Glyceraldehyde-3-phosphate dehydrogenase from Tetrahymena pyriformis: Enzyme purification and characterization of a gapC gene with primitive eukaryotic features

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Glyceraldehyde-3-phosphate dehydrogenase from *Tetrahymena pyriformis*: enzyme purification and characterization of a *gapC* gene with primitive eukaryotic features

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC.1.2.1.12) was purified to electrophoretic homogeneity from an amicronucleated strain of the ciliate *Tetrahymena pyriformis* using a three-step procedure. The native enzyme is an homotetramer of 145 kDa exhibiting absolute specificity for NAD. In its catalytic properties it is similar to other glycolytic GAPDHs. Chromatofocusing analysis showed the presence of only one basic GAPDH isoform with an isoelectric point of 8.8. Western blots using a monospecific polyclonal antibody raised against the *T. pyriformis* GAPDH showed a single 36-kDa band corresponding to the enzyme subunit in the cytosolic protein fraction of this strain and the closely related species, both from the class Oligohymenophorea, *Paramecium tetraurelia*. No bands were immunodetected in the ciliate *Colpoda inflata* (class Colpodea) and in the diverse eukaryotes and eubacteria tested. A 0.5-kb DNA fragment which corresponds to an internal region of a *gapC* gene was generated by polymerase chain reaction using cDNA of *T. pyriformis* as template. This gene codes for a basic GAPDH protein with eukaryotic-diplomonad signatures and exhibits a codon usage biased in the manner typical for *T. pyriformis* genes. Southern blots performed both under homologous and heterologous conditions using this amplified cDNA fragment as a probe, indicated that it should be the only *gapC* gene present in the macronuclear genome of this ciliate, its expression being confirmed by Northern blot analysis. These results are discussed in connection with the peculiar genomic organization of ciliates and in the context of protist evolution.

Keywords: Glyceraldehyde-3-phosphate dehydrogenase; *gapC*; Glycolysis; *Tetrahymena pyriformis*; Ciliates; Protist evolution; Chromatofocusing; RT-PCR; cDNA

1. Introduction

The NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC.1.2.1.12) is a key enzyme of the glycolytic pathway which catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate (G3P) into 1,3-bisphosphoglycerate [9]. All glycolytic GAPDHs so far studied are homotetrameric with 34–38 kDa subunits which have been remarkably conserved during evolution [5,8]. The primary structure of

Abbreviations: aa, amino acid(s); bp, base pair(s); *gapC*, gene (DNA, RNA) encoding glycolytic GAPDH; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GCG, Genetics Computer Group (Madison, WI); G3P, D-glyceraldehyde-3-phosphate; HPLC, high pressure liquid chromatography; kb, kilobase(s) or 1000 bp; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; p*I*, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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GAPDH over 50 species of the Bacteria, Archaea and Eukarya groups has been elucidated [8]. This extensive sequence information allowed the use of this enzyme as a marker in molecular evolution studies; the obtained phylogenetic trees showing good overall agreement with the known relationships of the organisms or organelles which contain them [5]. In this way, *gapC* genes encoding glycolytic GAPDHs of primitive unicellular eukaryotes are currently used in phylogenetic studies to provide clues in tracing the early evolution of eukaryotic central metabolism [12,18,19].

Ciliated protozoa form a large group of protists that, despite its great genetic diversity, remains united both by structural (presence of a complex cytoskeleton) and genetic (nuclear dimorphism) characteristics [15]. As an evolutionary group, the ciliates separated from the eukaryotic line that led to metazoa earlier than fungi and exhibit consequently features of primitive organisms [15]. The molecular genetics of only a few ciliates have been studied to date. *Tetrahymena* is one of them, being probably the most thoroughly studied ciliated protozoan, mainly because of its ability to grow in defined axenic media and the possibility to induce a very high degree of synchrony in its cultures [30]. It has been used, therefore, as a model cell system in many studies on morphogenesis, conjugation, gene mapping, cell division and growth kinetics [29].

We report here for the first time on a GAPDH from a ciliated protozoan. The enzyme of an amicronucleated strain of *Tetrahymena pyriformis* has been purified and characterized. Only one *gapC* gene encoding a protein with primitive eukaryotic features is present in the macronuclear genome of this ciliate.

2. Materials and methods

2.1. Organisms, growth conditions and cell-disruption procedure

The amicronucleated strain of the ciliate *T. pyriformis* used in this work (from Professor Eduardo Orias, University of California at Santa Barbara, CA) was grown axenically at 28°C in PPY medium [17]. The other ciliate strains used in this study, namely *Paramecium tetraurelia* (ATCC 30759, stock d4-2) and *Colpoda inflata* (provided by Professor Juan Carlos Gutiérrez, University of Madrid, Spain), were grown at the same temperature in cerophyl medium (Sigma, St. Louis, MO) inoculated the day before with *Klebsiella pneumoniae*. Prior to cell disruption, the cultures of these two latter ciliates were maintained to reach almost complete depletion of the bacterial nutrient. Cells were centrifuged (4000 *g* for 10 min) and washed with buffer at least three times to separate them from the remaining bacteria.

2.1.1. Preparation of cell-free extracts

Protozoan cells were resuspended at a ratio of about 3 ml g^{-1} (wet weight) in 20 mM Tris–HCl (pH 7.5) buffer containing 2 mM EDTA, 10 mM 2-mercaptoethanol, PMSF (2 mM), benzamidine (2 mM) and ε-amino-*n*-caproic acid (5 mM). Unless otherwise specified, the cells were then disrupted in the cold with a Branson model B12 Sonifier (40 w, 60 s). The supernatant (soluble protein fraction) obtained after centrifugation at 40000 *g* for 45 min at 4°C was considered as the crude extract.

2.2. Enzyme purification

All steps were performed at 4°C. Centrifugations were carried out at 40000 *g* for 45 min. Four purifications were carried out with similar results.

2.2.1. Precipitation and fractionation with ammonium sulfate

The crude extract was subjected to protein precipitation in the 66–88% (w/v) saturation range of ammonium sulfate. The final precipitate was dissolved in a minimal volume of 20 mM Tris–HCl (pH 7.5) containing 2 mM EDTA and 10 mM 2-mercaptoethanol (buffer A). The protein preparation was dialysed twice against 1 l of the same buffer.

2.2.2. Dye-affinity chromatography on Blue Sepharose CL-6B

The dialysed enzyme preparation was chromatographed on a Blue Sepharose CL-6B column (1.6×8 cm) equilibrated with 2 bed volumes of buffer A. The column was washed with 3 bed volumes of buffer A and 2 bed volumes of the same buffer adjusted to pH 8.5 (buffer B). The GAPDH activity was eluted with buffer B containing 10 mM NAD^+ at a flow rate of 10 ml h^{-1}. Active fractions were collected and concentrated by ultrafiltration (Microsep 10K, Pall-Filtron, Northborough, MA). SDS-PAGE analysis of this preparation showed a major 36-kDa protein band and several minor contaminating proteins.

2.2.3. Chromatofocusing

Concentrated GAPDH preparations were dialysed against 25 mM Tris–HCl (pH 9.8) containing 1 mM EDTA and 5 mM 2-mercaptoethanol (starting buffer). Column chromatofocusing in the pH range 9.0–5.5 was performed on a column (1×18 cm) of Polybuffer Exchanger PBE-94 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with starting buffer. After application of the concentrated GAPDH preparation, the column was washed with 5 ml of starting buffer. Elution of the enzyme was performed at a flow rate of 12 ml h^{-1} by washing the column with 10 bed volumes of a 10-fold diluted mixture of Polybuffer 96/Polybuffer 74 (30/70,

v/v) adjusted to pH 5.5 with acetic acid. The fractions corresponding to the only activity peak were concentrated and washed in standard buffer as above described. Analysis by SDS-PAGE revealed only one protein band of about 36 kDa thus indicating purification to electrophoretic homogeneity of the enzyme.

2.3. Analytical procedures

2.3.1. Enzyme assays

Enzymatic activity in the oxidative phosphorylation was determined spectrophotometrically at 30°C by monitoring NADH generation at 340 nm. The standard assay mixture contained 0.1 M triethanolamine-phosphate buffer (pH 8.9), 0.2 mM EDTA, 1 mM NAD^+ and 2 mM G3P. Only initial rates were determined. Kinetics parameters for NAD^+ and G3P were determined in the conditions described by Ferdinand [7]. For optimal pH determination, the enzymatic activity was measured over a wide range of pH (from 5 to 10) with different buffers (acetate, imidazole, Tris and carbonate/bicarbonate) adjusted to the same ionic strength as the standard reaction mixture. Ferdinand buffer [7], adjusted to pH 8.5 at each of the assayed temperature values, was used to determine optimal temperature. Reactions were carried out in the 5–65°C temperature range using a thermostated cuvette holder connected with a refrigerated bath circulator. Enzyme (1 U) is defined as the amount which catalyzes the formation of 1 μmol NADH min^{-1} under the conditions used. Esterase activity was determined spectrophotometrically using *p*-nitrophenylacetate as substrate [25]. Protein was determined with Coomassie blue [3]. Activity levels in cell-free extracts were expressed as specific activity (U mg^{-1} of protein).

2.3.2. Analytical HPLC gel filtration

About 50 μg of purified protein in a volume of 0.2 ml were applied onto a Waters Protein Pak 300 SW column (0.75 × 30 cm) equilibrated with buffer A supplemented with 0.1 M KCl using a Waters 650E Advanced Protein Purification System (Millipore, Bedford, MA). Elution was performed at a flow rate of 30 ml h^{-1}, A_{280} was recorded and fractions were collected and used for activity determination. The native molecular mass and the Stockes radius of the *T. pyriformis* GAPDH were estimated by interpolation using the following globular protein standards (Pharmacia Biotech; mol. mass and Stokes radius are indicated): catalase from bovine liver (232 kDa, 52.2 Å), aldolase from rabbit muscle (158 kDa, 48.1 Å), bovine serum albumin (67 kDa, 35.5 Å), and chicken ovalbumin (43 kDa, 30.5 Å).

2.3.3. Gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% (w/v) acrylamide gels (Mini-Protean, Bio-Rad, Richmond, CA) according to Laemmli [11]. Sample aliquots containing about 50 μg of protein were loaded in each lane. Proteins were stained with 0.2% (w/v) Coomassie brilliant blue R-250 in methanol/acetic acid/water (4:1:5) for about 45 min at room temperature. The subunit molecular mass of the *T. pyriformis* GAPDH was estimated by interpolation using (Sigma): bovine serum-albumin (66 kDa), chicken ovalbumin (45 kDa), rabbit muscle GAPDH (36 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), trypsinogen from bovine pancreas (24 kDa), and soybean trypsin inhibitor (20 kDa).

2.3.4. Western blotting

The GAPDH protein was immunodetected either in cell-free extracts or in purified preparations after SDS-PAGE and subsequent transfer to nitrocellulose. After blocking in non-fat milk, membranes containing blotted samples were exposed to a 1:200 dilution of a monospecific polyclonal antibody raised in rabbit against 350 μg of the chromatofocusing-purified GAPDH of *T. pyriformis*. Detection of the GAPDH subunit band was performed with a goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (1:1000 dilution) (Sigma).

2.4. Nucleic acid techniques

2.5. Reverse transcriptase-polymerase chain reaction

T. pyriformis poly(A)$^+$ mRNA was isolated using a Quick Prep Micro mRNA purification kit (Pharmacia Biotech). The GAPDH single strand cDNA was generated by primed reverse transcription (RT) of mRNA (1 μg), using AMV reverse transcriptase (Promega) and the oligonucleotide named GAP2 5′-GCC(T) T(A)C(G)C(T)TGC(T)ACG(C)ACG(C)AAC(T)TG-3′ during 1 h at 42°C. An aliquot from this template was used in a subsequent polymerase chain reaction (PCR) using *Taq* DNA polymerase (Eurobio, Les Ulis, France), GAP2 (antisense primer) and the oligonucleotide named GAP1 (sense primer) 5′-CCC(G)CAC(T)TCG(A)TTG(A)TCG(A)TA CCA-3′ (annealing temperature 45°C, 35 cycles). These two primers were degenerate oligonucleotides constructed from conserved regions present in all GAPDHs so far studied [8]. Agarose gel electrophoresis of the PCR-amplified cDNA from four independent amplification experiments showed a fragment of about 0.5 kb, the expected size of an internal *gapC* region comprising about half of the complete gene. Two PCR products

Table 1
Purification of GAPDH from *T. pyriformis*

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} of protein)	Purification factor (fold)	Yield (%)
Crude extract	672.0	230	0.3	1	100
Ammonium sulfate 66–88%	17.5	114	6.5	19	50
Blue Sepharose CL-6B eluate	8.0	80	10.0	29	35
Chromatofocusing eluate	1.5	42	32.0	94	18

from independent RT-PCR experiments were isolated after selective adsorption/desorption on glass beads (Gene Clean, Bio101, La Jolla, CA), cloned into the *Eco*RV site of pGEM-T vector (Promega) and eventually sequenced with identical results.

2.5.1. Cloning and sequencing

The two selected RT-PCR-amplified cDNA clones were sequenced in both strands (515 bp, EMBL/X99629) by the dideoxy chain termination method using a T7 DNA polymerase (Pharmacia Biotech). Reaction mixtures were analysed on polyacrylamide gels.

2.5.2. Southern blot analysis

T. pyriformis genomic DNA was digested to completion with several restriction endonucleases, fractionated (10 μg per lane) on 0.7% (w/v) agarose gel, blotted onto a Z-probe nylon membrane (Bio-Rad) by the capillary transfer method [20], hybridized with the cDNA probe labeled with [^{32}P]ATP by a random-primer kit (Ready-to-Go DNA labeling kit, Pharmacia Biotech), and finally washed at high stringency (65°C, 0.1 × SSC, homologous conditions) or low stringency (55°C, 2 × SSC, heterologous conditions).

2.5.3. Northern blot analysis

T. pyriformis total RNA was isolated by the guanidinium thiocyanate method [6]. Samples were quantified using a UV-visible densitometer, then denatured, subjected to electrophoresis in 1% agarose-formaldehyde gels (about 25 μg per lane) and finally blotted on nylon filters according to standard procedures [20]. The *gapC* mRNA was detected by sequencial hybridization of the filters using the random-priming ^{32}P-labeled cDNA probe and washing at high stringency (65°C, 0.1 × SSC).

2.5.4. Chemicals

NAD^+, G3P diethyl acetal, *p*-nitrophenyl acetate, iodoacetamide, PMSF, benzamidine, ε-aminocaproic acid, imidazole, trietanolamine, Tris, Tricine and EDTA were purchased from Sigma. Blue Sepharose CL-6B, Polybuffer Exchanger PBE 94, Polybuffer 96 and Polybuffer 74 were obtained from Pharmacia Biotech. All other chemicals were of analytical grade.

3. Results and discussion

3.1. Purification and physicochemical, catalytic and immunological properties of T. pyriformis GAPDH

Relatively high levels of GAPDH activity (0.3–0.5 U mg^{-1} protein) in comparison with other protists [13,14] have been found in the soluble protein fraction after sonication of cells of the different ciliated protozoa tested, namely *T. pyriformis*, *P. tetraurelia* and *C. inflata*. Similar values have been obtained when using gentler cell disruption methods (i.e. homogenization with a Sorvall blender or a Potter-Elvehjem device). Since no measurable activity was found associated with the membranous fraction obtained after centrifugation, it was assumed that virtually all the GAPDH should be located in the cytosol of these microorganisms, as was later confirmed by immunological studies (see below).

The GAPDH of an amicronucleated strain of *T. pyriformis* has been purified to electrophoretic homogeneity by a straightforward procedure involving two chromatographic steps, namely dye-affinity chromatography and chromatofocusing. Table 1 summarizes a representative purification. Values of about 30 U mg^{-1} protein were obtained for the purified enzyme, with a yield of 20% and a purification factor of about 100-fold. As other NAD^+-dependent GAPDHs [26,28] dye-affinity chromatography on Blue Sepharose was a very efficient purification step, although column chromatofocusing was needed in this case to eventually obtain the homogeneous enzyme. SDS-PAGE analysis of the different fractions obtained during the purification procedure showed a progressive enrichment in a 36-kDa protein (Fig. 1A). Only this protein band, which corresponds to the putative GAPDH subunit, was seen in the electrophoretically homogeneous final enzyme preparation (Fig. 1A, lane 4). Concerning to the physicochemical parameters of the purified *T. pyriformis* GAPDH, FPLC gel filtration yielded values of 145 kDa for the native molecular mass (Fig. 1B) and 42.6 Å for

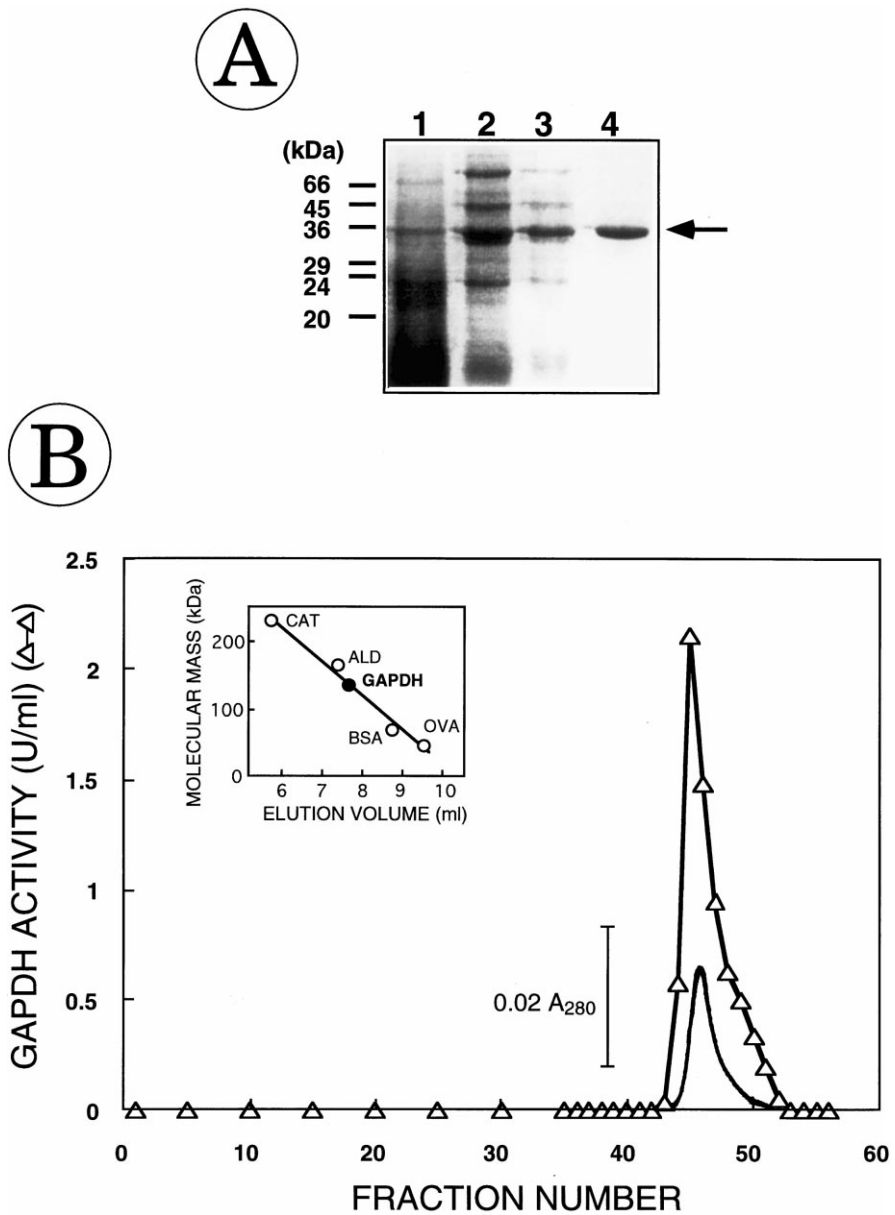

Fig. 1. (A) Coomassie-stained SDS-PAGE electrophoretogram showing the protein patterns of different fractions obtained during a GAPDH purification from *T. pyriformis*. Lane 1, crude extract (soluble protein fraction); lane 2, 66–88% ammonium sulfate protein fraction; lane 3, Blue-Sepharose eluate activity pool; and lane 4, chromatofocusing eluate activity pool. A similar amount of protein (about 50 μg) was applied to each lane. The arrow indicates the band corresponding to the 36-kDa GAPDH subunit which is observed even in the crude extract. The position and molecular masses of the protein markers are also indicated. (B) Determination of the native molecular mass of the purified *T. pyriformis* GAPDH by FPLC gel filtration. About 60 μg of protein were applied in a volume of 0.2 ml to a Waters Protein Pack 300 SW column. Elution was performed at a flow rate of 30 ml h^{-1}, absorbance at 280 nm was monitored and fractions were collected and used for activity determination. The native molecular mass, about 145 kDa, was estimated by interpolation using the protein standards catalase (CAT), aldolase (ALD), bovine seroalbumin (BSA) and ovalbumin (OVA).

the Stokes radius. SDS-PAGE of the purified enzyme showed, as stated above, a single stained band corresponding to a 36-kDa protein (Fig. 1A, lane 4), thus indicating that *T. pyriformis* GAPDH should have an homotetrameric structure like other GAPDHs [8]. Column chromatofocusing, a chromatographic technique of protein separation according to p*I*'s, showed a single protein peak which perfectly overlapped with that of GAPDH activity (Fig. 2). Maximal values of both parameters were found at pH 8.8, the p*I* of the native enzyme estimated by this method. Since chromatofocusing has been proved to efficiently resolve closely related GAPDH isoforms in other organisms [23,27], this result indicates that only one basic isoform of the enzyme occurs, and strongly suggests that a single *gapC* gene should be expressed in the amicronucleated *T.*

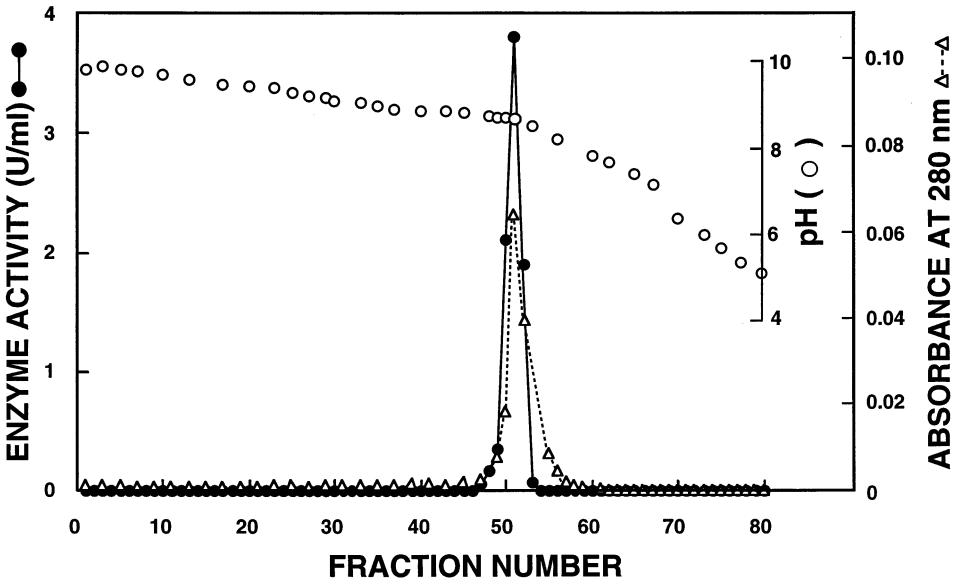

Fig. 2. Column chromatofocusing of GAPDH from *T. pyriformis*. A GAPDH preparation containing about 0.5 mg of protein was applied to a Polybuffer Exchanger PBE94 column (1 × 18 cm) and the enzyme was eluted by using a pH gradient (o) generated by 10 bed volumes of a 10-fold diluted mixture of Polybuffer 96/Polybuffer 74 (30/70, v/v) adjusted with acetic acid to pH 5.5. Fractions of 1 ml were collected. Absorbance at 280 nm and enzyme activity were measured. Only one peak of activity eluting around pH 8.8, the estimated p*I* of the protein, was found thus indicating the presence of only one basic enzyme isoform.

pyriformis strain used in this work (see below). Although a single GAPDH isoform has been found also in other microoorganisms, both prokaryotes (i.e. *E. coli*) and eukaryotes (i.e. *S. cerevisiae*) and in different animal tissues, it seems not to be a general rule since the presence of several enzyme isoforms with physiological significance has been reported in different organisms [5,13,14,27]. The attempts to obtain the amino-terminal sequence of the purified *T. pyriformis* GAPDH have been unsuccessful suggesting blocking of the amino-terminal group of the protein.

Since GAPDH catalyzes a two-substrate reaction, the K_m values for NAD^+ and G3P have been determined at saturating concentrations of the other substrate. The kinetic parameters were 5 ± 1, 150 ± 5 μM and 5.6 ± 0.8 μmol min^{-1} for $K_m(NAD^+)$, K_m(G3P) and V_{max}, respectively. Whereas the K_m(G3P) is similar to those found for cytosolic GAPDHs from human erythrocytes, rabbit muscle and *Trypanosoma brucei* [28], the $K_m(NAD^+)$ of the *T. pyriformis* GADPH is clearly lower and has, therefore, higher affinity for the nucleotide co-enzyme. An optimal pH value of about 8.5 and an apparent optimal temperature of 35°C have been determined. From the Arrhenius plot a value of 1.22 kcal mol^{-1} was calculated for the apparent E_a of the *T. pyriformis* GAPDH. Overall, these values are in agreement with those previously reported for dehydrogenase activity of cytosolic GAPDHs from unicellular eukaryotes and metazoa [27,28]. The GAPDH of *T. pyriformis* shows also esterase activity with *p*-nitrophenyl acetate as substrate [25], estimated values for K_m and V_{max} being 166 ± 7 μM and 6.4 ± 0.5 μmol min^{-1}, respectively. A strong inactivation by iodoacetamide (80–90% of total activity) was observed in the range of 0.01–0.1 mM, the kinetic of this process (k_2 at pH 7.5, 70 M^{-1} s^{-1}; $n = 1$) being consistent with the modification of only one highly reactive thiol group essential for the enzyme activity [25].

A polyclonal antibody raised against purified GAPDH of *T. pyriformis* was used for Western blot analysis of different crude protein preparations. This antibody clearly recognized the 36-kDa GAPDH protein subunit in both the purified enzyme preparation and the crude extract (soluble protein fraction) of *T.*

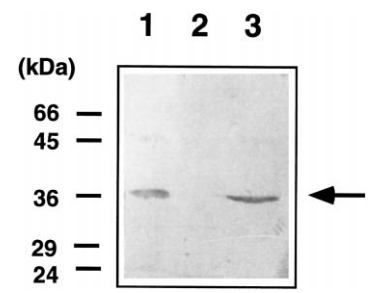

Fig. 3. Western blot analysis with a monospecific anti-GAPDH antibody raised against the *T. pyriformis* enzyme of crude extracts from the ciliated protozoa *T. pyriformis* (lane 1), *Colpoda inflata* (lane 2) and *P. tetraurelia* (lane 3). A similar amount of total protein (about 50 μg) was applied per lane. The arrow indicates the position of the protein band of 36 kDa, corresponding to the GAPDH subunit, immunodetected in lanes 1 and 3. The position and molecular masses of the protein markers are also shown.

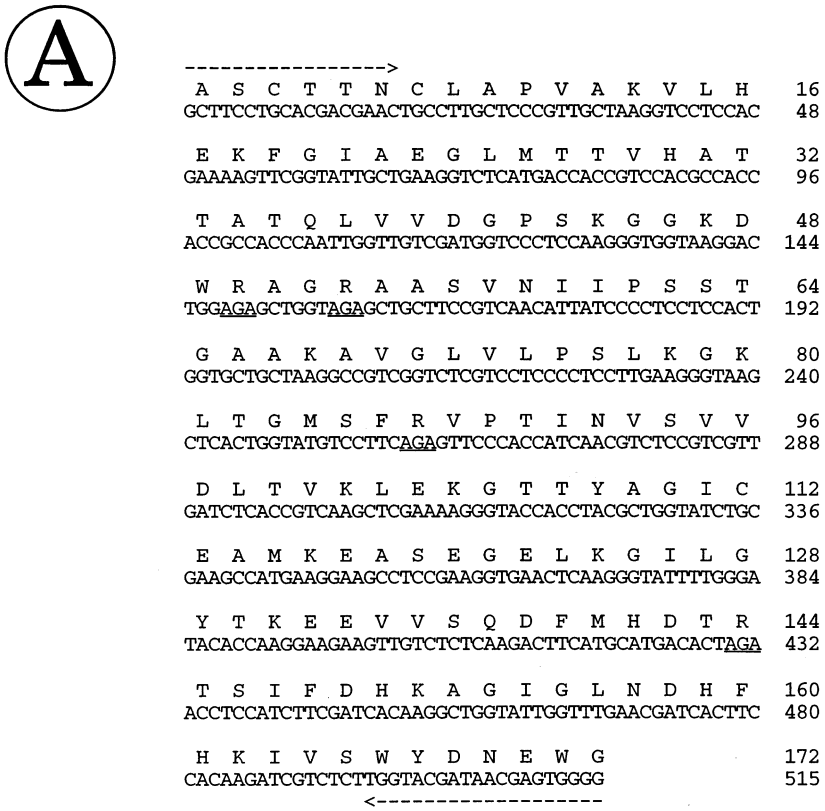

Fig. 5. (A) Nucleotide sequence of the partial *gapC* cDNA clone (515 bp, EMBL/X99629) generated by RT-PCR with *T. pyriformis* mRNA as template and deduced amino acid sequence (172 aa, about half of the complete predicted sequence). The two arrows indicate the primers used for the amplification reaction. Note that AGA (underlined) a codon which occurs with extreme rarity in eubacteria and other eukaryotes, encodes for all four arginines in the predicted protein sequence. The so-called S-loop region of the putative GAPDH protein comprises the aa residues between the positions 32 and 57. (B) Comparison of the amino acid residues forming the S-loop region of the GAPDH from the ciliate *T. pyriformis* and the enzyme from various organisms representative of eubacteria, groups of primitive eukaryotes and other eukaryotes. Numbering follows the putative translation of *G. lamblia gap 1*. Residues characterizing the eubacterial and eukaryotic enzymes are boxed.

gions produced a single cDNA fragment of the size (about 0.5 kb) expected for a region comprising about half of a *gapC* gene. Preparations of genomic DNA from *T. pyriformis* were subjected both to homologous and heterologous Southern blots using the partial *gapC* clone as a probe. Identical patterns of hybridizing bands were found in both conditions (Fig. 4). Only one band hybridized with the probe, except when the re-

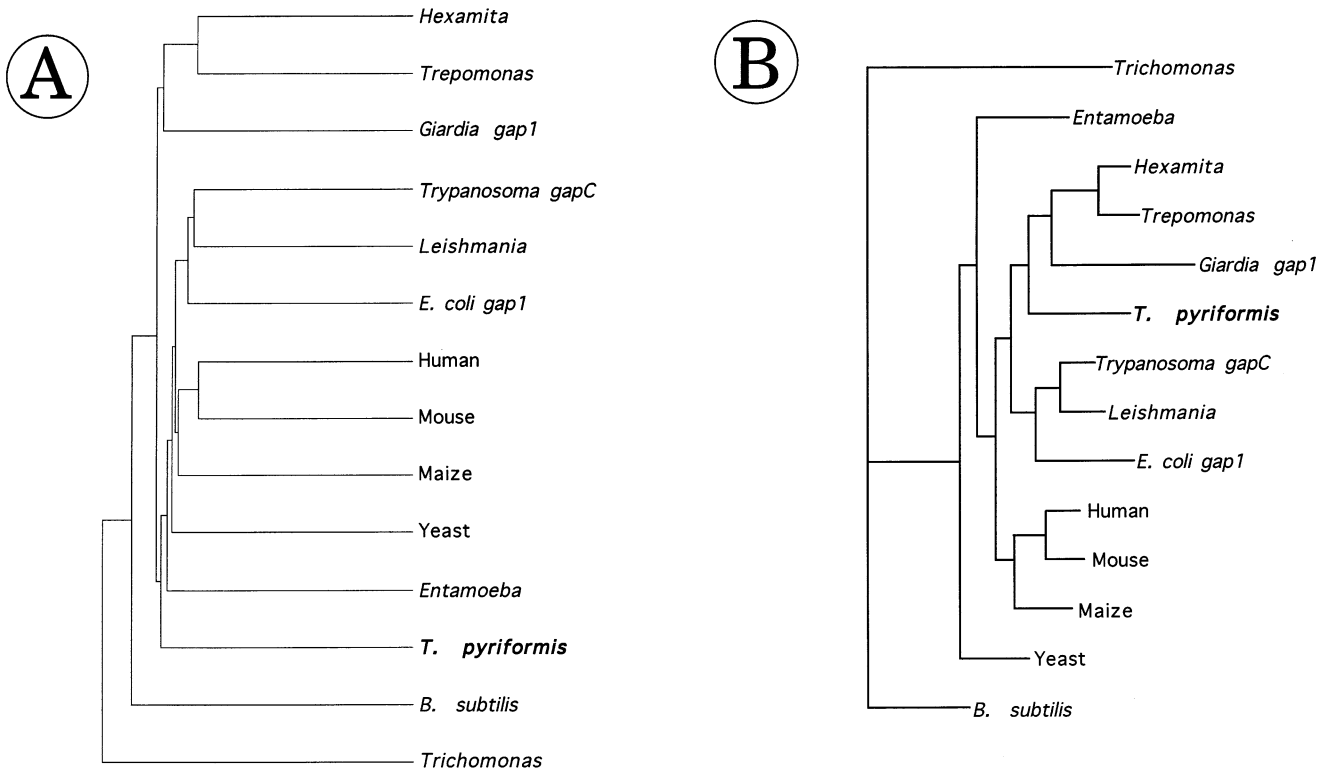

Fig. 6. (A) Sequence similarity tree for the partial GAPDH sequence (172 aa) deduced from the *T. pyriformis gapC* gene fragment reported in this work and the homologous sequence of the enzymes from other eukaryotic and prokaryotic organisms. Dendrogram was obtained from the multiple sequence alignment generated with the PILEUP program of the GCG package. (B) Phylogenetic tree for the partial aa sequence of the GAPDH of *T. pyriformis*. The Kimura distance matrix and the neighbor-joining method of the PHYLIP software package (version 3.5; J. Felsenstein, Department of Genetics, University of Washington) were used for alignments. The tree is unrooted using as outgroup the sequence of *B. subtilis* GAPDH.

strictase *Kpn*I, which has a cutting site into the clone, was used, in which case two bands of similar intensity were seen (Fig. 4). These results indicate that only one copy of the *gapC* gene should be present in the genome of the strain used. This is in agreement with the characteristics of the macronuclear genome of ciliates discussed above. Northern blot experiments confirmed that this *gapC* gene should be expressed, a single transcript of about 1.5 kb, the expected size for a *gapC*-containing poly(A)$^+$ mRNA, was detected with the 0.5-kb cDNA clone as a probe (data not shown). This gene should encode the only GAPDH isoform found in the amicronucleated *T. pyriformis* strain used in this work.

The nucleotide sequence determined for both strands of the PCR amplified cDNA fragment (515 bp, EMBL/X99629) is shown in Fig. 5A. Two clones from independent RT-PCR experiments were sequenced and found to be identical. The derived aa sequence showed all residues strictly conserved in GAPDHs from several organisms [8] and the codon usage is biased in the manner typical for *Tetrahymena* [22]. Thus, the cDNA clone contains AGA codons, which rarely occur in *E. coli* genes, encoding its four arginines (Fig. 5A); this is a feature common to most *Tetrahymena* genes [22]. When the deduced aa sequence was analyzed with the GCG software program PEPTIDESORT (Program Manual, 1994) a basic p*I* (7.6) was calculated. Although this is a theoretical value calculated from a partial aa sequence, this result suggests that the cloned DNA fragment may correspond to the *gapC* gene encoding the basic GAPDH isoform purified from *T. pyriformis* (see above). Unfortunately, since this protein seems to have its N-terminal blocked it was not possible to confirm this proposal by direct comparison of aa sequences.

When the aa sequence deduced from the PCR-amplified DNA fragment was compared to sequences in databases using the GCG software program FASTA, the highest degree of homology (about 75% identity) was with GAPDHs from the kinetoplastid *Leishmania mexicana* and with cytosolic GAPDHs from higher plants, such as *Zea mays* and *Pisum sativum*. High sequence homologies (about 70%) were also found with other unicellular protists including *T. brucei*, *Entamoeba histolytica* and the diplomonads *Giardia intestinalis* (*gap1*) and *Trepomonas agilis*. The fragment shared, in the overlapping region, only 50% identity with *Trichomonas vaginalis* GAPDH.

The predicted aa sequence from the cDNA clone includes the so-called S-loop region (Fig. 5A), located in the interface between the subunits forming the active tetrameric enzyme, a region that can serve as a signature motif distinguishing the eubacterial and eukaryotic GAPDHs [8]. Comparative analysis of the S-loop regions of the enzymes from primitive eukaryotes has been recently used for phylogenetic studies [19]. Fig. 5B shows a comparison of the aa residues forming the S-loop of the putative GAPDH from *T. pyriformis* and the enzymes from other eukaryotes and eubacteria. The ciliate enzyme shows a close affinity to the eukaryotic type but its S-loop is rather unusual both in length and details and shares some common features with the recently described GAPDHs from diplomonads, a group of amitochondrial protists that are considered one of the oldest eukaryotes [1,4,21]. A sequence similarity tree based on the partial 172-aa sequence reported in this work clusters *Tetrahymena* GAPDH in a peripheral position of the eukaryotic lineage group not far from the primitive amitochondrial protists (Fig. 6A). A phylogenetic tree shows also the relationship of *T. pyriformis* GAPDH with the homologous enzyme of primitive protists (Fig. 6B). It should be noted that molecular phylogeny based on analysis of the EF-1α protein [1,10] have shown that ciliates represent one of the earliest mitochondrion-containing branches and that they emerge after *Giardia* and Sporozoa and before Kinetoplastida and *Dictyostelium discoideum*. Moreover, both diplomonads [10] and ciliates [2] exhibit a non-canonical genetic code in which TAR codes for glutamine. The results with the *gapC* gene encoding the *T. pyriformis* GAPDH are in agreement with these studies and strongly suggest that *gapC* genes of ciliates may be used in molecular phylogeny studies to clarify their evolutive relationships with other groups of protists.

4. Note added in proof

During the editorial processing of this manuscript the complete sequence of the *gapC* gene of the ciliated protozoan *Tetrahymena thermophilia* has been reported (J. Euk. Microbiol. 1997;44:434–7). Although no protein studies were performed in that work, the molecular genetic data are in agreement with ours on *T. pyriformis*, namely a single copy of *gapC* gene was found and the predicted GADPH sequence is virtually identical to that reported here.

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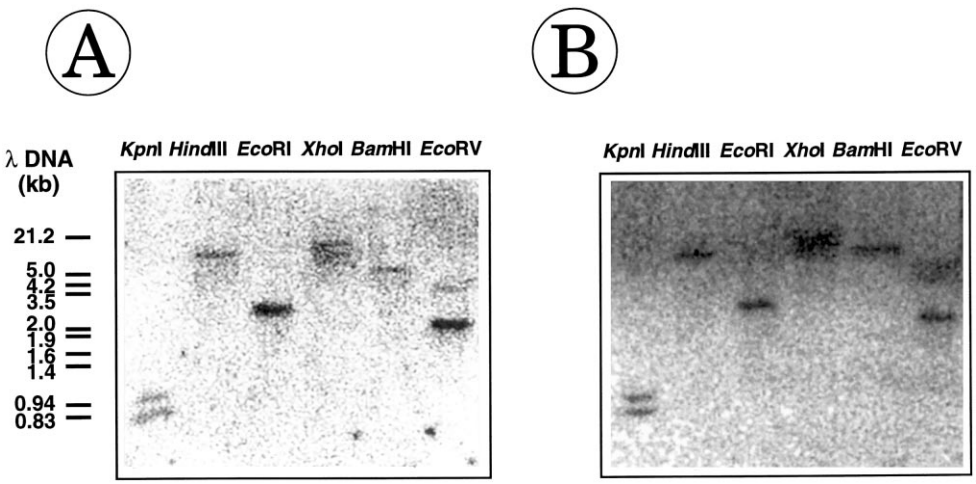

Fig. 4. (A) Southern blot analysis of restriction endonuclease-digested *T. pyriformis* genomic DNA (10 μg per lane) performed in homologous conditions using as probe the *gapC* cDNA clone of 0.5 kb described in this work. (B) Southern blot analysis performed in heterologous conditions of restriction endonuclease-digested *T. pyriformis* genomic DNA.

pyriformis. However, whereas this protein band was also immunodetected in cell-free extracts of the related ciliate *P. tetraurelia*, both strains belong to the same class Oligohymenophorea, nothing was seen with *C. inflata*, a ciliate of the class Colpodea (Fig. 3). No bands were immunodetected in cell-free extracts from all other sources tested, namely eubacteria (i.e. *E. coli* and different cyanobacteria), *S. cerevisiae*, and different mammalian tissues (data not shown). These cross-reaction experiments suggest structural differences between the GAPDHs from ciliates and the other organisms tested. The absence of cross hybridization between *Tetrahymena* and *Colpoda* may reflect, moreover, the large inter-class genetic distance observed in the phylum of the ciliates.

Since GAPDH is a key enzyme of the glycolytic/gluconeogenic pathways of central carbohydrate metabolism [8], we tested the effect of different trophic and environmental culture conditions on both enzymatic activity and immunodetected protein levels in cell-free extracts of *T. pyriformis*. During the normal evolution of cultures these parameters showed parallel patterns and marked changes, reaching maximal values at the end of the exponential phase of growth and a dramatic decline until reaching negligible values later on in the stationary phase (data not shown). These changes should be due to processes of synthesis/degradation of protein rather than to modulation of enzyme activity by activation/deactivation processes, as is suggested by its time scale. This behaviour in response to changes in environmental and trophic conditions is expected for an enzyme that, like GAPDH, is involved in central metabolism. These results are similar to those reported in photosynthetic protists for cytosolic GAPDHs involved in biosynthetic and degradative carbon metabolism [24]. The GAPDH of *T. pyriformis* is affected by both ionic and temperature stress. A simultaneous decrease of enzyme activity and protein, as well as lower growth rates, were observed after addition of increasing NaCl concentrations (up to 0.1 M) to the cultures, or when temperature was raised 10°C (data not shown). A marked inhibition of purified *T. pyriformis* GAPDH was also observed when increasing the ionic strength of the medium, with complete inhibition of the enzyme at about 0.2 M NaCl (data not shown). The only known salt-dependent GAPDH, that of the halophilic archaebacterium *Haloarcula vallismortis* [16] is a very acidic protein, and this physicochemical feature has been claimed to be involved in the tolerance to high ionic strength of the archaebacterial enzyme. The ciliate GAPDH with its marked sensitivity to salts is, in contrast, a rather basic protein.

3.2. Cloning and sequencing of a partial cDNA fragment of a gapC gene from T. pyriformis exhibiting primitive eukaryotic features

Nuclear duality, that is the presence of a germ line nucleus (micronucleus) and a somatic nucleus (macronucleus), is one key characteristic of ciliates, although amicronucleate strains are often encountered in the wild [15]. In this work, we used an amicronucleated strain of *T. pyriformis*, with only the macronuclear or vegetative genome. The macronuclear genome of *Tetrahymena* is a multiplied (about 46 times greater), fragmented and rearranged version of some fraction of the micronuclear genome (up to 20% of micronuclear DNA sequences are absent) [15]. Since only single but highly-amplified copies of those genes needed for vegetative growth are in the macronuclear genome, pseudogenes are absent. RT-PCR amplifications using primers constructed from two highly conserved GAPDH re-