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Probing the specificity of a trypanosomal aromatic α-hydroxy acid dehydrogenase by site-directed mutagenesis☆

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Abstract

The aromatic L-α-hydroxy acid dehydrogenase (AHDAH) from *Trypanosoma cruzi* has over 50% sequence identity with cytosolic malate dehydrogenases (cMDHs), yet it is unable to reduce oxaloacetate. Molecular modeling of the three-dimensional structure of AHADH using the pig cMDH as template directed the construction of several mutants. AHADH shares with MDHs the essential catalytic residues H195 and R171 (using Eventoff's numbering). The AHADH A102R mutant became able to reduce oxaloacetate, while remaining fully active towards aromatic α-oxoacids. The Y237G mutant diminished its affinity for all of the natural substrates, whereas the double mutant A102R/Y237G was more active than Y237G and had similar activity with oxaloacetate and with aromatic substrates. The present results reinforce our proposal that AHADH arose by a moderate number of point mutations from a cMDH no longer present in the parasite.

Keywords: *Trypanosoma cruzi*; Aromatic amino acid metabolism; Malate dehydrogenase; Aromatic L-α-hydroxy acid dehydrogenase

Pyridine nucleotide-specific α-hydroxy acid dehydrogenases acting on lactate (LDH) and malate (MDH) represent a ubiquitously distributed enzyme superfamily. The mechanism of action of these enzymes is identical. The low sequence similarities among the enzymes in this superfamily, their highly conserved 3-D structures, and phylogenetic analysis indicate that the LDH and MDH enzyme families diverged relatively early in evolution [1,2]. The MDH lineage forms two major groups, a group containing bacterial, eukaryotic cytosolic, and chloroplast enzymes (a subfamily designated cMDH in this paper), and a second group comprising bacterial, eukaryotic mitochondrial, and glyoxysomal enzymes (designated here mMDH) [1].

Although most members of the MDH family show high substrate specificity toward dicarboxylic substrates, recent studies on diverse unicellular eukaryotes revealed the existence of enzymes that belong to this family but are specific for monocarboxylic and not for dicarboxylic substrates. The parasitic kinetoplastid *Trypanosoma cruzi*, the agent causing Chagas disease, contains an aromatic L-α-hydroxy acid dehydrogenase (AHADH) and no cMDH [3]. AHDAH is specific for phenyllacetate and related compounds with no activity on either lactate or malate [3]. The amino acid sequence of this enzyme shows nearly 55% overall identity with several cMDHs; thus, it belongs to the cMDH subfamily [4]. Its closest known homolog is a typical cMDH in *T. brucei* [5] indicating that these two enzymes derive from a common ancestral molecule and that the change of specificity occurred in a kinetoplastid relatively recently. In another kinetoplastid, *Phytomonas* sp., an enzyme

☆ *Abbreviations:* AHADH, aromatic L-α-hydroxy acid dehydrogenase; MDH, malate dehydrogenase (cMDH and mMDH indicate cytosolic-type and mitochondrial MDH isozymes, respectively); LDH, lactate dehydrogenase.

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with almost identical substrate specificity was detected but its amino acid sequence placed it into the mMDH subfamily, again with the closest relatives being kinetoplastid mMDHs [6]. The amitochondriate parabasalid protist *Trichomonas vaginalis* and some other parabasalids harbor an enzyme with typical LDH activity [7,8], which belongs to the cMDH group [9]. These results indicate that a switch in substrate specificity from dicarboxylates to monocarboxylates has occurred more than once in the evolution of the MDH/LDH protein superfamily in eukaryotes. However, most of the available 3-D structures correspond to typical LDHs and MDHs thus the molecular mechanism responsible for aromatic monocarboxylic substrate specificity is not clearly understood yet.

AHADH in *T. cruzi* is responsible, together with tyrosine aminotransferase (TAT), for the catabolism of aromatic amino acids, which leads to the excretion of aromatic lactate derivatives into the culture medium [10]. This enzyme is a highly active cytosolic NAD-linked dehydrogenase, which reduces the aromatic α-oxo acids arising from transamination of aromatic amino acids to the corresponding L-α-hydroxy acids [3]. The *T. cruzi* AHADH is the first enzyme with such substrate stereospecificity to be purified to homogeneity [3], and fully sequenced [4].

Three ariginine residues, Arg-102, Arg-171, Arg-109; as well as His-195, and Asp-168 (residue numbering as proposed for *Escherichia coli* lactate dehydrogenase [11]) are known to be essential for substrate binding and catalysis in all MDHs (for a review see [1]). The charged guanidinium groups of Arg-102 and Arg-171 contribute to the binding and correct orientation of the substrates in the MDHs active site [1]. The similarity of *T. cruzi* AHADH to MDHs is most visible among the residues directly responsible for the catalytic mechanism, such as the pair His-195/Asp-168 and Arg-109, as well as the arginine residue involved in binding the C1 carboxylate of the substrate, Arg-171. At variance, an Ala residue in the *T. cruzi* AHADH [4] replaces the Arg-102 residue present in all MDHs (Fig. 1). MDHs and LDHs, which are specific for their respective substrates, differ in their highly similar active sites at only one critical residue in position 102: in most LDHs this is an uncharged Gln while in MDHs is a positively charged Arg, which forms a double ionic bond to the additional carboxyl group of oxaloacetate/malate [12–14]. Determination of three-dimensional structures for members of the LDHs/MDHs family, as well as site directed mutagenesis studies, showed that the amino acid residue at this position is of critical importance for conferring MDH or LDH substrate specificity [13–17]. Other residues are also believed to be responsible for the substrate specificity of these enzymes [2]. The Gln/Arg102 is part of a mobile loop that closes over the active site vacuole after the enzyme:coenzyme:substrate ternary complex has formed, isolating the substrates, as well as the catalytically important residues, from the solvent [1]. A shift in specificity from pyruvate to oxaloacetate was easily achieved in several eubacteria by introducing a mutation into the *ldh* gene, changing Gln-102 to Arg [18–20]. On the contrary, attempts to engineer MDHs into LDHs were more successful in some organisms [21] than in others [22], since the mutation R102Q changes the enzyme specificity for pyruvate but with lowered catalytic efficiencies. The best results were achieved with the MDH from *Haloarcula marismortui* [21]. Although most of the α-hydroxy acid dehydrogenases are highly specific for their substrates, lactate or oxaloacetate, a few less specific enzymes have been described in diverse prokaryotes such as *Mycoplasma genitalium* [23] and *Methanococcus jannaschii* [24].

We report here the molecular modeling of *T. cruzi* AHADH based on X-ray structures of cMDHs and the site-directed mutagenesis of those residues, which according to the model, seem to be involved in catalytic

		*			*			*		*			*		*			*		
Tc_AHADH	101	**P**	**A**	**K**	**P**	**G**	**T**	**L**	**R**	**R**	**D**	167	**L**	**D**	**H**	**N**	**R**	193	**G**	**N**	**H**	**S**	235	**R**	**G**	**Y**	**E**	**I**	**I**	**R**
Tb_cMDH		**P**	R	**K**	**P**	**G**	M	E	**R**	**R**	**D**		**L**	**D**	**H**	**N**	**R**		**G**	**N**	**H**	**S**		**R**	**G**	A	**E**	**I**	M	K
Mc_cMDH		**P**	R	**K**	E	**G**	M	E	**R**	K	**D**		**L**	**D**	**H**	**N**	**R**		**G**	**N**	**H**	**S**		**R**	**G**	A	A	**I**	**I**	K
Tf_MDH		**P**	R	**K**	A	**G**	M	E	**R**	**R**	**D**		**L**	**D**	**H**	**N**	**R**		**G**	**N**	**H**	**S**		**R**	**G**	A	A	**I**	**I**	Q
Mm_MDH		**P**	R	R	E	**G**	M	E	**R**	K	**D**		**L**	**D**	**H**	**N**	**R**		**G**	**N**	**H**	**S**		**R**	**G**	A	A	V	**I**	K
Tv_MDH		**P**	R	**K**	D	**G**	M	D	**R**	S	**D**		**L**	**D**	**H**	**N**	**R**		**G**	**N**	**H**	**S**		**R**	**G**	G	A	V	**I**	K
Tv_LDH		**P**	L	**K**	**P**	**G**	Q	V	**R**	A	**D**		**L**	**D**	Q	**N**	**R**		**G**	**N**	**H**	G		**R**	A	W	**D**	**I**	L	E
Ph_HADH		**P**	I	M	**P**	**G**	M	K	**R**	**D**	**D**		**L**	**D**	S	A	**R**		**G**	G	**H**	**S**		A	A	**Y**	**E**	V	**I**	D
Rn_LDH		R	Q	Q	E	**G**	E	S	**R**	L	N		**L**	**D**	I	I	**R**		**G**	E	**H**	G		S	A	**Y**	**E**	V	**I**	K
Ec_MDH		A	R	**K**	**P**	**G**	M	D	**R**	S	**D**		**L**	**D**	I	I	**R**		**G**	G	**H**	**S**		A	**G**	T	**E**	V	V	E
Tb_gMDH		**P**	R	**K**	**P**	**G**	M	S	**R**	**D**	**D**		**L**	**D**	G	L	**R**		**G**	G	**H**	**S**		A	**G**	T	**E**	V	V	K
Tb_mMDH		**P**	R	**K**	**P**	**G**	M	T	**R**	**D**	**D**		**L**	**D**	G	L	**R**		**G**	G	**H**	**S**		A	**G**	T	**E**	V	V	K

Fig. 1. Comparison of the amino acid sequence of the *T. cruzi* AHADH with those of selected MDHs and LDHs. Partial sequences of the MDHs from *T. brucei* (cytosolic) (Tb_cMDH, AF287299), (glycosomal) (Tb_gMDH, AF079110); *Mesembryanthenum crystallinum* (cytosolic) (Mc_cMDH, X96539); *Thermus flavus* (Tf_MDH, J02598); *Mus musculus* (cytosolic) (Mm_cMDH, P14152); *T. vaginalis* (Tv_MDH, U38692); *E. coli* (Ec_MDH, U04742); *T. cruzi* (glycosomal) (Tc_gMDH, AF051893); and of the LDHs from *T. vaginalis* (Tv_LDH, AF060233); *Rattus norvegicus* (Rn_LDH B, U07181) and of the 2-hydroxyacid dehydrogenase from *Phytomonas* sp (glycosomal) (Ph_HADH, AF284096) were selected for comparison. AHADH sequence and residues identical to those in AHADH are in bold type. Ala-102, Arg-109, Asp-168, Arg-171, His-195, Tyr-237, and Ile-239 are indicated by ∗.

mechanism and substrate binding. The results support our proposal [4] that AHADH belongs to the cMDH subfamily, since a single mutation, A102R, broadened the *T. cruzi* AHADH substrate specificity, allowing the enzyme to acquire MDH activity while remaining fully active towards its natural substrates.

Experimental procedures

Molecular modeling. Three-dimensional (3D) models of the *T. cruzi* AHDAH were built by comparative protein structure modeling with the program Modeller-6 [25]. The models calculated in this study passed the tests of PROSAII [26] and PROCHECK [27] programs. The closest template structure found with the SEQUENCE_SEARCH command of Modeller and Psi-Blast [28] was *Sus scrofa* cMDH (PDB 4mdh), which shared more than 50% identity to the target AHADH sequence. The loop in 4mdh (Asp92–Leu100) that forms a considerable part of the active site cleft was not well defined by the crystallographic analysis [13], consistent with a poor PROSAII energy profile for the region. Thus, this loop segment was built by using *E. coli* MDH structure as template [17] (PDB 1emd; residues 76–98), while the remaining parts of the AHADH sequence were built based on 4mdh. *p*-Hydroxyphenylpyruvate from the structure of Macrophage Migration Inhibitory Factor (PDB code 1ca7) was included in the model building to obtain an AHADH model in complex with its physiological substrate. The model also includes the NAD cofactor, inherited from the 4mdh template. The substrate and the cofactor were docked as rigid bodies mimicking the corresponding conformations and positions in the template structures as much as possible. Several distance restraints, in addition to those automatically derived by Modeller, were imposed on the relative orientation of the substrates and the enzymes to incorporate previous knowledge about the substrate–enzyme interactions. The superposition of representative and structurally highly similar structures 1bu8 (MDH) and 3ldh (LDH) revealed the conservation of conformation of residues His-195, Arg-171, Arg-109. Additional upper bound distance restraints were extracted on atoms of the conserved residues and the substrate from 1bu8 and 3ldh. Once the overall model was built, the active site loop was refined by restrained loop modeling, which retained the conformation of the loop but optimized its global orientation to improve local packing [29].

Site-directed mutagenesis. A clone of the AHADH gene (accession number AF112259) has been established in pET24a(+) vector (Novagen) [4]. The Quickchange Site-Directed mutagenesis system (Stratagene) was used to mutate selected codons in the clone of the AHADH gene. The following primers were used: (i) to change Ala-102 to Arg: 5′-CC ATT CTC TGC GGC TCG TTT CCA ***CGT*** AAA CCC GGC ACG CTGCG-3′ and 5′-CG CAG CGT GCC GGG TTT ***ACG*** TGG AAA CGA GCC GCA GAG AAT GG-3′, (ii) to change His-195 to Ala: 5′-C TGT ATT ATT TGG GGA AAC ***GCG*** AGC GGT ACA CAA GTC CC-3′ and 5′-GG GAC TTG TGT ACC GCT ***CGC*** GTT TCC CCA AAT AAT ACA G-3′, (iii) to change Arg-171 to Lys: 5′-CGA CTG GAC CAT AAC ***AAA*** TCG CTT GCG CTT GTG GC-3′ and 5′-GC CAC AAG CGC AAG CGA ***TTT*** GTT ATG GTC CAG TCG-3′, (iv) to change Ile-239 to Val: 5′-CGC GGG TAT GAG ***G***TT ATT CGG TGG C-3′ and 5′-G CCA CCG AAT AA***C*** CTC ATA CCC GCG-3′, and (v) to change Tyr-237 to Gly: 5′-CT GTG CAG CAA CGC GGG ***GG***T GAG ATT ATT CGG TGG C-3′ and 5′-G CCA CCG AAT AAT CTC A***CC*** CCC GCG TTG CTG CAC AG-3′ (bold face italics denote the changed nucleotides). The same approach has been used to obtain further multiple mutations. The mutant plasmids were transformed into *E. coli* (XL1-Blue) and their sequences were verified.

Expression of AHADH. The recombinant pET24a(+) vector was used to transform *E. coli* BL21-CodonPlus. Transformed cells were selected by kanamycin resistance. *E. coli* cells harboring the pET24a(+) plasmid were inoculated into 10 ml of LB broth containing 12.5 μg ml^{-1} tetracycline and 30 μg ml^{-1} kanamycin. Overnight cultures were transferred into 500 ml of the same medium and were grown at 37 °C until an OD value of 0.9 at 600 nm was reached. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 200 μM, and the cultures were further grown at 28 °C for 15 h. Wild-type and mutant enzymes were purified by Ni-nitrilotriacetic acid resin chromatography according to the supplier's instructions (Qiagen, Valencia, CA). The purity of the enzyme preparations was assessed by SDS–PAGE.

Enzyme assays. Apparent kinetic constants in the direction of α-hydroxy acid reduction were determined as previously described [3], by monitoring NADH oxidation at 37 °C and 340 nm, and using a computer program to fit the data to a hyperbola by applying the Gauss–Newton algorithm [30]. Protein content was determined in cell-free extracts by the method of Bradford [31], using bovine serum albumin as standard. All K_m values reported hereafter are apparent K_m values.

Results

Molecular modeling

Three-dimensional molecular modeling helped to define the active site environment of *T. cruzi* AHADH (Fig. 2) and to compare it to cMDH. Several insights into the reasons for the differences in substrate specificity of these two highly similar enzymes could be obtained by comparing the active site environments. The most notable difference in the active site was the switch from Arg-102 in MDHs to Ala-102 in AHADH. This change is explained by the role of Arg-102 that coordinates the C4 carboxyl group of the malate substrate in MDHs. A further significant difference was the change from Gly-237 in most MDHs to the more bulky Tyr-237 in AHADH, explored further in Discussion.

Expression constructs

Based on the above mentioned differences and their presence in the close environment of the active site pocket (Fig. 2) the following singly, doubly, and triply mutated constructs were developed from the *T. cruzi* AHADH gene: A102R, H195A, R171K, I239V, Y237G, A102R/I239V, A102R/Y237G, Y237G/I239V, and A102 R/Y237G/I239V. Sequencing these constructs verified the replacements.

Expression and biochemical characterization

Total extracts of induced bacteria that carried the various constructs contained highly similar amounts of the corresponding heterologous proteins. The histidine-tagged recombinant enzymes were purified by nickel–agarose affinity chromatography. The wild-type protein, as well as each of the purified mutant enzymes, corresponded to a single band on SDS/PAGE, with an

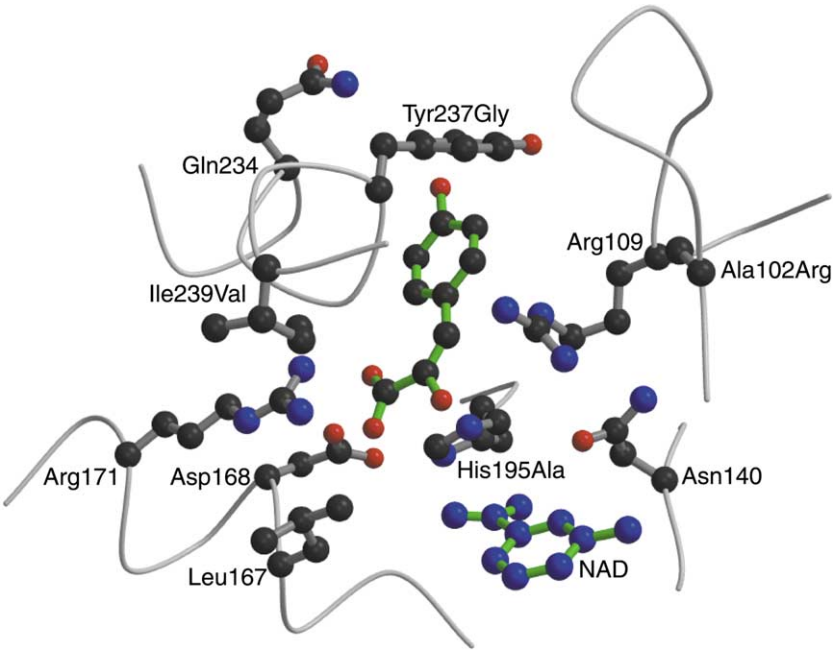

Fig. 2. The model of the active site pocket of AHADH. The following residues are plotted: (i) residues previously shown to be important for catalysis, (ii) residues that are within 6 Å of any of the substrate atoms and are different between AHADH (*T. cruzi*), LDH, and MDH (*T. vaginalis*). The labels for the four residues from the latter set that were mutated indicate the mutants explored. The *p*-hydroxyphenylpyruvate substrate is in green, and a fraction of the NAD cofactor (in green) is also included in the lower left corner.

apparent molecular mass of 35 kDa, which were recognized in Western blots by anti-AHADH polyclonal antiserum (not shown). This was the case not only for the active mutants, but also for the inactive enzymes (R171K and H195A).

Five single point mutants for the *T. cruzi* AHADH were obtained and characterized, in order to address the functionality of these residues in the parasite enzyme: H195A, R171K, A102R, Y237G, and I239V (Table 1). The first two mutations yielded inactive enzymes, showing the critical role of both residues in AHADH catalytic mechanism and substrate binding. On the other hand, the exchange of Ala-102 to Arg gave *T. cruzi* AHADH a new substrate specificity, now able to reduce oxaloacetate, with a K_m value of 99 μM. This mutation also affected the enzyme affinity for α-ketoisocaproate and phenylpyruvate as it is reflected in the nearly 7- and 5-fold decrease in the K_m values for both substrates, respectively, while its ability to reduce *p*-hydroxyphenylpyruvate improved, with a 50% increase in the k_{cat}/K_m ratio (Table 1).

The removal of the bulky side chain of Tyr in the AHADH Y237G mutant increases the K_m values for *p*-hydroxyphenylpyruvate, phenylpyruvate, and α-ketoisocaproate, respectively, which became 4-, 25-, and 29-fold higher than those for the wild type enzyme (Table 1). The double mutant A102R/Y237G, however, not only presented the highest MDH activity (as shown by a k_{cat}/K_m ratio of 8000), but in addition exhibited remarkably lower K_m values for reduction of the AHADH natural substrates, when compared with the values obtained for AHADH-Y237G (Table 1); moreover, the k_{cat}/K_m ratio for the substrate *p*-hydroxyphenylpyruvate, 9700, was again about 50% higher than that shown by the wild-type enzyme.

The AHADH I239V mutant, when compared with the wild type enzyme, presented a nearly 3-fold increase in the K_m values for the natural substrates (Table 1). The double mutant A102R/I239V, on the other hand, presented K_m values for the natural substrates of AHADH actually lower than those of the wild-type enzyme, and had the lowest MDH activity. The triple mutant A102R/Y237G/I239V, when compared with the double mutant A102R/Y237G, showed an increase in the K_m values (nearly 5-fold) for the natural substrates, resulting in the lowest k_{cat}/K_m ratios, and also for oxaloacetate (Table 1).

Discussion

The substrate specificity and enzymatic efficiency of *T. cruzi* AHADH can be modulated easily by mutating one or few amino acid residues, similarly to results obtained for LDHs and MDHs from other prokaryotic and eukaryotic microorganisms [9,18–22]. The most dramatic change is elicited by changing the uncharged Ala-102 to arginine. The introduction of the additional positive charge into the substrate binding pocket without any further changes in the active site environment confers a significant activity on the dicarboxylate

Table 1
Apparent kinetic constants in the direction of α-oxoacid reduction of wild type and mutated *T. cruzi* AHADH

Substrate	Kinetic parameters	Wild type	I239V	Y237G	I239V Y237G	A102R	A102R I239V	A102R Y237G	A102R I239V Y237G
p-OH-phenyl pyruvate	k_{cat} (s^{-1})	447 ± 21	399 ± 22	669 ± 16	513 ± 39	190 ± 4	182 ± 6	565 ± 33	210 ± 9
	K_m (mM)	0.069 ± 0.010	0.188 ± 0.025	0.287 ± 0.019	0.261 ± 0.026	0.019 ± 0.002	0.041 ± 0.005	0.058 ± 0.006	0.300 ± 0.033
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	6473	2120	2330	1967	10,002	4437	9734	701
Phenyl pyruvate	k_{cat} (s^{-1})	352 ± 17	421 ± 10	488 ± 32	442 ± 27	34 ± 2	46 ± 2	567 ± 53	164 ± 4
	K_m (mM)	0.045 ± 0.007	0.120 ± 0.008	1.140 ± 0.188	1.347 ± 0.194	0.008 ± 0.001	0.024 ± 0.003	0.197 ± 0.040	0.915 ± 0.062
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	7812	3507	428	328	4243	1928	2878	179
α-Ketoisocaproate	k_{cat} (s^{-1})	236 ± 8	190 ± 7	47 ± 4	40 ± 1	156 ± 4	107 ± 4	95 ± 2	32 ± 1
	K_m (mM)	0.330 ± 0.035	0.682 ± 0.060	9.548 ± 1.233	10.524 ± 0.707	0.048 ± 0.005	0.159 ± 0.022	2.289 ± 0.135	11.914 ± 1.373
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	714	279	5	4	3252	672	42	3
Oxaloacetate	k_{cat} (s^{-1})	[a]	[a]	[a]	[a]	88 ± 4	112 ± 5	376 ± 18	125 ± 4
	K_m (mM)	[a]	[a]	[a]	[a]	0.098 ± 0.009	0.261 ± 0.028	0.047 ± 0.007	0.181 ± 0.016
	k_{cat}/K_m ($s^{-1}mM^{-1}$)	–	–	–	–	900	428	8010	689

The kinetic constants are the means of five determinations ±SE and were obtained as described in Experimental procedures.
[a] No activity detected.

substrate, oxaloacetate. In essence, the enzyme gained MDH activity without loosing its AHADH specificity. The ease with which dicarboxylate specificity can be conferred to diverse α-hydroxy acid dehydrogenases is well documented for typical LDH molecules [18–20] and also for the LDH of the eukaryote *T. vaginalis*, which is a cMDH homolog [9].

Although the major difference between dicarboxylate specific and monocarboxylate specific α-hydroxy acid dehydrogenases is the presence or absence of the charged arginine at position 102 [14], replacement of this residue with uncharged ones was rarely successful in experimentally converting various MDHs to LDHs [23]. In most organisms such conversions probably will require a more extensive remodeling of the MDH molecule. A recent report of changing an MDH into an enzyme acting on phenyllactate shows the direction for such remodeling [32].

Interestingly, the substitution of Arg-102 for Ala-102 not only broadened the substrate specificity of *T. cruzi* AHADH but it even improved the enzyme affinity for its natural substrates. There are not many straightforward explanations for the need of a charged guanidinium group at the position 102 to enhance the binding of the natural substrates with non-polar and bulky side groups. However, it is tempting to speculate that this substitution places the highly conserved external loop in a better position to perform its crucial role in closing the active site and isolating the already formed ternary complex from the solvent. This and the overall high sequence similarity of the *T. cruzi* enzyme with MDHs from different organisms support our proposal that AHADH arose by a moderate number of point mutations from a cytosolic MDH no longer found in this parasite [4].

In addition to the critical Ala-102 or Arg-102, *T. cruzi* AHADH shares with MDHs and LDHs the invariant Arg-109 and Arg-171, as well as the His-195/Asp-168 pair [4]. The latter pair of residues linked by a hydrogen bond, has been shown to form a proton relay system in the active site of α-hydroxy acid dehydrogenases superfamily [33]. This proton relay system was additionally proposed to explain the relatively stronger binding of the reduced coenzyme form versus its oxidized form by cMDHs [33]. The lower K_m values for NADH versus NAD observed in *T. cruzi* AHADH are thus in good agreement with the functionality of this His–Asp pair in this enzyme [4]. Arg-171 has been proposed to act as a counterion for the C1 carboxylate group [1]. The complete lack of enzymatic activity in the AHADH H195A and R171K mutants strongly suggests that the parasite enzyme operates through a similar catalytic mechanism.

The single substitution Y237G in *T. cruzi* AHADH had a detrimental effect on the mutated enzyme affinity for its natural substrates, mainly for phenylpyruvate and

α-keto-isocaproate. The presence at position 237 of a large aromatic residue resembles the LDHs structure, and suggests that *T. cruzi* AHADH could use Tyr-237 to accommodate in the active site, by means of hydrophobic interactions, its less polar natural substrates. Interestingly, both MDH and LDH of *T. vaginalis* are able to reduce phenylpyruvate. This activity is higher for the LDH that has Trp-237 and can be increased 8-fold in the MDH by replacing Gly-237 by Tyr, indicating a special role for the aromatic group in the interaction with phenolic substrates (unpublished results).

The kinetics of the reaction catalyzed by the AHADH I239V mutant showed a less important effect on the enzyme affinity for its natural substrates, although the K_m values were also higher than for the wild-type enzyme. The K_m values for the double mutant Y237G/I239V were similar to those obtained for the Y237G mutant. These results again suggest that Y237 may be more directly involved in binding the natural substrates than I239. Probably due to the absence of Arg-102 in AHADH, the smaller and less hydrophobic residue, Gly-237, usually present in most MDHs, is replaced in the parasite enzyme by this Tyr residue to contribute, by means of non-polar interactions, to the correct localization of its natural substrates in the active site. Double mutants containing also the A102R mutation had significantly lower K_m values for all natural AHADH substrates, in addition to the newly acquired MDH activity, as compared with the I239V and Y237G single mutants. This may suggest that the bulkier Arg residue may compensate for the effect of the latter mutations, by allowing a better interaction with the substrates. The triple mutant, however, had the lowest k_{cat}/K_m ratio for all the AHADH natural substrates, probably meaning that the sum of these mutations induces some conformational change in the active site resulting in a less active enzyme.

The fact that activities on monocarboxylic α-hydroxy acids emerged at least three times in different protist lineages and in each case from a different MDH subgroup (mMDH in *Phytomonas* sp. [6] and separate cMDH lineages in *T. cruzi* [4] and *T. vaginalis* [9]) indicates that on an evolutionary timescale this transition is relatively easy. It remains to be seen whether such functional changes will be observed also in other protist groups. The physiological significance of these changes for the protist lineages where they occurred remains to be determined. A common selective force is probably responsible for the independent emergence of AHADH in different kinetoplastid groups [4,6]. Pyruvate reduction by LDH expectedly contributes to the redox balance of the parabasalid *T. vaginalis* [34], but it does not explain why LDH did not appear in all subgroups of this lineage [35]. At the same time the existence of several closely related MDH/LDH pairs in various groups of unicellular eukaryotes provides a promising area for detailed studies of the evolution and determination of specific substrate specificities.

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