; protein purification; oligomerization state; disulfides

INTRODUCTION

The protozoan parasite *Giardia lamblia* merits extensive studies for several reasons. From the evolutionary point of view, *G. lamblia* has been considered a rather remarkable organism because its morphological, metabolic, and molecular features correspond to those of one of the earliest branching lineages of eukaryotes.[1–5] Indeed, the G+C content, gene complementation, and rRNA subunits of *G. lamblia* are markedly similar to those of prokaryotic organisms. In addition, the parasite is the causative agent of human giardiasis, a worldwide disease that affects millions of people, in particular children.[6] Although metronidazole is effective in giardiasis,[7] it exerts strong side effects in the host,[8] and there is clear evidence that strains of *G. lamblia* resistant to metronidazole are now existent.[9] This finding underlines the need for developing drugs that are effective against giardiasis. In this regard, it is noted that an additional feature of *G. lamblia* is that it lacks mitochondria and oxidative phosphorylation.[10] Thus, in *G. lamblia*, the main source of ATP is the glycolytic pathway.[11] This finding suggests that the enzymes of glycolysis could be potential targets for drug design. The nucleotide sequence of the triosephosphate isomerase gene from *G. lamblia* has been previously reported.[12] However, there are no data on the kinetics and structure of triosephosphate isomerase from *G. lamblia*. Therefore, because of its importance in the evolutionary processes and its potential as a target for drug design, we characterized triosephosphate isomerase from *G. lamblia* (GlTIM) and also determined its distribution within the parasite.

TIM is a ubiquitous glycolytic enzyme that catalyzes the reversible isomerization between (R)-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). The kinetics and energetics of the catalytic reaction are well established,[13–16] and the three-dimensional (3D) structure of the enzyme from 13 different species has been determined. The active site residues of all known TIMs are located in the central region of the α/β-barrel and belong to the same main-chain. Most TIMs so far described are homodimers in which each of the two monomers exhibits an α/β-barrel structure. However, some TIMs from hyperthermophilic organisms have a different quaternary structure, which apparently is related to their thermal stability. For example, TIMs from *Pyrococcus woesei*[17,18] and *Methanothermus fervidus*[19] are tetrameric enzymes. In *Thermotoga neapolitana* and *T. maritima,* the enzyme is a tetramer fused with a phosphoglycerate kinase.[20,21] TIM from *T. maritima* was separated from phosphoglycerate kinase and crystallized.[21] The modified TIM was a tetramer in which two dimers were in close contact through hydrophobic and polar residues. However,

Grant sponsor: Consejo Nacional de Ciencia y Tecnología, México; Grant number: J37071-B.

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Received 7 August 2003; Accepted 11 December 2003

Published online 1 April 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/prot.20097

it is relevant to this work that the dimers of *T. maritima* were joined through two disulfide bridges, between Cys 142 of each monomer. Our studies on GlTIM show that it is an enzyme with characteristics different from those of TIMs from other mesophilic organisms.

MATERIALS AND METHODS

Parasites

Trophozoites of *G. lamblia* WB strain were cultured in TYI-S-33 as previously described.[22] Cultures were grown at 37°C for 72 h to semiconfluence and washed with phosphate buffered saline (PBS), pH 7.3.

Amplification of Triosephosphate Isomerase Gene From *Giardia lamblia*

Genomic DNA from *G. lamblia* trophozoites (WB strain) was purified by the phenol-chloroform method with slight modifications.[23] The polymerase chain reaction (PCR) conditions were those described by Gibco-BRL (Taq polymerase supplier) using the following oligonucleotides: sense 5′-AATAACATATGCCTGCTCGTC-3′ and antisense 5′-CCAGGATCCTATGTACGGG-3′, which contain *Nde*I and *Bam*HI restriction sites at the 5′ end, respectively. The reaction mixture contained 400 ng of gDNA, 1.5 mM $MgCl_2$, 0.8 mM of dNTP, and 2.5 units of Taq Polymerase (Gibco-BRL). Amplification was performed by using 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C.

Cloning of the GlTIM Gene and Sequence Analysis

The amplified DNA fragment was purified by electrophoresis using an extraction kit (Concert; Gibco-BRL) and cloned into the pCR2.1 vector, as recommended (Novagen). *E. coli* TOP10 competent cells were transformed for cloning. The cloned fragment was subcloned into the pET3a vector after digestion with *Nde*I and *Bam*HI enzymes and used to transform *E. coli* TOP10 competent cells. Enzyme restriction assays and electrophoresis were performed to confirm the correct fragment insertion into the vector.

The plasmid from positive clones was purified by using an extraction plasmid kit (Concert; Gibco-BRL) and sequenced by the enzymatic method with an automatic sequencer. A clone named 13* showed the correct sequence and orientation of the gene of *G. lamblia* TIM. This was used for the production of wild-type recombinant GlTIM.

Overexpression of GlTIM in *E. coli*

The plasmid from clone 13* was extracted and used to transform competent *E. coli* BL21(DE3)pLys cells. BL21 cells contain the gene codifying for the T7 RNA pol needed for expression of the recombinant enzyme. The transformed cells were grown in Luria-Bertani medium with 100 μg/mL ampicillin at 37°C to an A_{600nm} of 0.2–0.5. To induce overexpression, 0.4 mM isopropyl β-D-thiogalactopyranoside was added, and growth was continued overnight at 30°C.

Purification of Recombinant GlTIM

Transformed bacteria from 1 L culture were suspended in 80 mL of a buffer containing 25 mM Tris, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.9. The cells were disrupted by sonication at 4°C, six cycles of 45 s with 75 s of resting intervals. The sonicate was centrifuged at 45,000 rpm for 1 h, and the supernatant was taken to 45% saturation ammonium sulfate. The suspension was allowed to stand for 3 h at 4°C and thereafter was centrifuged at 10,000 rpm for 30 min. The pellet was discarded, and the concentration of ammonium sulfate in the supernatant was increased to 75% saturation. The suspension was incubated at 4°C overnight. It was then centrifuged at 10,000 rpm for 30 min, and the supernatant was discarded. The pellet was suspended in 5 mL of a buffer containing 25 mM Tris, 1 mM EDTA, 1 mM NaN_3, pH 8, and filtered (0.45-μm pore diameter). The dissolved protein was applied to a G-75 Sephadex (2.5 × 100 cm) column equilibrated with the same buffer. The proteins were eluted at a flow rate of 1 mL/min. Fractions of 2.5 mL were collected. GlTIM eluted after 140 mL had passed through the column. The fractions with TIM activity were pooled and concentrated in Amicon filters (YM 10) to a volume of 10 mL. The protein was dialyzed against 0.6 L of a buffer containing 10 mM Tris, 1 mM EDTA, pH 8.7. The enzyme was then applied to a Q-Sepharose FF (1.5 × 12 cm) column equilibrated and washed with the same buffer. GlTIM was eluted with a gradient of 30–100 mM NaCl. Fractions with activity were pooled and concentrated.

TABLE I. Purification of Recombinant Triosephosphate Isomerase From *G. lamblia*[†]

Step	Total protein (mg)	Total activity (μmol GAP min^{-1})[a]	Specific activity (μmol min^{-1} mg^{-1})
Homogenate (sonicated)	336	559,440	1794
G-75 column	60	457,523	3305
Q-Sepharose column	20	76,000	3800

[†]The enzyme was purified as described in Materials and Methods, starting from 1-L culture.
[a]GAP, glyceraldehyde 3-phosphate.

To increase the purity of the enzyme, GlTIM was applied to a SW300 (7.5 × 300 mm) column equilibrated with a buffer containing 50 mM triethanolamine hydrochloride, 10 mM EDTA, 150 mM NaCl, 30% glycerol, pH 7.5. Fractions with activity were pooled, concentrated, and stored at 4°C as a 50% glycerol solution in the same buffer. For the studies, the enzyme was dialyzed against a buffer containing 100 mM triethanolamine hydrochloride, 10 mM EDTA, pH 7.4. Densitometric analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions showed that GlTIM was >95% pure. This protocol was followed several times, and the enzyme yield ranged between 18 and 23 mg/L of culture. Table I summarizes the purification of GlTIM.

T. brucei and *T. cruzi* recombinant TIMs were purified as previously described.[24,25] Protein concentration of pure TIM was determined spectrometrically at 280 nm. The molar extinction coefficients at 280 nm of TcTIM and TbTIM were 36440 and 34950, respectively.[25] The molar

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                    *
GlTIM  MPARRPFIGG  FKCNGSLDF IKSHVAAIAA ---HKIPDSV DVVIAPSAVH LSTAIAANTS 57
EhTIM  MGAGKFVVGG  WKCNGTLAS IETLTKGVAA SVDAELAKKV EVIVGVPFIY IPKVQQILAG 60
PfTIM  M-ARKYFVAA  WKCNGTLES IKSLTNSFNN --LDFDPSKL DVVVFPVSVH YDHTRKLLQS 57
TbTIM  MSKPQPIAAA  WKCNGSQQS LSELIDLFNS ---TSINHDV QCVVASTFVH LAMTKERLSH 57
TcTIM  --KPQPIAAA  WKCNGSESL LVPLIETLNA ---ATFDHDV QCVVAPTFLH IPMTKARLTN 55
TmTIM  -ITRKLILAG  WKMHKTISE AKKFVSLLVN --ELHDVKEF EIVVCPPFTA LSEVGEILSG 57
HuTIM  APSRKFFVGG  WKMNGRKQS LGELIGTLNA ---AKVPADT EVVCAPPTAY IDFARQKLD- 56

                                                  *
GlTIM  ----KQLRIA AQNVYLEGNG AWTGETSVEM LQDMGLKHVI VGHSERRRIM GETDEQSAKK 113
EhTIM  EANGANILVS AENAWTKS-G AYTGEVHVGM LVDCQVPYVI LGHSERRQIF HESNEQVAEK 119
PfTIM  -----KFSTG IQNVSKFGNG SYTGEVSAEI AKDLNIEYVI IGHFERRKYF HETDEDVREK 112
TbTIM  ----PKFVIA AQNAIAKS-G AFTGEVSLPI LKDFGVNWIV LGHSERRAYY GETNEIVADK 112
TcTIM  ----PKFQIA AQNAITRS-G AFTGEVSLQI LKDYGISWVV LGHSERRLYY GETNEIVAEK 110
TmTIM  ----RNIKLG AQNVFYEDQG AFTGEISPLM LQEIGVEYVI VGHSERRRIF KEDDEFINRK 113
HuTIM  ----PKIAVA AQNCYKVTNG AFTGEISPGM IKDCGATWVV LGHSERRHVF GESDELIGQK 112

                                                          *
GlTIM  AKRALEKGMT VIFCVGETLD ERKANRTMEV NIAQLEALGK ELGESKMLWK EVVIAYEPVW 173
EhTIM  VKVAIDAGLK VIACIGETEA QRIANQTEEV VAAQLKAINN AIS--KEAWK NIILAYEPVW 177
PfTIM  LQASLKNNLK AVVCFGESLE QREQNKTIEV ITKQVKAFVD LID----NFD NVILAYEPLW 168
TbTIM  VAAAVASGFM VIACIGETLQ ERESGRTAVV VLTQIAAIAK KLK--KADWA KVVIAYEPVW 170
TcTIM  VAQACAAGFH VIVCVGETNE EREAGRTAAV VLTQLAAVAQ KLS--KEAWS RVVIAYEPVW 168
TmTIM  VKAVLEKGMT PILCVGETLE EREKGLTFCV VEKQVREGFY GLD--KEEAK RVVIAYEPVW 171
HuTIM  VAHALAEGLG VIACIGEKLD EREAGITEKV VFEQTKVIAD NV----KDWS KVVLAYEPVW 168

GlTIM  SIGTGVVATP EQAEEVHVGL RKWFAEKVCA EGAQHIRIIY GGSANGSNCE KLGQCPNIDG 233
EhTIM  AIGTGKTATP DQAQEVHQYI RKWMTENISK EVAEATRIQY GGSVNPANCN ELAKKADIDG 237
PfTIM  AIGTGKTATP EQAQLVHKEI RKIVKDTCGE KQANQIRILY GGSVNTENCS SLIQQEDIDG 228
TbTIM  AIGTGKVATP QQAQEAHALI RSWVSSKIGA DVAGELRILY GGSVNGKNAR TLYQQRDVNG 230
TcTIM  AIGTGKVATP QQAQEVHELL RRWVRSKLGT DIAAQLRILY GGSVTAKNAR TLYQMRDING 228
TmTIM  AIGTGRVATP QQAQEVHAFI RKLLSEMYDE ETAGSIRILY GGSIKPDNFL GLIVQKDIDG 231
HuTIM  AIGTGKTATP QQAQEVHEKL RGWLKSNVSD AVAQSTRIIY GGSVTGATCK ELASQPDVDG 228

GlTIM  FLVGGASLKP -EFMTMIDIL TKTRT 257
EhTIM  FLVGGASLDA AKFKTIINSV SEKF- 261
PfTIM  FLVGNASLKE -SFVDIIKSA M---- 248
TbTIM  FLVGGASLKP -EFVDIIKAT Q---- 250
TcTIM  FLVGGASLKP -EFVEIIEAT K---- 248
TmTIM  GLVGGASLKE -SFIELARIM RGVIS 255
HuTIM  FLVGGASLKP -EFVDIINAK Q---- 248
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Fig. 1. Sequence alignment of GlTIM with other TIMs. The sequence of EhTIM, PfTIM, TbTIM, TcTIM, TmTIM, and HuTIM are shown. The active site residues are marked (*).

extinction coefficient of GlTIM was 26600, calculated according to Pace et al.[26]

To ascertain the best conditions for storage of GlTIM, the purified enzyme was incubated in buffers with different pH, maintaining a constant ionic strength. After this, aliquots were taken and diluted in a buffer containing 100 mM triethanolamine, 10 mM EDTA at pH 7.4. The activities of all samples were measured at this pH. The activity as a function of pH showed a bell-shaped curve, after it was incubated for 2 h, with a maximum activity between pH 6.5–9.0. The activity dropped significantly outside of this pH range. At pH 7.4, the activity of the enzyme was unaffected for at least 2 weeks.

Activity Assays

Enzyme activity in the direction of GAP to DHAP was measured at 25°C by following the decay in absorbance at 340 nm of 1 mL reaction mixture at pH 7.4 that contained 100 mM triethanolamine, 10 mM EDTA, 0.2 mM NADH, 0.9 U α-glycerol-3-phosphate dehydrogenase, 1 mM GAP (except when catalytic constants were determined), and 10 ng TIM per mL. The kinetic constants were calculated from initial velocities (v_i) obtained at different substrate concentrations.

For the determination of Km, k_{cat}, and K_i values for 2-phosphoglycolate (PG), the concentration of GAP ranged between 0.5 and 4 mM. The determination of catalytic constants in the direction of DHAP to GAP were determined at 25°C in 100 mM triethanolamine, 10 mM EDTA, 1 mM NAD, 4 mM arsenate, 120 μM dithiothreitol, 1 U glyceraldehyde 3-phosphate dehydrogenase per mL, 100 ng GlTIM per mL, and 0.3–10 mM DHAP.

Polyclonal Anti-GlTIM Antibodies

Chickens (16-week-old Delkab-Warren) were immunized subcutaneously with 240 μg of highly purified GlTIM dissolved in complete Freund's adjuvant. The immunization dose was repeated two times at intervals of 1

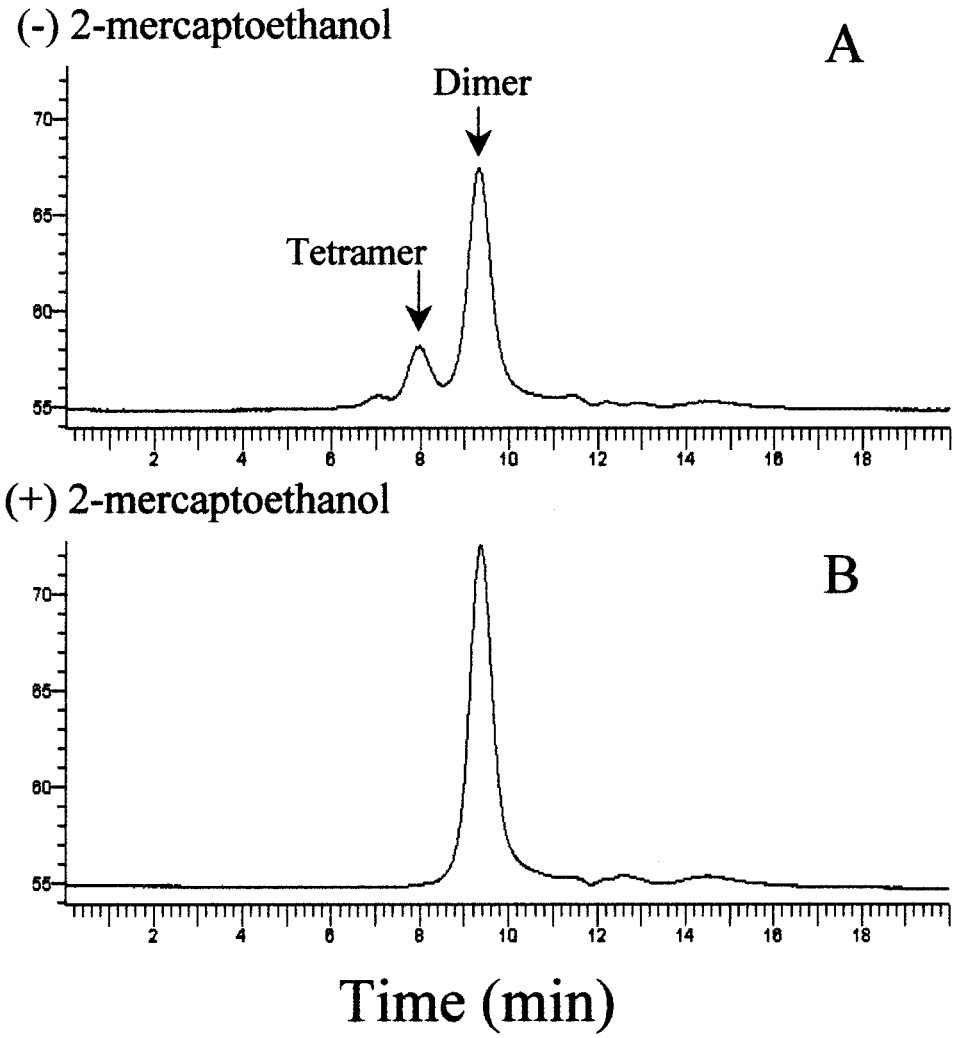

Fig. 2. Size exclusion chromatography of GlTIM. The chromatographic analysis under nonreducing (**A**) and reducing (**B**) conditions were performed by using a SW300 column equilibrated with buffer containing 50 mM triethanolamine, 10 mM EDTA, 150 mM NaCl, 30% glycerol, pH 7.5. The proteins (25 μg) were eluted at a rate flow of 1 mL/min. For calibration, the following molecular weight markers were used: thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B_{12}.

week. After the final boost, eggs were collected daily and processed individually. Chicken polyclonal antibodies (IgY) were purified from the egg yolk by using the method described by Polson et al.[27] The IgY proteins were dialyzed extensively against PBS buffer and applied to a size exclusion column equilibrated with the same buffer. The fractions enriched with IgY were pooled and concentrated. Western blot analysis was used to evaluate antibody specificity.

Light and Electron Microscopy

For light microscopy, cells were grown over glass coverslips into six-well cell culture clusters (Costar). For electron microscopy, pellets of *G. lamblia* trophozoites with ~10^6 cells were obtained by centrifugation and embedded in acrylic resin Lowicryl K4M. Samples were fixed by using freshly prepared 4% paraformaldehyde in PBS.

Immunocytochemistry

Coverslips with a monolayer of trophozoites were incubated in 0.5% Triton X-100 for 5 min at 4°C to permeate cells. Samples were rinsed with PBS and incubated on TBS buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 20 mM NaN_3, 1% Tween 20, 5% BSA, 5% normal goat serum) for 1 h. Samples were incubated with anti-GlTIM IgY diluted 1:4000 in PBS at 4°C overnight, rinsed with PBS, and incubated with rabbit anti-chicken IgG at 1:500 in PBS for 2 h at room temperature. Anti-rabbit IgG coupled to fluorescein isothiocyanate diluted 1:500 in PBS was used. Nuclei were stained by using DAPI (4′,6-diamidino-2-phenylindole) for specific DNA localization. Mounted samples were photographed with a fluorescence microscope (Axiovert 200; Carl Zeiss).

Ultrathin sections (~70 nm wide) were placed on nickel grids. Grids were floated on TBS buffer for 1 h and

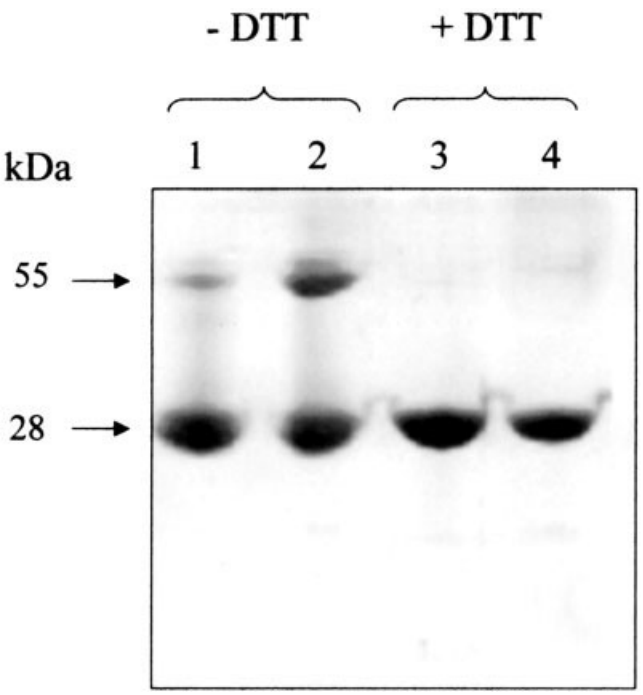

Fig. 3. Denaturing electrophoresis of dimeric and tetrameric GlTIM. The samples that corresponded to tetrameric and dimeric GlTIM were obtained from the chromatography fractions shown in Figure 2(A). Four micrograms of dimer (lanes 1 and 3) or 4 μg of tetramer (lanes 2 and 4, respectively) were analyzed by SDS-PAGE (12% acrylamide) according to Shägger and von Jagow,[51] under nonreducing (lanes 1 and 2) or reducing conditions (lanes 3 and 4).

incubated at 4°C overnight with a 1:4000 solution of anti-GlTIM antibody. Grids were rinsed with PBS and incubated for 1.5 h with a secondary antibody against IgY (Jackson Immunoresearch) at 1:200 in PBS. Anti-rabbit IgG coupled to 10-nm colloidal gold particles (ICN) diluted 1:100 was added and incubated for 2 h. Samples were stained with 0.5% uranyl acetate and lead citrate for 1 min each. Photographs were taken in a transmission electron microscope (EM109; Carl Zeiss).

RESULTS

Sequence Analysis

In consonance with the data of Mowatt et al.,[12] we found that the gene of GlTIM is formed by 774 pb with a predicted sequence of 257 amino acid residues and a molecular mass of 27,903 Da. Figure 1 shows the sequence alignment of TIMs from *G. lamblia* (GlTIM), *Entamoeba histolytica* (EhTIM), *Plasmodium falciparum* (PfTIM), *T. brucei* (TbTIM), *T. cruzi* (TcTIM), *T. maritima* (TmTIM), and human (HuTIM). The identity of GlTIM with the enzymes from these organisms is 42, 41, 44, 46, 42, and 45%, respectively. From the amino acid sequence of GlTIM, a pI of 7.05 was calculated. This is around 1 unit higher than the pI of TIM from *E. coli* (5.89). This allowed the separation of the two enzymes by ion exchange chromatography (see Materials and Methods). GlTIM has five cysteines per monomer; human TIM has the same number of cysteines, but in different positions. The other enzymes have a lower number of cysteines. It is noted that except for TmTIM and HuTIM, the rest of enzymes have a cysteine in their interface (Cys 14 in GlTIM). This region of the interface formed by the side-chain of Cys14 and its surrounding loop 3 of the other subunit has been described as a potential target for drug design.[28,29]

Hydrodynamic Parameters and SDS-PAGE of GlTIM

To explore the oligomerization state of GlTIM, the enzyme was incubated overnight with or without the thiol-reducing agent 2-mercaptoethanol and then applied to an analytical SEC column (Fig. 2). The chromatographic profile of the enzyme incubated without 2-mercaptoethanol showed a minor peak with the Stokes radius of a protein with a molecular mass of 108 kDa and a major peak of 55 kDa. The enzyme that had been exposed to 2-mercaptoethanol showed only the 55-kDa protein. The peak that corresponded to the 55 kDa was larger for the enzyme treated with 2-mercaptoethanol. The same data were obtained when GlTIM was incubated with dithiothreitol (data not shown).

To further examine the nature of the 55- and 108-kDa proteins, their electrophoretic profile in denaturing gel was determined in the *absence* of reducing agents (Fig. 3). In SDS-PAGE, the protein that had a mass of 55 kDa in size exclusion chromatography exhibited a main band of about 28 kDa and a minor band of about 55 kDa (Fig. 3, lane 1), which is probably a contamination with the 108-kDa protein. The 108-kDa protein showed two bands of Mw of about 55 kDa and 28 kDa. Under reducing conditions (dithiothreitol added), the high and low molecular weight proteins exhibited a single band of 28 kDa (Fig. 3, lanes 3 and 4). Both the 108- and 55-kDa proteins exhibited catalytic activity (see below).

Taken together, the results in Figures 2 and 3 indicate that recombinant GlTIM may exist as a tetramer and a dimer of identical subunits. Moreover, the loss of the 108-kDa peak and the concomitant appearance of the 55-kDa protein under reducing conditions illustrate that in the tetramer, two GlTIM dimers are joined by a disulfide bridge or bridges.

Kinetics

The kinetics of GlTIM were determined at different enzyme concentrations (0.1 or 0.2 nM). The traces of activity versus time were linear until NADH became limiting. Thus, the enzyme did not dissociate into monomers during the assay. As expected, the kinetic constants were independent of GlTIM concentration. Lineweaver–Burk plots of the activity at different glyceraldehyde 3-phosphate concentrations (0.5–4 mM) were linear. The Km and k_{cat} values of dimeric GlTIM were similar to those reported for TIM from other species (Table II). The Km value of tetrameric enzyme was also in the range observed in other TIMs; however, its k_{cat} was about half of that of the dimer (Table II). Using dihydroxyacetone as substrate (0.3–10 mM), the kinetic constants of the GlTIM dimer were also similar to those reported for other TIMs. Be-

TABLE II. Kinetic Constants of Recombinant G1TIM Compared With TIM From *T. brucei* (TbTIM) and Yeast (YtTIM)[†]

TIM	Km (GAP) mM	*kcat* (GAP) min^{-1}	Km (DHAP) mM	*kcat* (DHAP) min^{-1}	*Ki* (PG) mM
G1TIM dimer	0.53 ± 0.03	$(2.9 \pm 0.2) \times 10^5$	2.2 ± 0.2	$(1.6 \pm 0.04) \times 10^4$	0.043 ± 0.005
G1TIM tetramer	0.87 ± 0.07	$(1.47 \pm 0.04) \times 10^5$	n.d.	n.d.	n.d.
TbTIM[a]	0.35	2.6×10^5	1.9	2.7×10^4	0.024
YtTIM[b]	1.27	1×10^6	1.23	4.9×10^4	0.03

[†]Activities were determined with glyceraldehyde 3-phosphate (GAP) in the range of 0.5–4 mM; concentration ranged between 0.3 and 10 mM for dihydroxyacetone phosphate (DHAP); PG is 2-phosphoglycolate. The conditions of the reaction are described in Materials and Methods. The values are the averages (±SE) of three independent experiments.
[a]Data from Hernández-Alcántara et al.[29]
[b]Data from Lambeir et al.[31]

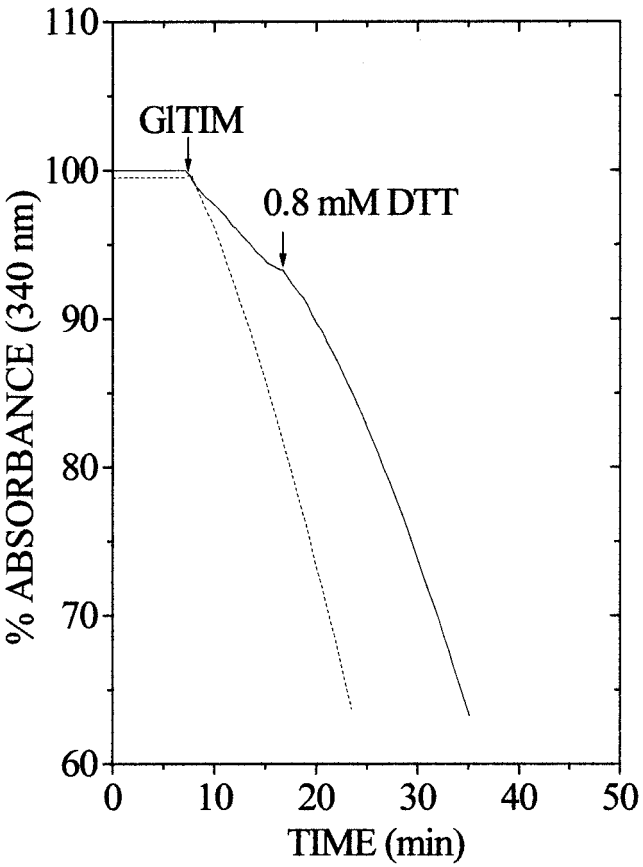

Fig. 4. Effect of dithiothreitol on the activity of the tetramer. The activities of the dimer (dashed line) and the tetramer (solid line) were followed spectrophotometrically at 340 nm. The arrows indicate the addition of 2.5 ng of dimer or tetramer to 1 mL reaction mixture (see Materials and Methods). The addition of 0.8 mM dithiothreitol is also indicated.

cause of its sensitivity to reducing agents (see Figs. 2 and 3), the kinetics of the tetramer in the direction of dihydroxyacetone to glyceraldehyde 3-phosphate could not be determined. Because the k_{cat} of the tetramer was lower than that of the dimer, we explored if the conversion of tetramer to dimers brought about an increase in activity. Indeed, we observed that the addition of dithiothreitol to tetrameric GlTIM undergoing active catalysis induced an increase in the rate at which the enzyme converts GAP to DHAP (Fig. 4). Under the conditions of the experiment, a rate equal to that of the dimer was attained in ~15 min after the addition of dithiothreitol.

The inhibition constant (K_i) of the transition-state analog 2-phosphoglycolate was determined for the dimeric enzyme (Table II). The K_i value was close to that reported for trypanosomal and yeast TIMs.[30]

Stability of GlTIM at Different Concentrations

The interface of dimeric TIM is formed by noncovalent interactions between TIM monomers; the association constant of the two subunits is in the order of 10^{-10} to 10^{-16} M.[31–35] Because in its monomeric state the enzyme is catalytically inactive,[30,36,37] the loss of the dimeric state can be followed by the activity of the enzyme after it has been incubated at different concentrations. The apparent dissociation constant (K_D^{app}) between the two monomers can be calculated from the data. Therefore, to gain insight into the dissociation constants between the monomers of GlTIM, the tetramer and the dimer were incubated at concentrations that ranged from 0.0025 to 500 μg/mL. After 2 h of incubation, the specific activity of the two enzymes was determined.

The curves of percent of specific activity versus enzyme concentration of the dimer and tetramer of GlTIM were sigmoid (Fig. 5), as observed with other TIMs.[38] At low protein concentrations, the specific activity of tetramer and dimer of GlTIM was low; however, as the concentration of protein was raised, the specific activity of the two enzymes increased until it reached a constant level. From the data, the K_D^{app} was calculated. As shown in Figure 5, the apparent dissociation constant between the monomers of dimeric ($50.6 \pm 12 \times 10^{-9}$ M) and tetrameric ($9.8 \pm 0.6 \times 10^{-9}$ M) GlTIM was slightly lower than that of TcTIM ($44 \pm 2.3 \times 10^{-9}$ M) and TbTIM ($43.8 \pm 4 \times 10^{-9}$ M). It is also noteworthy that the K_D^{app} of the dimer and tetramer of GlTIM was in the same range. These findings suggest that the tetramer results from the covalent attachment of two dimers that conserve similar association constants between their constituent monomers.

A point that is worth noting is that enzyme concentrations of the order of 0.2 nM were used for measurements of activity. According to the data of Figure 5, it could be expected that at concentrations of 0.2 nM, there would be dissociation of the subunits of GlTIM. However, it is recalled that the occupancy of the catalytic site increases the stability of TIM.[25,39,40] Moreover, in the conditions used for Figure 5, loss of activity is a rather slow process.

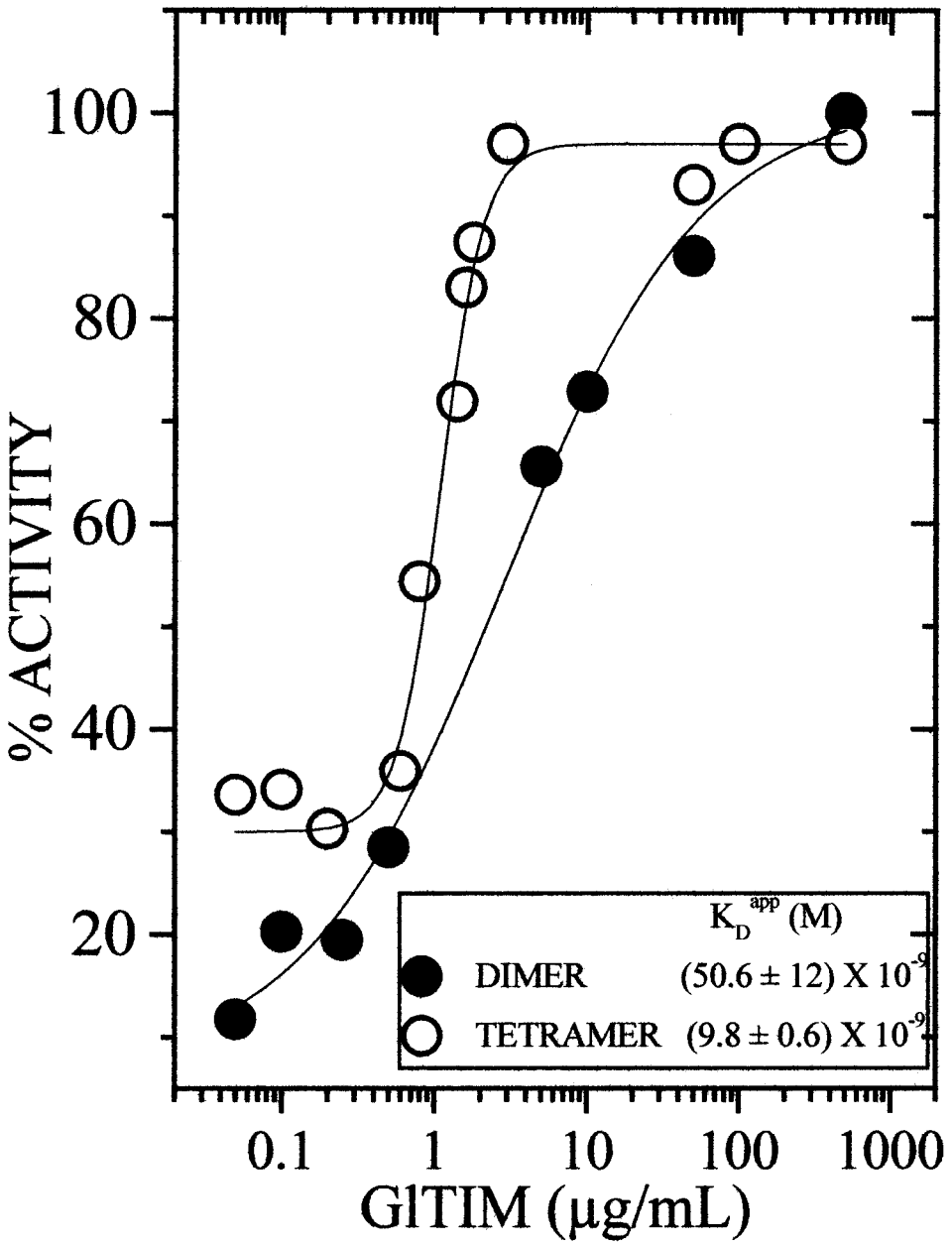

Fig. 5. Stability of the dimer and tetramer of GlTIM at different concentrations. The dimer and tetramer were incubated at concentrations that range from 0.0025 to 500 μg/mL at 40°C in a buffer containing 100 mM triethanolamine, 10 mM EDTA, pH 7.4. After 2-h samples were incubated for 1 min at 25°C, subsequently the residual activity was determined with 10 ng of protein/mL reaction mixture. The percent of specific activity versus enzyme concentration is shown; 100% of activity was 1700 and 714 μmol min^{-1} mg^{-1} for the dimer and tetramer, respectively. The apparent K_D (±SE) of three independent experiments were calculated and fitted with nonlinear regression plots.

Thus, in the times in which activity was measured, no dissociation to monomers took place. Indeed, we always observed that the activity traces were linear with time.

Stability to Temperature

The thermostabilities of the dimer and the tetramer of GlTIM were determined from measurements of the decay of activity at different temperatures. The data were compared with those of TcTIM and TbTIM. In all cases, inactivation followed a simple exponential decay. Thus, the data were expressed as k_{obs} (Table III). At 45 and 55°C, the GlTIM dimer exhibited the highest decay rate. At 60°C, however, the rate of inactivation was similar in the four enzymes. It is noted that the loss of activity in the tetramer was not markedly different from that of the other enzymes tested, albeit at 45 and 55°C, it was slightly more stable than the dimer.

TABLE III. First-Order Rate Constants for Inactivation as a Function of Temperature for G1TIM, TcTIM, and TbTIM[†]

	k_{obs} (h^{-1})		
TIM	45°C	55°C	60°C
G1TIM dimer	1.9 ± 0.1	65 ± 3	173 ± 9
G1TIM tetramer	0	43 ± 5.4	169 ± 7.5
TcTIM	$(4 \pm 0.37) \times 10^{-2}$	36 ± 4.3	198 ± 17
TbTIM	$(9 \pm 0.72) \times 10^{-2}$	23.04 ± 1.44	120 ± 2.4

[†]The enzymes were incubated at 100 μg/mL in 100 mM triethanolamine, 10 mM EDTA buffer (pH 7.4) at the indicated temperatures. At different times of incubation, aliquots were withdrawn and activities measured at 25°C. The k_{obs} (±SE) were calculated from nonlinear regression plots.

In Situ Localization of GlTIM

Fluorescence immunolocalization of cellular GlTIM showed that the enzyme was quite abundant in all the cytoplasm of *G. lamblia* trophozoites [Fig. 6(A), anti-GlTIM panel]. Its distribution was uniform, albeit the nuclei were not labeled [Fig. 6(A), DAPI panel]. Electron microscopy corroborated the homogeneous cellular distribution of GlTIM [Fig. 6(B)]; that is, the enzyme is not contained in membranous vesicles (as in the glycosomes of trypanosomes).[41]

DISCUSSION

The kinetics, the association constant between monomers, and the thermostability of GlTIM are similar to those of other TIMs. Nonetheless, in size exclusion chromatography, two catalytically active proteins with molecular masses of 108 and 55 kDa were clearly evident. The same results were obtained when the catalytically active proteins were analyzed in nonreducing native gel electrophoresis. It is also noteworthy that only the 28-kDa protein was observed in SDS-PAGE under reducing conditions, whereas in the absence of reducing agents, two proteins with molecular masses of 55 and 28 kDa were detected. Taken together, these findings indicate that TIM from *G. lamblia* can exist as a tetramer or a dimer of identical subunits. Moreover, because reducing agents induce the transformation of tetramers to dimers, it may be concluded that two GlTIM dimers are covalently linked by disulfide bonds in the tetramer. In this regard, it is noted that in the tetramer, the linkage of two dimers must be through an -S-S- bridge between only one of the monomers of each dimer; otherwise, the tetrameric form in SDS-PAGE under nonreducing conditions would have exhibited only the 55-kDa protein; instead, the gel showed bands of 55 and 28 kDa.

GlTIM has cysteines at positions 14, 127, 202, 222, and 228. PfTIM has cysteines at positions 13, 126, 196, and 217. The cysteines of PfTIM and GlTIM have different positions in the primary sequence. This is because GlTIM has an insertion of seven residues. However, the positions of the cysteines between both organisms are equivalent (Fig. 1). Therefore, the crystal structure of the PfTIM[42] was used to model the accessible solvent area (ASA) of the five cysteines of GlTIM. The predicted ASA of the five lateral chains of the cysteines from GlTIM[43] showed that Cys 202, located in helix 6, could be the residue most exposed to solvent (47.1 $Å^2$). Alternatively, GlTIM possesses a Cys 228 residue, but the residue that PfTIM contains at this equivalent position is Gln 223. Consequently, Cys 228 of GlTIM would be located on loop 8 with also a highly predicted ASA (26.7 $Å^2$). Therefore, it is probable that the cysteines involved in the disulfide of the tetramer were either Cys 202 or Cys 228 from each dimer.

As noted, TIM from *T. maritima* is a tetramer fused with phosphoglycerate kinase.[44] The crystal structure of the tetrameric TIM after it was separated from glycerate kinase by molecular engineering has been reported.[21] In this tetramer, the two dimers are linked through contacts between hydrophobic and polar residues; however, the predominant linkages in the tetramer are the two disulfide bonds that are established between cysteines 142 of each monomer. In regard to the structural features of TIM from *T. maritima,* it has been proposed that through tetramerization, the enzyme acquires a higher thermostability.[44] Here, we found that the dimer and tetramer of GlTIM do not exhibit important differences in thermostability nor in their association constant between monomers. Therefore, it is possible that in the mesophile *G. lamblia,* the occurrence of tetrameric and dimeric forms serves a different purpose.

In the latter respect, the comparison of the kinetics of the GlTIM dimer and tetramer may be illustrative. The data show that although the two enzymes have the same Km for glyceraldehyde 3-phosphate, the k_{cat} of the tetramer is about half of that of the dimer. This suggests that the tetramer is an enzyme with only two catalytically competent sites. In this connection, it is relevant to point out that TIM dimers, in which one of its two catalytic sites has been poisoned with a covalently linked inhibitor, express 50% of its maximal activity without important changes in Km,[45,46] indicating that the catalytic sites of two monomers work independently. However, Biemann and Koshland[47] reported that a protein with two potential binding sites exhibited Michaelis–Menten behavior with a Hill coefficient of 1 and that, nonetheless, the protein expressed what they called half of the site reactivity. That is, the occupancy of one binding site suppressed the function of the other site. With the present data, it is not possible to distinguish between the two alternatives. However, it is noted that the kinetics of the dimer and tetramer exhibited classical Michaelis–Menten behavior with Hill coefficients of 1.1 and 1.02, respectively.

In sum, our data on TIM from *G. lamblia* show that it has characteristics that set it apart from all the other TIMs so far reported. Indeed, to our knowledge, GlTIM is the first example of a TIM from a mesophile that may acquire a tetrameric structure. Likewise, GlTIM is also the only known eukaryotic TIM that can acquire a tetrameric structure. Along this line, it may not be a coincidence that *G. lamblia* is one of the earliest branches of eukaryotes. In addition, from the point of view of catalytic mechanisms, GlTIM also seems rather unique, because the kinetics of

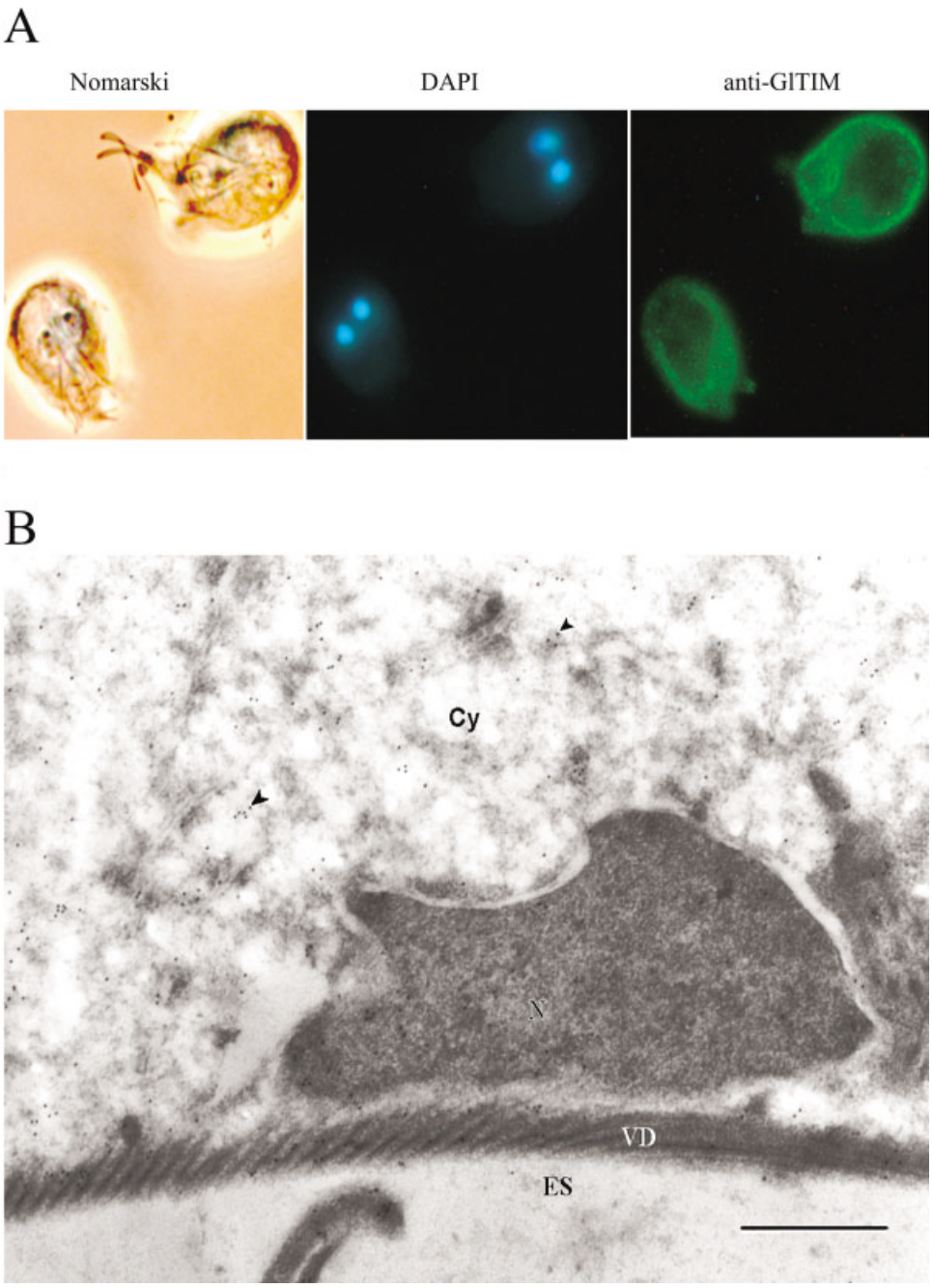

Fig. 6. (**A**) Immunofluorescence of cellular GlTIM and (**B**) its distribution at the electron-microscopical level. Colloidal gold particles (arrow heads) show the localization of GlTIM. ES, extracellular space; VD, ventral disc; Cy, cytoplasm; N, nucleus. Bar 0.5 μm.

the enzyme showed that in the tetramer only two of its four catalytic sites are catalytically competent.

The aforementioned considerations raise the question of whether the characteristics of GlTIM, particularly in its ability to tetramerize with a concomitant decrease in catalytic efficiency, are of physiological significance. Implicit in this question is the possibility that tetramerization of GlTIM is an "artifact" of laboratory manipulations during its purification or storage. Indeed, when we carry out the purification procedure with solutions supplemented with DTT, we only observed GlTIM dimers. This is what would be expected if the tetrameric structure were maintained by disulfide bounds. Nonetheless, regardless of whether tetramerization is of physiological significance, the fact remains that dimers of GlTIM are prone to tetramerization. In fact, we have observed that in a few hours at room temperatures, ~5% of the dimers are transformed into tetramers. Conversely, under reducing conditions, tetramers are converted into dimers with a corresponding gain in catalytic activity. Thus, the possibil-

ity that tetramerization is relevant to the life of the parasite should be considered. The life cycle of *G. lamblia* involves transformation of cysts into trophozoites; therefore, it could be that the transition of GlTIM tetramers to dimers, or vice versa, is guided by the intracellular conditions in a given physiological state. The tetramer could be a "storage conformation," which participates in a transition from a state with a low metabolism toward a higher metabolic state. Thus, it seems clear that the reversible reaction between GlTIM dimers and tetramers deserves further studies.

Before closing, we call attention to two points that arose from the data of this work. The first is that, although the bulk of eukaryotic genome appears to share common ancestry with archaeobacteria,[48,49] the phylogenetic analysis of the TIM gene from several species (including *G. lamblia*) supports the notion that eukaryotic TIM has an alpha-proteobacterial origin.[50] Therefore, it would be interesting to determine if the different oligomerization states of GlTIM are a consequence of parasitic adaptations or whether they are an archaebacterial relic. Finally, the data on the intracellular localization of GlTIM show that it is an enzyme that is evenly distributed throughout the cytoplasm and separated from the extracellular milieu only by the cytoplasmic membrane. Therefore, the penetration of drugs that target on GlTIM would not be hindered by internal permeability barriers.

ACKNOWLEDGMENTS

D.M.-O. is the recipient of a fellowship from CONACyT. The authors are indebted to Dr. M. Tuena de Gómez-Puyou and Dr. A. Gómez-Puyou for their advice on the improvement of the manuscript. We also thank Dr. E. Chávez Cosio for invaluable help on IgY development, Dr. Antonio Lazcano-Araujo for his comments of the manuscript, and Janet Flores for providing us with HPLC resources. The technical assistance of Carmen Ortiz, Sara Navarrete, and Amparo Tapia is acknowledged.

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