Phytomonas sp. contains two malate dehydrogenase isofoms, a mitochondrial isoenzyme with a high specificitity for oxaloacetate and a glycosomal isozyme that acts on a broad range of substrates (Uttaro, A. D., and Opperdoes, F.R. (1997) Mol. Biochem. Parasitol. 89, 51–59). Here, we show that the low specificity of the latter isoenzyme is the result of a number of recent gene duplications that gave rise to a family of glycosomal 2-hydroxyacid dehydrogenase genes. Two of these genes were cloned, sequenced, and overexpressed in Escherichia coli. Although both gene products have 322 amino acids, share 90.4% identical residues, and have a similar hydrophobicity profile and net charge, their kinetic properties were strikingly different. One isoform behaved as a real malate dehydrogenase with a high specificity for oxaloacetate, whereas the other showed no activity with oxaloacetate but was able to reduce other oxoacids, such as phenyl pyruvate, 2-oxoisocaproate, 2-oxovalerate, 2-oxobutyrate, 2-oxo-4-methylbutyrate, and pyruvate.

Malate dehydrogenase (MDH) belongs to the enzyme family of 2-hydroxyacid dehydrogenases and is a classical example of a broadly distributed enzyme. In eukaryotes it is present in different subcellular compartments and takes part in different metabolic pathways (1, 2). MDH utilizes either the cofactor NAD⁺ or NADP⁺ to catalyze the reversible oxidation of malate to oxaloacetate. MDHs are generally active as dimers with identical subunits. Exceptions are the MDH of various Bacillus species, in which the enzyme is tetrameric (2–4). Recently Reynoso-Hunter et al. (5) reported the purification of both a mitochondrial and glycosomal MDH from Trypanosoma cruzi. As in Bacillus, the latter glycosomal isoenzyme appeared to be tetrameric, thus representing the first example of an eukaryotic tetrameric MDH. The cloning and sequencing of genes coding for the mitochondrial and glycosomal MDH isoenzymes from Trypanosoma brucei has been reported (6, 7), but no information is available on the quaternary structure of these enzymes.

We previously described that Phytomonas sp., a trypanosomatid of plants, has two isofoms of MDH, a mitochondrial form and a glycosomal form (8). Phytomonas sp. do not contain an active citric acid cycle, and the mitochondrial MDH is believed to be part of a system transferring reducing equivalents from the glycosome to the mitochondrion (8–10), which regenerates NAD⁺ for glycolysis.

We have purified the glycosomal MDH from Phytomonas. By contrast with glycosomal MDH from T. cruzi, Phytomonas MDH behaves as a homodimer with a low substrate specificity. In in vitro assays, it can also accept oxaloacetate, phenylpyruvate, 2-oxocaproate, and 2-oxoisocaproate as substrates (8). Because of the presumed important role of this enzyme in Phytomonas’ energy metabolism and its unusual properties, we decided to clone its gene and overexpress the corresponding protein. This allowed us to study the nature of the apparent low substrate specificity in detail.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Phytomonas strain was isolated in France from Euphorbia characias (11). Phenyl-Sepharose CL-4B, CM-Sepharose, and blue-Sepharose were from Amersham Pharmacia Biotech. Oxaloacetic acid, malic acid, phenylpyruvic acid, 2-oxoacrylic acid, 2-oxoisocaproic acid, 2-oxovaleric acid, 2-oxobutyric acid, 2-oxo-4-methylbutyric acid, and pyruvic acid were from Sigma. Restriction enzymes were from New England BioLabs. Hybond-N nylon membrane was from Amersham Pharmacia Biotech. Pancreatic bovine DNase (type IV) was from Sigma.

**Purification of Wild-type and Recombinant Enzymes**—Wild-type MDH was purified from 1 liter of Phytomonas culture exactly as described before (8). Briefly, a method was used involving hydrophobic interaction chromatography on phenyl-Sepharose at pH 7.8 followed by ion-exchange chromatography on CM-Sepharose and affinity chromatography on blue-Sepharose at pH 6.8. Recombinant enzymes were purified from 1 liter of E. coli cells. Cells were resuspended in buffer A (Tris-HCl 25 mM, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide). DNase I (1 mg/ml) and MgCl₂ (10 mM) were added immediately before the preparation of extracts using a French press. Cell debris was removed by centrifugation (10,000 × g, 10 min). Ammonium sulfate and Triton X-100 were added to the supernatant to a final concentration of 1 M and 0.05%, respectively, and the supernatant was clarified by centrifugation at 40,000 × g and loaded onto a phenyl-Sepharose column (1.4 × 25 cm) equilibrated in 1 M ammonium sulfate in buffer A. The column was washed with 50 ml of the same buffer and eluted with a decreasing ammonium sulfate gradient (1 M–50 mM, 300 ml), followed by a wash with 50 ml of 50 mM ammonium sulfate and a gradient of ethylene glycol from 0 to 70% in buffer A (300 ml). Fractions of 7 ml were collected, and those containing activity were pooled and loaded onto a blue-Sepharose column (1 × 7 ml) equilibrated with 200 mM NaCl in buffer A. The column was washed with the same buffer followed by a 60-ml gradient of NaCl (0.2–1 M).
Protein Determination—Protein concentration was determined by the method of Bradford (12) using bovine serum albumin as a standard. SDS-PAGE—Analytical SDS-PAGE was performed in 10% (w/v) polyacrylamide gels according to Chua (13). Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

Partial Amino Acid Sequence Determination—The glycosomal MDH was purified by SDS-PAGE. Bands (corresponding to about 20 μg of protein) were cut from the gel, concentrated by the agarose-based gel concentration system, and digested with trypsin (14). Peptides were separated by reversed-phase HPLC (14), and selected peptides were sequenced on a 477A protein sequencer (Applied Biosystems, Foster City, CA).

Screening of a Phytomonas Genomic Library—A genomic library made in E. coli MB406 using the phage vector λGEM-11 (Promega) was screened by hybridization with a T. brucei glycosomal MDH probe (7) labeled by nick translation. Positive recombinant λGEM-11 phages were purified and screened. High titer phage lysates were prepared, and DNA was purified from phages as described (15).

DNA Sequence Determination—Restriction fragments from the recombinant phages were subcloned into the phagemid vectors pBluescript SIISK (+/-) and pBluescript SIISK (+/-) from Stratagene. E. coli strain XL-1-blue was used for propagation of the recombinant phagemids. Both strands were sequenced using the T7 DNA polymerase kit from Amersham Pharmacia Biotech and specific primers.

Expression in E. coli—2-Hydroxyacid dehydrogenase genes were amplified by PCR from phage DNA using the oligonucleotide U1 (5’-GGAATTCCTATGCGCCATGTGGTGTGTT-3’) which contains a 5’ Ndel artificial restriction site with 18 nucleotides that correspond to the 5’ end of the gene, and D1 (5’-CCGGGCTTCCAGCCCTAGAGCCGCA-3’) which contains 20 nucleotides complementary to the 3’ end including the TAA stop codon and an artificial BamHI restriction site. The amplified DNA fragments were cloned in the pT7-7.1 expression vector (16) and digested with Ndel and BamHI. The sequences were verified before introduction into E. coli BL-21 containing the plasmid pLysS. The transformed cells were grown at 30 °C in M9 minimal medium supplemented with 1% glucose, 100 μg/ml ampicillin, and 25 μg/ml chloramphenicol. Expression was induced at 25 °C.

Determination of the 2-Hydroxyacid Dehydrogenase Gene Copy Number—Southern blots were carried out on Hybond-N membranes with 4 μg of total DNA from Phytomonas sp. digested with different restriction enzymes. To determine the copy number of genes, the complete gene was amplified by PCR using the U1 and D1 oligonucleotides, radioactively labeled, and used as probes. To estimate the relative number of gMDH and gHADH genes, a probe was obtained by PCR amplification of a noncoding region downstream of the gene (from base pairs 1472 to 2030; see Fig. 1) using the oligonucleotides U6 (5’-CTTTATAATGCCCTTGTA-3’) and D3 (5’-TCTGGCAGAATGCTGTCG-3’). All experiments were repeated three times. Using the autoradiograph as a template, the radioactive bands were cut from the nylon membrane and counted for radioactivity.

Kinetic Characterization—Dehydrogenases were assayed spectrophotometrically by following the 2-oxoacid-dependent oxidation of NADH or the 2-hydroxyacid-dependent reduction of NAD+ at 340 nm and 25 °C. Optimal pH and buffers for forward and reverse reactions were as described previously (8). One unit of activity is the amount of enzyme catalyzing the conversion of 1 μmol of substrate/min at 25 °C.

Phylogenetic Analyses—All available glycosomal MDH protein sequences, as well as the E. coli MDH, which served as an outgroup, were aligned using the program ClustalX (17). Positions with gaps were removed, and pairwise distances were calculated using a correction for multiple hits and back mutations according to Kimura (18) as implemented in the Protst program (19). Phylogenetic analyses were carried out using the programs Puzzles 4.01 (20) (maximum likelihood), Protst together with Neighbor (distance matrix analysis), and Protpars (maximum parsimony) (19). All methods used gave the same tree topology. Also, using the program Puzzle, the possibility of the functioning of a constant molecular clock was analyzed.

RESULTS

Partial Amino Acid Sequence Determination of Purified Glycosomal 2-Hydroxyacid Dehydrogenases—Glycosomal MDH was purified from Phytomonas cells as described (8). Following gel purification, concentration in agarose, and trypsin digestion, peptides were separated by reversed-phase HPLC. Five peptides were chosen for Edman sequencing and were found back in the gMDH sequence (Fig. 2A). In a primary search of the GenBankTXT, peptides 1, 3, and 4 showed high similarity with other MDHs.

Isolation and Sequence of Glycosomal 2-Hydroxyacid Dehydrogenase Clones—All attempts to amplify specific fragments from genomic Phytomonas DNA by PCR using degenerate oligonucleotides based on the gMDH peptide sequences were unsuccessful. Therefore, we decided to clone the gene by making use of a heterologous probe. A 2.4-kb fragment containing the T. brucei glycosomal MDH cDNA (7) was radiolabeled and used to screen a genomic library from Phytomonas. We screened 1.5 × 105 plaques resulting in the isolation of 100 positive clones. Eight of 10 selected clones presented a similar restriction pattern with HindIII and PstI and, upon hybridization with the probe, their 0.5- and 1.3-kb HindIII and 1.0-kb PstI fragments were recognized. Subsequent subcloning and sequencing of these fragments from phase 2 revealed an uninterrupted open reading frame (ORF) of 966 nucleotides, containing a deduced sequence of 322 amino acids (Fig. 2A) with 70% positional identity with T. brucei glycosomal MDH (7) and a calculated molecular weight of 33,728. Three of five peptides matched 100% with this sequence (Fig. 2A). The putative ATG start codon of this ORF was found at position 443 of the nucleotide sequence. The TAAA terminator codon was found at position 1409, immediately after three codons encoding a potential carboxyl-terminal peroxisomal targeting signal type 1 (PTS-1, SKL, 21).

PCR amplification was carried out on DNA from each of the eight phage clones and genomic DNA using an upstream oligonucleotide (U1) designed on the first 18 bases of the ORF present in phase 2 and a downstream degenerate (5’-TADGTYTACCNCNCTRCTXCA) oligonucleotide (Deg) complementary to the deduced DNA sequence from peptide 3 (Figs. 1 and 2A). Only genomic DNA and phases 1, 3, and 5 allowed the amplification of a 670-bp DNA fragment. Subcloning and sequencing of the HindIII and PstI fragments from phase 3 revealed an identity of 96.3% at the DNA level with phase 2 and the presence of an ORF of 966 nucleotides. The deduced amino acid sequence was 90.4% identical to that encoded in phase 2 and also presented a PTS-1 with SKL.

Overexpression in E. coli and Purification of Recombinant Enzymes—The dehydrogenases derived from clones 2 and 3 were cloned into the pT7-7.1 vector and expressed in E. coli. The overexpression of recombinant enzyme was possible only when the cells were grown in minimal medium plus glucose and after induction with isopropyl-β-D-thiogalactosamine at 30 °C. Under these conditions, the expression of endogenous bacterial MDH was reduced (22) and crude extracts from E. coli transformed with the clone 3 gene exhibited activity only with
oxaloacetate as substrate, indicative of an authentic MDH. However, when the cells were transformed with the gene derived from clone 2, extracts showed a high activity with other 2-oxoacids such as phenylpyruvate, 2-oxocaproate, or 2-oxoisocaproate, whereas the activity with oxaloacetate was very low and comparable with that found for nontransformed *E. coli*. Therefore, we conclude that this gene codes for the *Phytomonas* gHADH. Hydrophobic interaction chromatography of crude extracts from transformed *E. coli* on phenyl-Sepharose chromatography yielded two peaks of dehydrogenase activity. One peak eluted with 50 mM ammonium sulfate and was specific for oxaloacetate as substrate, coinciding with the endogenous *E. coli* MDH. The second peak of activity eluted in a gradient of ethylene glycol and was specific for oxaloacetate as substrate when transformation was carried out with the gMDH gene and specific for the other 2-oxoacids when the gHADH gene was used for the transformation. This peak was absent in crude extracts of nontransformed *E. coli*.

The second peak of activity was purified by blue-Sepharose affinity chromatography for further characterization. In both cases, a protein with an apparent molecular weight of 35,000 was obtained on SDS-PAGE.

**Characterization of Recombinant 2-Hydroxyacid Dehydrogenases**—Kinetic parameters were determined with recombinant enzymes purified from *E. coli* transformed with *Phytomonas* gHADH and gMDH genes. gMDH was highly specific for oxaloacetate (\(K_m = 117 \mu M, V_{max} = 163 \text{ units/mg of protein}\)) in the reductive reaction and malate (\(K_m = 1.93 \text{ mM}, V_{max} = 42 \text{ units/mg of protein}\)) in the oxidative reaction. gHADH did not show activity with either of these substrates but was able to reduce a series of other 2-oxoacids such as phenylpyruvate (\(K_m = 185 \mu M, V_{max} = 147 \text{ units/mg of protein}\)), 2-oxoisocaproate (\(K_m = 875 \mu M, V_{max} = 140 \text{ units/mg of protein}\)), 2-oxocaproate, 2-oxovalerate, 2-oxobutyrate, 2-oxo-4-methylbutyrate, and pyruvate.

**Determination of the Gene Copy Number of the 2-Hydroxyacid Dehydrogenase Family**—Total genomic DNA from *Phytomonas* sp. was digested with restriction enzymes, submitted to Southern blot analysis, and hybridized with a probe obtained by PCR amplification with primers U1 and D1 on total DNA. This probe represented a mixture of full-length 2-hydroxyacid dehydrogenase genes. Fig. 3A shows the result of using enzymes (BamHI, KpnI, NdeI, XbaI, and XhoI) for which no recognition sites were found in the sequenced fragments. Of these, only NdeI and KpnI showed a pattern of bands that could be because of a polymorphic distribution of repeated gHADH and gMDH genes in the genome of *Phytomonas* sp. The experiment was repeated three times to rule out the possibility of partial digestion. The relative intensity of each band for NdeI was 1:1:1:3:6:1:15 and for KpnI, 1:27. From this ratio we estimated the total copy number of the genes to be 28.

As shown in Fig. 1, the gMDH gene has a SacI restriction site at position 1334 that is absent in gHADH. We used this difference to estimate the relative copy number of each type of gene.
Fig. 3. Southern blot analysis. Phytomonas total DNA (4 μg) was digested with (by lane): 1, BamHI; 2, KpnI; 3, NdeI; 4, XbaI; 5, XhoI; 6, HindIII and 7, HindIII and SacI. After electrophoresis on an 0.8% agarose gel followed by Southern blotting, membranes were hybridized with radiolabeled probes consisting of: A, a 1-kb fragment obtained by PCR on genomic DNA using the oligonucleotides U1 and D1; and B, an 0.55-kb fragment obtained by PCR using the oligonucleotides U6 and D3. In panel B, A1 and A2 represent the 1305-bp HindIII restriction fragment; B1 indicates the 837-bp HindIII-SacI restriction fragment.

Fig. 3B shows a Southern blot experiment in which a HindIII-SacI double digestion is compared with a single HindIII digestion of genomic DNA hybridized with a probe that represents the noncoding region downstream of the gene (between U6 and D3 in Fig. 1). The remaining radioactivity in band A1 (the 1305-bp HindIII fragment) corresponded to 29% of band A2 or 23% of the total radioactivity present in band A1 plus B1 (the 837-bp HindIII-SacI fragment, Fig. 3B), indicating that one-quarter (6–7 of 28 copies) of the genes are gHADH.

Phylogenetic Relationship between Glycosomal MDH in Trypanosomatids—A BLAST search using Phytomonas gMDH showed maximum positional identity with Leishmania major (74%), T. brucei (72%), and T. cruzi (70%) glycosomal MDHs.2 The next most similar sequence corresponded to E. coli MDH, which was used as an outgroup for a phylogenetic analysis. As shown in Fig. 4, the resulting tree is in good agreement with our previous data based on another glycosomal enzyme, glyceraldehyde-phosphate dehydrogenase (23), and with data presented by others using ribosomal RNA sequences (24). The data also indicate that Phytomonas, despite the fact that its metabolism resembles that of the bloodstream form of the African trypanosome (9, 10), is more related to Leishmania on the basis of the gMDH comparison.

DISCUSSION

In a previous work, we detected two MDH isoforms in Phytomonas sp., a mitochondrial isoenzyme with a high specificity for oxaloacetate and a glycosomal isozyme that could act on a broad range of substrates (8). As shown here, the low specificity of the latter enzyme may be attributed to the presence of a family of glycosomal 2-hydroxyacid dehydrogenase genes. Two of these genes were cloned, sequenced, and overexpressed in E. coli. Both enzymes have 322 amino acids and have 90.4% positional identity. They have similar hydrophobicity profiles and net charges. However, the kinetic properties of these recombinant enzymes are strikingly different. gMDH behaves as a real MDH with a high specific activity for oxaloacetate. In contrast, the overexpressed enzyme from clone 2 (gHADH) exhibits no activity toward oxaloacetate but is able to reduce other oxoacids such as phenyl pyruvate (K_m = 185 μM) and 2-oxoisocaproate (K_m = 875 μM), in good agreement with our previous results (8).

The copurification of both activities from glycosomes of Phytomonas as a homogeneous protein as reported in our previous paper (8) could be because of the formation of heterodimers between both types of polypeptides or because of the highly similar physicochemical properties of both homodimers. Our results could not distinguish between these possibilities. Coexpression experiments with one of the genes containing tagged sequences (as polyhistidine tails) are in progress.

As shown before, the specific activity of the dehydrogenases in crude extracts of Phytomonas with phenylpyruvate as a substrate is one order of magnitude lower than with oxaloacetate (8). However, we estimate here that from the approximately 28 copies of dehydrogenase genes, there must be three

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2 Following is a list of MDH sequences used in this paper with their respective GenBank™ accession numbers: Leishmania major, emb AL117268.1 LMF1A520; Trypanosoma brucei, gb AF079110.1; Trypanosoma cruzi, gb AF051893.1; Escherichia coli K-12 chromo, gb U18997.1; E. coli K-12, gb AE000463.1.
gMDH genes for each gHADH gene, indicating that other types of regulation than gene dosage should be operational in Phytomonas to explain the observed level of relative expression. However, it cannot be excluded that part of the gHADHs represent pseudogenes, or genes of which the products have a lower specific activity than that of the one we describe in this paper.

Both genes present a high degree of identity at the DNA level (94%), and this identity increases to 96%, also taking into account the extragenic sequence. This high identity is in agreement with the low degree of restriction enzyme polymorphism shown in Fig. 3. The presence of this family of HADH genes is probably the result of multiple events of duplication of an ancestral MDH gene, followed by the accumulation of mutations. Why all these genes remained and what could be the respective functions of the isoenzymes in the glycosome is still a matter of speculation. This gene family appears to be a typical characteristic of Phytomonas sp., since we have found evidence for it in 15 of 20 isolates analyzed (25). The various members of this gene family could be involved in the utilization of different amino acids from the insect gut or from plant fluids, thus allowing for the regeneration of glycosomal NAD⁺. Indeed, we have detected leucin-, alanine-, aspartate- and methionine-pyruvate aminotransferases in the cytosolic fractions of Phytomonas sp. (data not shown), the oxo-acids of which may all serve as substrates for the gHADH.

Phylogenetic analysis (Fig. 4), using all known glycosomal sequences together with E. coli MDH as an outgroup, shows that gene duplication leading to the appearance of the Phytomonas-specific gHADH must have been a relatively recent event. Assuming the functioning of a constant molecular clock, and using a value of 100 million years as the supposed time of divergence between salivarian and stercorarian trypanosomes (24, 26), it can be estimated that this event must have taken place approximately 45 million years ago. This is very recent indeed and suggests that a metabolic adaptation of Phytomonas, perhaps in response to the appearance of a new plant or insect host, must have occurred at that time. Separation of Leishmania and Phytomonas took place some 144 million years ago, and the trypanosomes diverged some 185 million years ago, which is in good agreement with previous estimations (24, 26).

The high gene copy number of gMDH parallels the high specific activity of this enzyme in Phytomonas, which represents 80% (approximately 2.6 units/mg of protein) of the total MDH activity present in this organism (8). This high activity relates to its important function in the regeneration of glycosomal NAD⁺, thus maintaining the oxidative power of the organelle required for the continuation of glycolysis. In Phytomonas, the latter pathway is essential because the citric acid cycle is absent. The situation is different in T. cruzi, where the mitochondrion is fully functional and the trypanosome utilizes predominantly amino acids as an energy source. Reynoso-Hunter et al. (5) reported that the glycosomal MDH isoenzyme represents only 25% of the total MDH activity, which amounts to 3 units/mg of protein, similar to that present in the Phytomonas organelle. The higher total specific activity of MDH in T. cruzi (12 units/mg of protein) is due to the high activity of the mitochondrial isozyme, in agreement with the full functionality of this organelle and its involvement in the citric acid cycle.

Only 31 of 322 amino acids differ between both gene products. Arginine 89 of gMDH is changed to isoleucine in gHADH. Both residues are in an amino-terminal region believed to form a mobile loop at the top of the active site pocket. This Arg-102, in the numbering of the residues of 2-hydroxyacid dehydrogenase (27), is conserved in all known MDHs and forms a hydrogen bond with the second carboxyl group of oxaloacetate (28, 29). The smaller isoleucine could result in an enlargement of the active site pocket to accommodate bigger substrates, as is the case for gHADH. Moreover, this mutation reduces the net positive charge of the active site pocket and its water solvation. As shown in Fig. 2A, the loop region appears to be a hot spot for mutation including, in addition, the replacement of valine 85 to glycine, alanine 88 to proline, and lysine 90 to methionine. All of these changes go in the same direction of reducing the net positive charge and increasing the volume of the active site pocket. The other residues known to be essential for dehydrogenase activity (Arg-95, Asp-158, Arg-161, and His-184) are all conserved in both proteins (29). The fact that only a limited number of amino acid replacements is involved would facilitate the identification of residues that are involved in determining the substrate specificity. Experiments to identify such amino acids are in progress.

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