Molecular and structural characterization of NADPH-dependent D-glycerate dehydrogenase from the enteric parasitic protist *Entamoeba histolytica*

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Putative NADPH-dependent GDH (D-glycerate dehydrogenase) of the protozoan parasite *Entamoeba histolytica* (EhGDH) has been characterized. The EhGDH gene encodes a protein of 318 amino acids with a calculated isoelectric point of 6.29 and a molecular mass of 35.8 kDa. EhGDH showed highest identities with GDH from ε-proteobacteria. This close kinship was also supported by phylogenetic analyses, suggesting possible lateral transfer of the gene from ε-proteobacteria to *E. histolytica*. In contrast with the implications from protein alignment and phylogenetic analysis, kinetic studies revealed that the amoebic GDH showed biochemical properties similar to those of mammalian GDH, i.e. a preference for NADPH as cofactor and higher affinities towards NADPH and β-hydroxybutyrate than towards NADP⁺ and D-glycerate. Whereas the amino acids involved in nucleotide binding and catalysis are totally conserved in EhGDH, substitution of a negatively charged amino acid with a non-charged hydroxy-group-containing amino acid is probably responsible for the observed high affinity of EhGDH for NADP⁺/NADPH. In addition, the amoebic GDH, diSSimilar to the bacterial and mammalian GDHs, lacks glyoxylate reductase activity. Native and recombinant EhGDH showed comparable subunit structure, kinetic parameters and elution profiles on anion-exchange chromatography. We propose that the GDH enzyme is likely to be involved in regulation of the intracellular concentration of serine, and, thus, also in controlling cysteine biosynthesis located downstream of the serine metabolic pathways in this protist.

Key words: anaerobic protist, cysteine biosynthesis, *Entamoeba histolytica*, gluconeogenesis, glycerate dehydrogenase, serine biosynthesis.

INTRODUCTION

D-Glycerate dehydrogenase (GDH; EC 1.1.1.29) catalyses the NADH- or NADPH-dependent reduction of HP (β-hydroxybutyrate) as a committed step of serine degradation in mammals [1]. The product of this reaction, D-glycerate, is channelled into the gluconeogenic pathway [2]. Therefore GDH has a critical role to link serine metabolism and gluconeogenesis in mammalian organisms. GDH also catalyses the NAD⁺- or NADPH⁺-linked oxidation of D-glycerate in the direction of serine biosynthesis in plants [3]. Therefore the plant GDH apparently functions in both the forward and reverse orientations, and consequently has two roles, i.e. degradation of serine leading to gluconeogenesis and serine biosynthesis [3]. In humans, GDH is expressed in the various organs, but the highest GDH activity and mRNA level were found in the liver [4,5], suggesting GDH is involved primarily in serine degradation, leading to gluconeogenesis in mammals. The physiological importance of GDH has been demonstrated by its deficiency in humans. Primary hyperoxaluria type 2 is a genetic metabolic disease attributable to a deficiency in GDH activity, accompanied with compensatory high lactate dehydrogenase activity, which causes excretion of excessive L-glycerate and oxalate in the urine leading to kidney dysfunction [4,6,7]. Although GDH has been shown to be present in a wide variety of organisms from bacteria to mammals and plants, and its physiological importance in higher eukaryotes is well understood, neither its presence nor its biochemical properties has been demonstrated in unicellular eukaryotes.

*Entamoeba histolytica*, the causative agent of human amoebiasis, is an enteric protozoan parasite and causes amoebic colitis and extraintestinal abscesses in approximately 50 000 000 inhabitants of endemic areas [8]. Sulphur-containing amino acid metabolism in *E. histolytica* is unique in a variety of aspects, including: (1) a lack of both forward and reverse trans-sulphuration pathways; (2) a lack of enzymes responsible for cysteine and homocysteine degradation in mammals, including cysteine dioxygenase and phosphopantetheinylcysteine synthase; and (3) the presence of the *de novo* sulphur-assimilatory cysteine-biosynthetic pathway [9–11a]. Together with unique metabolism of sulphur amino acids in this parasite, a physiological requirement of cysteine has also been shown [12,13]. The major, and probably sole, route of cysteine biosynthesis is the condensation of OAS (O-acetylseryine) with sulphide, mediated by the *de novo* cysteine-biosynthetic pathway. OAS is produced by a trans-acetylation reaction (the addition of an acetyl moiety to serine), which is probably obtained via *de novo* serine biosynthesis. To understand better sulphur-containing amino acid metabolism and cysteine biosynthesis in protozoan parasites, we attempted to identify and characterize putative serine metabolic pathways. We have identified in the *E. histolytica* genome database genes encoding GDH, GK (glycerate kinase), PGDH (phosphoglycerate dehydrogenase) and PSAT (phosphoserine aminotransferase)
and Xho of the sequential reactions catalysed by L-serine:pyruvate aminotransferase, d-glycerate dehydrogenase and d-glycerate kinase [1,3]. In the present work, we describe cloning and enzymological characterization of a gene encoding GDH from E. histolytica. As far as we are aware, this is the first report on GDH in unicellular eukaryotes.

EXPERIMENTAL

Chemicals

All chemicals of analytical grade were purchased from Wako (Tokyo, Japan) or Sigma–Aldrich (Tokyo, Japan), unless stated otherwise. Pre-packed Mono Q 5/5 HR and Sephacryl S-300 HR Hiprep columns were purchased from Amersham Biosciences (Tokyo, Japan).

Parasite culture

Trophozoites of the E. histolytica clonal strain HM1:IMSS cl6 [14] were cultured axenically in TYI-S33 medium at 35 °C, as described previously [15].

Bacterial expression and purification of recombinant EhGDH (rEhGDH)

A plasmid was constructed to produce rEhGDH possessing the N-terminal histidine tag. A fragment corresponding to an ORF (open reading frame) of EhGDH was amplified by PCR using a cDNA library [9] as the template and the following oligonucleotide primers: 5′-caGGATCCaagatagttgtattagacgca-3′ and 5′-caCTCGAGtagactattctattttc-3′, where capital letters indicate BamHI or XhoI restriction sites. The cycling parameters were: (1) denaturation at 94 °C for 30 s; (2) annealing at 55 °C for 30 s; (3) elongation at 72 °C for 60 s; and (4) 30 cycles. An approx. 1.0 kb PCR fragment was digested with BamHI and XhoI, electrophoresed, purified with Geneclean kit II (BIO 101; Vista, CA, U.S.A.), and cloned into BamHI- and XhoI-double-digested pET-15b (Novagen) to produce pET–EhGDH. The nucleotide sequence of the amplified EhGDH ORF was verified by sequencing, and was found to be identical with that of contig 317757 in the E. histolytica genome database (nt 11 110–12 066). The pET–EhGDH construct was introduced into Escherichia coli BL21(DE3) cells (Novagen). Expression of the rEhGDH protein was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) for 4–5 h at 30 °C. The bacterial cells were harvested, washed, lysed in 50 mM Tris/HCl, pH 8.0/300 mM NaCl containing 10 mM imidazole, 0.1% (v/v) Triton X-100, 100 µg/ml lysozyme and complete mini EDTA-free protease inhibitor cocktail (Roche, Tokyo, Japan), and then sonicated. The rEhGDH protein was purified from the supernatant fraction using an Ni2+-nitrilotriacetate column (Novagen) according to the manufacturer’s instructions. The eluted rEhGDH protein was dialysed extensively in 50 mM Tris/HCl, pH 8.0/300 mM NaCl containing 10% (v/v) glycerol and the protease inhibitors described above overnight at 4 °C, before storage at −80 °C with 50% glycerol. Enzyme remained active for more than 1 month when stored at −80 °C under these conditions.

Enzyme assays

The enzymic activity of GDH was assayed in both the forward and reverse directions using either a spectrophotometer or a fluorimeter. The GDH activity in the forward reaction was measured spectrophotometrically using a BeckmanDU530 spectrophotometer by following the decrease in absorbance at 340 nm due to HP-dependent oxidation of NADPH or NADH for 2–4 min at 25 °C. The reaction mixture contained 50 mM sodium phosphate, pH 6.5, 300 mM NaCl, 0.2 mM NADPH or NADH, 0.2 mM DTT (dithiothreitol), 500 µM HP and the enzyme. Kinetic parameters for NADPH in the forward direction were also estimated fluorimetrically (using a Fluorimeter F-2500; Hitachi, Tokyo, Japan) by measuring the rate of change in fluorescence (emission wavelength 470 nm; excitation wavelength 340 nm). D-Glycerate-dependent production of NADPH in the reverse reaction was measured fluorimetrically. Since the reverse reaction showed an optimum pH of 8.5, all reactions were allowed to proceed at this pH. The assay mixture contained 50 mM Tris/HCl, pH 8.5, 300 mM NaCl, 0.1 mM DTT, 0.2 mM NADP+, 1.5 mM glycerate and 1.0 µg of the purified rEhGDH. Kmax and Vmax values were estimated with Hanes–Woolf and Lineweaver–Burk plots.

Chromatographic separation of native EhGDH from E. histolytica lysate

E. histolytica trophozoites (∼107; 200 mg wet weight) suspended in 1.0 ml of 100 mM Tris/HCl, pH 8.0, 1.0 mM EDTA, 2.0 mM DTT and 2.0 M glycerol containing 10 µg/ml E64 [trans-epoxysoyuccinyl-l-leucylamido-(4-guanidino)butane] and complete mini-EDTA-free protease inhibitor cocktail were subjected to three cycles of freezing and thawing and sonication. After centrifugation at 45 000 g for 15 min at 4 °C, the supernatant was filtered through a 0.45 µm cellulose acetate membrane and concentrated to a volume of 250 µl by rotating at 72 °C for 60 s. This concentrate was loaded onto a Mono Q 5/5 HR column that had been pre-equilibrated with binding buffer [100 mM Tris/HCl, pH 8.0 containing 1.0 mM EDTA, 2.0 mM DTT, 2.0 M glycerol and 1 µg/ml E64] on AKTA Explorer 10S system (Amersham Biosciences, Tokyo, Japan). After extensive washing, bound proteins were eluted with a linear gradient of 0–1 M NaCl with a flow rate of 1 ml/min. Each fraction (0.5 ml) was analysed for GDH activity. The rEhGDH was fractionated on the same column under identical conditions; native EhGDH and rEhGDH were also separated by gel-filtration chromatography using a 60-cm-long, 1.6-cm-diam. Sephacryl S-300 HR Hiprep pre-packed column. The column was pre-equilibrated, loaded, washed and eluted with the gel-filtration buffer [0.1 M Tris/HCl (pH 8.0)/0.1 M NaCl] at a flow rate of 0.5 ml/min.

Amino acid comparison and phylogenetic analysis

Amino acid sequences that showed significant similarity to EhGDH were obtained from the DDBJ/GenBank/EBI databases by using a BLASTP search. These sequences included GDH, PGDH, HP reductase, glyoxalate reductase and putative D-2-hydroxyacid dehydrogenase from various organisms. Sequence alignments were generated using the program CLUSTAL W version 1.81 [16] with the BLOSUM matrix. Phylogenetic analysis using the Neighbor-Joining method with Kimura’s correction was also performed using CLUSTAL W. Phylogenetic
trees were drawn using the Tree View PPC program. The branch lengths in these trees were obtained from the PHYLIP analysis with bootstrap values in 1000 replicates.

RESULTS

Features of the deduced protein primary structure of EhGDH

We obtained a contig sequence (contig 317 757) by a homology search of the *E. histolytica* genome database with GDH from bacteria, plants and mammals. The putative GDH gene contained a 957 bp ORF, which encodes a protein of 318 amino acids, with a predicted molecular mass of 35.8 kDa and a pI (isoelectric point) of 6.29. No other independent contig was found to contain the GDH gene (results not shown), suggesting that this GDH gene is present as a single copy. We searched thoroughly for other possible GDH genes using this amoebic GDH gene in the *E. histolytica* genome database. However, no other possible GDH-related sequence was found, except for a putative PGDH gene, which we will report separately.

The amino acid sequence of the *E. histolytica* GDH showed 24–40% identities with those from bacteria, mammals and plants. The *E. histolytica* GDH showed the highest amino acid identities (38–40%) with GDH from *ε*-proteobacteria, including *Campylobacter jejuni* and *Helicobacter pylori*, and the lowest identities (24–26%) with GDH from higher eukaryotes, including plants and humans (specifically, EhGDH showed identities of 40% with *C. jejuni* GDH, 38% with *H. pylori* GDH, 34% with *Neisseria meningitidis* GDH, 32% with *Methyllobacterium extorquens* GDH, 31% with *Archaeoglobus fulgidus* GDH, 28% with mouse and *Schizosaccharomyces pombe* GDHs, 27% with *Bacillus subtilis* and *E. coli* GDHs, 26% with human GDH and 24% with *Hyphomicrobium methyllovorum* and cucumber GDHs). The amoebic GDH, similar to GDH from *ε*- and *β*-proteobacteria, possesses a 5-amino-acid insertion in the central region (amino acids 130–134 of EhGDH) that is absent in other members of GDH, but lacks two internal insertions (between amino acids 33 and 34 and between amino acids 49 and 50 of EhGDH) that are found in some of other organisms (Figure 1). EhGDH also lacks both an internal 17-amino-acid insertion at the centre and an approximately 27-amino-acid C-terminal extension found in the plant GDH. We also searched for putative GDH genes in the genome and expressed sequence tag databases of other parasitic protozoa, including *Leishmania*, *Plasmodium*, *Giardia*, *Trypanosoma* and *Trichomonas*, and the non-parasitic protozoan *Dictyostelium discoideum*, but did not find orthologues in these databases, suggesting that GDH is exclusively present only in this anaerobic enteric parasite among the protists. The consensus sequence Gly-Xaa-Gly-Xaa-,Gly-Xaa,-Asp (where ‘Xaa’ denotes ‘any amino acid’), involved in the binding of the adenosine portion of NAD⁺ [17], was located at residues 154–177 of EhGDH. Asp [77], which was conserved among *A. fulgidus*, *H. methyllovorum* and cucumber GDHs, was replaced with serine in EhGDH, as observed in GDH from *C. jejuni* and *H. pylori*. All important residues implicated in pairing in the active-site-histidine–carboxylate couple, as predicted from the crystal structure of *H. methyllovorum* GDH (Arg [241], Glu [270], His [285] and Asp [268]) [18], and also an arginine residue (Arg [241]) involved in substrate orientation, were totally conserved in EhGDH (Arg [235], Glu [264], His [285] and Asp [269]). This type of catalytic arrangement is also found in the other enzymes possessing 2-hydroxyacid dehydrogenase activities, i.e. lactate dehydrogenase and malate dehydrogenase, and other D-isomer-specific dehydrogenases (but not formate dehydrogenase) [18,19], serine proteases, thermolysin [20] and phospholipase A₂ [21].

Phylogenetic analysis

Phylogenetic reconstruction was performed with 16 GDH and three PGDH protein primary structures, which were used as the ‘out-group’, from various organisms using the CLUSTAL W program. The phylogenetic tree (Figure 2) demonstrates that two major groups of GDH represent individual clades, which are well supported by high bootstrap proportions at the nodes (98–99%). One group comprises *E. histolytica* and proteobacteria, including *α-, γ- and ε*-proteobacteria; the other group comprises mammals, plants, *B. subtilis*, *E. coli* and *H. methyllovorum*. Within the first clade, a group including *E. histolytica* and *ε*-proteobacteria forms a sister group with *α- and γ*-proteobacteria; both clades were statistically well supported (96–97%). A close phylogenetic association between EhGDH and GDHs from *ε*-proteobacteria, together with the shared insertions and deletions of amino acids described above among these GDHs, suggest that amoebic GDH was probably obtained from an ancestor of modern *ε*-proteobacteria by lateral transfer, as suggested for other metabolic enzymes in this parasite [22,23].

Purification and characterization of rEhGDH

The rEhGDH protein revealed an apparently homogeneous band of 38 kDa on SDS/PAGE analysis (results not shown), which is consistent with the predicted size of the deduced EhGDH protein primary structure with an extra 20 amino acids added at the N-terminus. The purified rEhGDH protein was evaluated as being >95% pure, as determined from the Coomassie-Blue-stained SDS/PAGE gel. We first optimized the conditions for enzymic assay, i.e. pH, salt concentrations, requirement for cofactors, bivalent metal ions, DTT and stabilizing reagents. rEhGDH was found to be unstable: the enzyme was totally inactivated when stored without any preservative or additive at room temperature, 4 °C or −20 °C overnight. When rEhGDH was stored in 50 mM Tris/HCl buffer, pH 8.0, containing 50% (v/v) glycerol at −80 °C, rEhGDH remained fully active for more than 1 month. The maximum activity of rEhGDH for the forward reaction was observed at pH 6.0–6.5, which decreased substantially at higher measurements of pH (results not shown). The GDH activity in the reverse reaction was less affected by variations in pH; the activity was found to be highest at a slightly basic pH (pH 8.0–8.5). Substrate inhibition by HP (at 0.2 mM and higher concentrations) was alleviated by the addition of salt (100–400 mM NaCl), as reported for bovine liver GDH [24,25]. Substrate inhibition by HP was more pronounced with NADPH than with NADH for mammalian GDH [7]. In contrast, inhibition of rEhGDH by HP (1.0 mM) was not observed in the presence of higher NaCl concentrations (e.g. 300 mM) at a wide range of NADPH/NADH concentrations (between 40 and 200 μM). The maximum stimulatory effect (2–3-fold) was observed with 250–300 mM NaCl or KCl. The univalent salts, i.e. Na⁺ and K⁺, were found to be more effective than bivalent salts, such as Mg²⁺ and Ca²⁺ (results not shown), for rEhGDH. rEhGDH showed a 2–3-fold-higher activity with NADPH as compared with NADH when 0.1–0.5 mM HP, 0.2 mM cofactors and 300 mM salt were added, as shown for the mammalian GDH. The addition of neither DTT nor EDTA resulted in any significant change in the activity of rEhGDH.

Kinetic properties of rEhGDH

Owing to the apparent stimulatory effect of salt on rEhGDH activity, as described above, we conducted further kinetic studies...
in the presence of 300 mM NaCl. At saturating concentrations of the substrate, rEhGDH showed a two orders of magnitude (≈100-fold) higher affinity for NADPH than for NADH (Table 1). However, the specific activity was 3.9-fold higher with NADH than with NADPH. The $K_m$ values for D-glycerate and NADP$^+$ in the reverse reaction were calculated to be one order of magnitude higher than those for HP and NADPH in the forward reaction. We did not observe utilization of NAD$^+$ as a substrate in the reverse reaction, even in the presence of high concentrations of NAD$^+$ (0.3 mM) and D-glycerate (5–10 mM). Although GDH from mammals and plants was shown to utilize glyoxylate as a substrate to produce glycolate, the amoebic enzyme did not catalyse this reaction (results not shown), and thus appears to be specific for the conversion of HP into glycerate. Both serine and cysteine at 5 mM inhibited recombinant GDH activity by 20–25%, with a maximum inhibition of approx. 70–80% observed in the presence of these amino acids at concentrations of 20–25 mM. Other structurally related amino acids (alanine, glycine, valine, methionine and threonine) did not show any inhibition up to 10 mM.

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**Figure 2**  Phylogenetic analysis of GDH proteins from a variety of organisms

A phylogenetic tree was constructed using the CLUSTAL W program and drawn using the Treeview PPC program. A rooted tree with three PGDH sequences used as the out-group is shown. Numbers at the nodes represent bootstrap values of 1000 replicates. Species names and accession numbers of these sequences are indicated in the tree. The scale bar indicates 0.1 substitutions per each amino acid position.

**Table 1**  Kinetic properties of recombinant EhGDH

Mean values ± S.D. for independent measurements (n = 3–5) are shown. ND, not detected.

<table>
<thead>
<tr>
<th>Substrate/cofactor</th>
<th>pH</th>
<th>$K_m$ (mM)</th>
<th>Specific activity (mmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxypyruvate</td>
<td>6.5</td>
<td>$61.1 \pm 4.86^*$</td>
<td>15.6 ± 2.28*</td>
</tr>
<tr>
<td>NADPH</td>
<td>6.5</td>
<td>$1.55 \pm 0.44^+$</td>
<td>1.69 ± 1.29†</td>
</tr>
<tr>
<td>NADH</td>
<td>6.5</td>
<td>$147 \pm 4.35^+$</td>
<td>11.4 ± 2.34‡</td>
</tr>
<tr>
<td>α-Glyceric acid</td>
<td>8.5</td>
<td>$483 \pm 58.4^§$</td>
<td>64.1 ± 26.8§</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>8.5</td>
<td>$27.5 \pm 2.12^{</td>
<td></td>
</tr>
<tr>
<td>NAD⁺</td>
<td>8.5</td>
<td>ND^{¶}</td>
<td>ND^{¶}</td>
</tr>
</tbody>
</table>

* 0.2 mM NADPH was used.
† 0.1 mM HP was used.
‡ 0.5 mM HP was used.
§ 0.2 mM NADP⁺ was used.
¶ 5 mM α-glyceric acid was used.
¶ 0.3 mM NAD⁺ and 5–10 mM α-glyceric acid was used.

**Chromatographic separation of the native and recombinant EhGDH activities**

In order to correlate native GDH activity in the *E. histolytica* lysate with that of the recombinant enzyme, the lysate from the trophozoites and rEhGDH were subjected to chromatographic separation on a Mono Q anion-exchange column (Figure 3). The *E. histolytica* lysate showed a GDH activity of approx. 3.94 ± 0.25 mmol/min per mg of lysate protein. Thus native GDH represents 0.14–0.2 % of the total soluble protein, assuming that native EhGDH and rEhGDH possess comparable specific activities. GDH activity was eluted as a single peak at an identical salt concentration for both native EhGFH and rEhGDH. This finding, together with the fact that the GDH gene is present as a single copy, indicates that the *EhGDH* gene we have cloned represents at least the dominant, and probably the sole, gene responsible for GDH activity in the axenic trophozoites. In order to gain an insight into its multimeric composition, the recombinant GDH enzyme was subjected to gel-filtration chromatography.
GDH activity was eluted at the predicted molecular size of 75–80 kDa (results not shown). This observation is consistent with the notion that rEHGDH exists as a dimer (monomer 35.8 + 2.6 kDa). In addition, separation of the amoebic crude lysate on the gel-filtration column showed that native GDH activity was also eluted at the position compatible with the native GDH being a dimer (results not shown). These data suggest that both the native and recombinant enzymes exist in their homodimeric forms, as found in other species [26–28]. Our attempt to purify the native GDH to homogeneity in order to perform peptide sequencing failed due to instability of the enzyme (results not shown).

**DISCUSSION**

**Presence of GDH and the non-phosphorylated pathway in serine degradation in the anaerobic parasitic protist**

In the present study, we have demonstrated that the enteric protozoan parasite *E. histolytica* possesses one of the key enzymes of serine metabolism. As far as we are aware, this is the first demonstration of GDH in unicellular eukaryotes, including parasitic and non-parasitic protists. GDH has been implicated in having an essential role in the serine degradation pathway in humans, as demonstrated by genetic diseases caused by its deficiency [4,29].

We propose, on the basis of the following bioinformatic and biochemical evidence, that this enzyme also has a key role in maintenance of the intracellular serine concentration in this anaerobic parasitic protist.

First, on the basis of the presence of orthologues of GDH, GK, PGDH, and PSAT in the genome database (results not shown), *E. histolytica* probably possesses both phosphorylated and non-phosphorylated pathways for serine metabolism. A gene encoding GDH is absent in other parasitic and non-parasitic protists, including *Leishmania, Plasmodium, Giardia, Trypanosoma, Trichomonas* and *D. discoideum*. The presence of a non-phosphorylated pathway might be unique for *E. histolytica,* or a group of anaerobic protists including *E. histolytica.* This presents the possibility that GDH and the non-phosphorylated serine pathway may be involved in cellular metabolism associated with anaerobic metabolism (see below). Disclosure of the entire genome data for other anaerobic protists, e.g. *Trichomonas* and *Giardia,* should address this question.

Secondly, kinetic parameters determined for EhGDH also support the premise that the amoebic GDH functions in the direction of serine degradation. This amoebic GDH showed a strong preference towards NADPH and HP as substrates as compared with NADP+ and D-glycerate. Furthermore, the amoebic GDH showed a ≈100-fold-higher affinity for NADP+/NADPH than for NAD+/NADH. Assuming that the cytosolic NADPH:NADP+ ratio (approx. 100:1) [30] and the HP concentration (≈ 5 µM) [7] are comparable between mammals and *E. histolytica,* we conclude that the forward reaction, which leads to serine degradation, is favourable in the amoeba. In *E. histolytica* trophozoites, most of the NADH in the cell is probably converted into NADPH during glycolysis by conversion of phosphoenolpyruvate into pyruvate via oxaloacetate and malate in a reaction catalysed by malate dehydrogenase and malic enzyme [31]. *E. histolytica* also possesses pyridine nucleotide transhydrogenase (EC 1.6.1.1) [32], which catalyses the hydrogen-exchange reaction between NADH and NADP+, thereby reinforcing the predominance of NADPH over NADH in this parasite. Taken together, these findings support our premise that the amoebic GDH acts in vivo as an NADP+-dependent HP-reducing enzyme to produce D-glycerate, and is thus likely have a role in serine degradation, but not in serine biosynthesis.

**Possible biological role of GDH in *E. histolytica***

The presence of a non-phosphorylated serine metabolic pathway in *E. histolytica* might be associated with the unique metabolism in this anaerobic/microaerophilic parasite. Since *E. histolytica* does not possess a functional tricarboxylic acid cycle and pentosephosphate pathway, the major source of energy is from glycolysis. In addition, due to the absence of lactate dehydrogenase, NADH formed during glycolysis is not reoxidized by the conversion...
of pyruvate into lactate. Instead, acetyl-CoA is anaerobically reduced to ethanol and CO₂ [33,34], where NADH is reoxidized. NADH is also converted into NADPH by pyridine nucleotide transhydrogenase, as described above. Serine, together with pyruvate, is located at the ‘gateway’ of glycolysis, fermentation and amino acid metabolism in *E. histolytica*. Serine dehydratase is implicated in the microaerophilic energy metabolism of this parasite, since serine stimulates oxygen consumption and is converted into pyruvate in living cells and extracts [35]. This prompted us to dissect the serine metabolic pathway at the molecular level for a comprehensive understanding of energy metabolism in this parasite.

The physiological role of GDH has been well demonstrated by l-glyceric aciduria (hyperoxaluria type II) [4] in humans. GDH deficiency causes accumulation of HP and glyoxylate, which are converted into L-glycerate and oxalate respectively by lactate dehydrogenase, resulting in the abnormal excretion of L-glycerate and oxalate in the urine [4,6,7]. Therefore the key role of GDH in mammals is to maintain the intracellular concentration of both HP and glyoxylate during the catabolism of serine. Since *E. histolytica* lacks lactate dehydrogenase, and the amoebic GDH does not catalyse glyoxylate reduction, the fate of HP, when GDH is absent, is not known. However, it is conceivable that a high concentration of HP may be detrimental to the amoeba. To support this premise, serine (at a concentration of 5–20 mM) inhibited the trophozoite growth in vitro by 50–70% (M. Tokoro and T. Nozaki, unpublished work). Taken together, we propose that, in *E. histolytica*, GDH may have an important role in the maintenance of intracellular serine and HP concentrations, and therefore also in the control of cysteine biosynthesis located downstream of the serine metabolic pathway.

**Peculiarity of the amoebic GDH**

Whereas some oxidoreductases use both NADH and NADPH coenzymes, others are very specific for either NADH or NADPH. For example, lactate dehydrogenase and malate dehydrogenase only utilize NAD⁺/NADH [20], whereas glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and malic enzymes specifically require NADP⁺/NADPH. Similarly, EhGDH and the rat-liver GDH [7] are very specific for NADP⁺/NADPH, whereas bacterial GDH utilizes only NAD⁺/NADH [29]. It is conceivable that the replacement of a charged amino acid (aspartate or glutamate), which is well conserved in GDH from plants (e.g. Asp¹⁹⁶ of cucumber GDH), archaea (Asp¹⁷⁹ of *A. fulgidus* GDH) and α- and β-proteobacteria (Asp¹⁷⁸ of *H. methyllovorum*; Asp¹⁷⁷ of *N. meningitidis* GDH) with a non-charged hydroxy amino acid (Ser²⁷⁷ of EhGDH; Thr³⁸³ of human GDH) [5,7] is responsible for the observed high affinity of these enzymes towards NADP⁺/NADPH. It should also be noted that the PGDH enzymes from a variety of organisms [3,36] possess the conserved aspartate residue, and that all of the PGDHs for which biochemical information is available have a strong preference for NAD⁺/NADH. An extra phosphate group present in NADP⁺/NADPH might interfere with the interaction between the mammalian and amoebic GDHs and NADP⁺/NADPH. The carboxylic group of aspartate and negative charge of phosphate group at neutral pH produce a repulsion force that may reduce proper binding. Replacement of aspartate with an uncharged polar amino acid, as seen in the amoebic and mammalian GDHs, might enable the phosphate group of NADP⁺/NADPH to interact with this amino acid. Mutational analysis of the amoebic and mammalian GDHs should either prove or disprove this hypothesis.

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