Catalysis by N-Acetyl-D-glucosaminylphosphatidylinositol De-N-acetylase (PIG-L) from Entamoeba histolytica

NEW ROLES FOR CONSERVED RESIDUES

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Background: E. histolytica PIG-L is active even in absence of metal, unlike other homologs. Metal stimulation of activity alters V_max, not K_m. Metal does not alter optimum pH of catalysis. What explains these differences?

Results: Conserved Asp-46 and His-140 participate in a general acid-base pair mechanism, unusual for de-N-acetylases.

Conclusion: PIG-L of amoeba is significantly different from mammalian PIG-L.

Significance: We have identified a probable drug target for selective delivery.

We showed previously that Entamoeba histolytica PIG-L exhibits a novel metal-independent albeit metal-stimulated activity. Using mutational and biochemical analysis, here we identify Asp-46 and His-140 of the enzyme as being important for catalysis. We show that these mutations neither affect the global conformational of the enzyme nor alter its metal binding affinity. The defect in catalysis, due to the mutations, is specifically due to an effect on V_max and not due to altered substrate affinity (or K_m). We propose a general acid-base pair mechanism to explain our results.

The biosynthesis of the glycosylphosphatidylinositol (GPI) anchor is an essential and ubiquitous pathway in eukaryotes. GPI-anchored proteins are known to be involved in infection and virulence of many eukaryotic pathogens, including Trypanosoma, Leishmania, Candida, and Entamoeba (1–4).

The GPI anchor is synthesized in the endoplasmic reticulum in a stepwise process and transferred as a whole onto the C termini of proteins that possess the GPI-anterioring signal sequence (5). The PIG-L enzyme functions at the second step of GPI anchor biosynthesis, converting N-acetylgalactosaminylphosphatidylinositol (GlcNAc-PI) to glucosaminylphosphatidylinositol (GlcN-PI) (5, 6). This step is conserved in the GPI biosynthesis pathway, and deletion mutations of the gene are known to be lethal in all eukaryotes studied so far (7). Besides being essential, it is one of the few steps of the GPI biosynthetic pathway that involves a single enzyme rather than a multiprotein complex and takes place on the cytoplasmic face of the endoplasmic reticulum. The enzyme shows species-specific variations as well and may therefore be an attractive target for pathogen-specific drugs (8).

PIG-L of Entamoeba histolytica (EhPIG-L) has been reported to be important for amoebic pathogenesis (4). In a previous report we showed that unlike rat PIG-L and other known de-N-acetylases, EhPIG-L is actually capable of low activity in the absence of metal, and the catalysis is stimulated by divalent cations (9). We also showed that, unlike other known PIG-L enzymes, EhPIG-L preferred divalent cations such as Mg^{2+}, Mn^{2+}, or Co^{2+} rather than Zn^{2+}. Also unusual was the fact that the enzyme had an optimum pH of 5.5. Interestingly, this pH was not altered by the presence or absence of externally added metal, suggesting that the metal did not play a direct role in catalysis as has been proposed for other deacetylases. We also showed that the role of the metal appeared to be in altering catalytic rates by inducing a catalytically efficient conformation of the enzyme rather than in altering the affinity of the enzyme for its substrate. Thus, metal altered V_max rather than the K_m of the enzyme for its substrate (9).

All in all, it would appear that EhPIG-L has a significantly different catalytic pocket from those described so far. We wondered whether conserved residues within the catalytic pocket may also have taken on new functions in the EhPIG-L enzyme compared with the enzyme from other sources. Therefore, in this report we investigated the role of conserved aspartate and histidine residues in the activity of the protein. As before, we used the cytoplasmic catalytic domain of E. histolytica PIG-L (EhΔTMPiG-L). We show here that residues Asp-46 and His-140 within the putative catalytic pocket are important for the activity of EhΔTMPiG-L and provide a probable model for the catalysis.

EXPERIMENTAL PROCEDURES

Materials—The YPH-501 yeast strain was procured from Institute of Microbial Technology (Chandigrah, India) and DH5α cells from Bangalore Genie. UDP-[6-3H]GlcNAc and acetic anhydride were procured from Sigma, amylose resin from New England Biolabs (NEB), and Factor Xa from Novagen. The restriction enzymes and DNA polymerases were purchased either from Bangalore Genie, MBI Fermentas, or NEB.
All other materials were purchased either from Merck, Qualigens, or Sisco research laboratories.

**Creation of the Site-specific Mutants**—Using primers carrying site-specific mutations (see [supplemental Table 1](#)) we amplified the vector, pMALEhATMPIG-L (pMAL-c2X plasmid bearing EhATMPIG-L, a truncation mutant of full-length EhPIG-L lacking the first 24 N-terminal residues (9)). The PCR product was then digested by DpnI restriction enzyme and used to transform DH5α cells. Colonies obtained after transformation were screened by colony PCR using gene-specific primers. The mutations were further confirmed by DNA sequencing.

**Expression and Purification of MBP-tagged Proteins**—The TB1 strain of *Escherichia coli* was transformed with pMALEhATMPIG-L (which expresses EhATMPIG-L with a MBP tag at the N terminus) or its mutant variants and grown at 37 °C to an A_{600}nm of 0.5–0.6 in Luria-Bertani medium containing 0.3% (w/v) glucose. Protein expression and purification were carried out essentially as described previously (9). Briefly, protein expression was induced with 0.25 mM isopropyl-β-D-galactopyranoside, and the cells were grown at 16 °C for another 16 h. The proteins were affinity-purified from amylose beads and used without removal of the MBP tag for all the enzyme assays. We have previously shown that the MBP tag does not significantly alter the activity of the enzyme (9).

**Assays for GlcNAc-PI De-N-acetylation Activity**—The substrate for the assays was prepared by exogenously providing UDP-[6-3H]N-acetylglucosamine (UDP-[6-3H]GlcNAc) to yeast (YPH-501) microsomes, as described previously (9). This generally results in transfer of [6-3H]GlcNAc from the donor, UDP-[6-3H]GlcNAc, to phosphatidylinositol (PI) by the GPI- N-acetylglucosaminyltransferase enzyme involved in the first step of GPI biosynthesis. Normally a significant amount of this desired substrate is also de-N-acetylated to [6-3H]glucosaminyl-PI ([6-3H]Glc-N-PI) by the endogenously present yeast PIG-L (GPI12) in the microsomes. Therefore, for our assays, the [6-3H]Glc-N-PI generated was reacylated back using acetic anhydride to provide us with sufficient amount of the substrate for the assays (9).

The GlcNAc-PI de-N-acetylation assays were carried out with no modifications to our previously reported protocol (9). In brief, the dried glycolipids containing [6-3H]GlcNAc-PI were resuspended in 20 μl of acetate buffer, pH 5.5, containing 50 mM KCl, 10 mM MgCl₂, 10 mM MnCl₂. For enzyme assays carried out “in the absence of metal,” MgCl₂ and MnCl₂ were left out from the assay mixture. It is possible that the enzyme picks up some amount of metal from the cellular environment. However, as we show in this paper, the enzyme and its mutants continue to be able to bind to externally added metal and show approximately similar K_{m} values for divalent metal, suggesting that the intrinsic bound metal, if any, is not very high in our enzyme preparations. Approximately 4 μg of protein was added to the lipid suspension and mixed gently by vortexing. This was then sonicated briefly and incubated at 37 °C for 2 h in a total reaction volume of 40 μl. The glycolipids were extracted in water-saturated butanol, dried, and resuspended in 10 μl of the same solvent before being resolved on HPTLC plates and analyzed by BioScan AR2000 TLC scanner. For the steady-state assays, the substrate was quantified by plotting a standard curve using different known amounts of UDP[6-3H]GlcNAc as described previously (9). The endogenously present unlabeled GlcNAc-PI in the assay is not estimated by this method. So the K_{m} and V_{max} values for the catalytic activity correspond to “apparent” rather than “absolute” values. However, the method is valid for comparative analysis.

**Far-UV Circular Dichroism (CD) Spectroscopy**—For CD spectroscopic studies, the MBP tag on the protein was cleaved with Factor Xa followed by dialysis and passage through amyllose column to remove free MBP as described previously (9). The far-UV CD spectra (average of three scans) of the wild type and mutant variants (−0.03 mg/ml in 10 mM acetate buffer, pH 5.5, with 200 mM NaCl, 10% glycerol) were recorded between 260 and 200 nm at 25 °C in a 1-mm path length cuvette as described previously (9).

**RESULTS**

PIG-L proteins belong to the family of metal-dependent deacetylases. A sequence alignment with >100 homologous proteins from archaea, bacteria, protozoa, and other eukaryotes, including PIG-L from mammals, suggested the presence of two conserved motifs with the consensus sequences (P/A)-H-(P/A)-DD and HXHXH (10). Structural and biochemical studies too pointed to the importance of the aspartate and histidine residues of these conserved motifs in metal binding and catalysis by different metal-dependent deacetylases (10–12). In a previous study we showed that EhPIG-L too possesses an AHADD motif along with a HPNH motif corresponding to these conserved motifs (9). Studying the role of the histidine and aspartate residues of these two motifs, therefore, seemed to be a good starting point for our analysis.

**Site-directed Mutagenesis**—Using site-directed mutagenesis, we mutated the conserved histidine and aspartate residues of the AHADD and HXHXH motifs to alanine to generate the mutants EhATMPIG-L H43A, EhATMPIG-L D45A, EhATMPIG-L D46A, EhATMPIG-L H140A, and EhATMPIG-L H143A (Fig. 1A). In addition we generated the EhATMPIG-L D47A mutant (Fig. 1A). The most common residue at this position in eukaryotic GlcNAc-PI de-N-acetylases is glutamate, but in other close homologs there is considerable variability at this position (10). Further, to probe the role of other negatively charged residues in metal binding, we mutated three other conserved acidic residues to alanine to generate the mutants EhATMPIG-L E79A, EhATMPIG-L D102A, and EhATMPIG-L D133A (Fig. 1A).

The MBP-tagged mutant proteins were affinity-purified using an amyllose column by the protocol described previously (9) (data not shown).

**Secondary Conformation of the Mutant Proteins**—To ascertain whether the specific site-directed mutants significantly affected the global conformation of the protein, we carried out far-UV CD spectroscopic studies on the mutant variants of the protein after removal of the MBP tag. We observed that the mutations did not significantly alter the global conformations of the proteins (Fig. 1B).

**Catalytic Activity of the Mutants and Identification of Residues Important for Activity**—To test whether the specific site-directed mutations significantly affected the enzymatic activity...
of the protein, we carried out GlcNAc-PI de-N-acetylase activity assays both in the absence and presence of externally added metal (Fig. 1C). It must be noted that the addition of divalent cations stimulates the catalytic activity of the wild type (WT) enzyme by approximately 1.6-fold compared with activity in the absence of metal. The mutant proteins possessed varying amounts of activity. We classified them into three major groups based on the catalytic activity that they exhibited (Table 1).

The first group (Class I) comprised mutants that possessed a 60% activity or higher even in the absence of externally added metal (40% activity or higher even in the absence of externally added metal). These included the mutants EhΔTMPIG-L H43A, EhΔTMPIG-L E79A, and EhΔTMPIG-L H143A. Of these, both EhΔTMPIG-L H43A and EhΔTMPIG-L H143A showed only ~20% of the activity of the wild type EhΔTMPIG-L in the absence of externally added metal. However, EhΔTMPIG-L H43A showed an approximately 6-fold stimulation in catalytic activity upon addition of metal whereas EhΔTMPIG-L H143A showed a 5-fold enhancement in activity under similar conditions. Thus, the catalytically active conformation of the enzyme is attained in both these mutants upon the addition of metal. In other words, these mutants are impaired only in attainment of the catalytic conformation in the absence of added metal. Thus, both His-43 and His-143 appear to be important for the integrity of the active site conformation but not for metal binding or catalysis itself. In comparison to these mutants, EhΔTMPIG-L D46A and EhΔTMPIG-L D102A showed a higher level of activity in the absence of added metal (34%). This mutant too was well stimulated (2-fold) by the addition of metal. Thus, Asp-47 also does not appear to be a catalytic residue.

In the second group (Class II), we placed those mutants that showed significantly impaired catalytic activity in the absence of added metal, but whose activity could be stimulated by the addition of metal. These included the mutants EhΔTMPIG-L H43A, EhΔTMPIG-L D47A, and EhΔTMPIG-L H143A. Of these, both EhΔTMPIG-L H43A and EhΔTMPIG-L H143A showed only ~20% of the activity of the wild type EhΔTMPIG-L in the absence of externally added metal. However, EhΔTMPIG-L H43A showed an approximately 6-fold stimulation in catalytic activity upon addition of metal whereas EhΔTMPIG-L H143A showed a 5-fold enhancement in activity under similar conditions. Thus, the catalytically active conformation of the enzyme is attained in both these mutants upon the addition of metal. In other words, these mutants are impaired only in attainment of the catalytic conformation in the absence of added metal. Thus, both His-43 and His-143 appear to be important for the integrity of the active site conformation but not for metal binding or catalysis itself. In comparison to these mutants, EhΔTMPIG-L D46A and EhΔTMPIG-L D102A showed a 5-fold enhancement in activity under similar conditions. Thus, the catalytically active conformation of the enzyme is attained in both these mutants upon the addition of metal. In other words, these mutants are impaired only in attainment of the catalytic conformation in the absence of added metal. Thus, both His-43 and His-143 appear to be important for the integrity of the active site conformation but not for metal binding or catalysis itself. In comparison to these mutants, EhΔTMPIG-L D46A and EhΔTMPIG-L D102A showed a higher level of activity in the absence of added metal (34%). This mutant too was well stimulated (2-fold) by the addition of metal. Thus, Asp-47 also does not appear to be a catalytic residue.

In the third group (Class III) we placed those mutants that were significantly impaired in catalytic activity both in the absence of externally added metal and upon addition of metal. The mutants EhΔTMPIG-L D46A and EhΔTMPIG-L H140A belonged to this category. To determine whether Asp-46 and His-140 could be catalytic residues, we investigated metal binding as well as the steady-state kinetic parameters for the de-N-acetylation of [6-3H]GlcNAc-PI by these mutants, as described below.

**Metal Binding by the Mutant Proteins**—We have previously shown that binding of Mn2+ to EhΔTMPIG-L results in a significant alteration in the global conformation of the protein that could be monitored by far-UV CD spectroscopy (9). We therefore used CD spectroscopy to monitor whether EhΔTMPIG-L D46A and EhΔTMPIG-L H140A had significantly altered metal binding. For comparison, we also analyzed mutants from Class I and Class II. All of the mutants showed approximately similar extents of global conformational

**TABLE 1 Classification and steady-state kinetic parameters of EhΔTMPIG-L mutants.**

| Mutants      | Activity vis-à-vis EhΔTMPIG-L |
|--------------|-------------------------------|
| Class-I      |                               |
| EhΔTMPIG-L D45A | good activity in absence of metal; well stimulated upon addition of metal |
| EhΔTMPIG-L E79A |                               |
| EhΔTMPIG-L D102A |                               |
| EhΔTMPIG-L D133A |                               |
| Class-II     |                               |
| EhΔTMPIG-L H43A | low activity in absence of metal; well stimulated upon addition of metal |
| EhΔTMPIG-L D47A |                               |
| EhΔTMPIG-L H143A |                               |
| Class-III    |                               |
| EhΔTMPIG-L D46A | low activity in absence of metal; no stimulation upon addition of metal |
| EhΔTMPIG-L H140A |                               |
FIGURE 2. Metal binding is unaffected in the mutants of EhΔTMPiG-L. A, far-UV spectra of WT EhΔTMPiG-L and its mutants. The spectra in the absence of externally added metal (no metal) or in the presence of externally added 3 μM MnCl₂ (+ metal) were recorded after removal of the MBP fusion tags as described under “Results.” B, binding plots showing affinity of EhΔTMPiG-L and its mutants for Mn²⁺. The proteins were titrated with (0–3.0 μM MnCl₂) and incubated at 25 °C for 5 min after each addition before recording the CD spectra. Normalized changes in CD signal at 220 nm were used to obtain the binding plot for the proteins as a function of ligand concentration. The data were fit using Sigma Plot 8.0 assuming a one-site binding model. The average of two independent data sets done in duplicate was taken for Kᵣ estimation. Bₘᵡₚ represents the value at saturation. R² values corresponding to the goodness of the fits ranged from 0.95 to 0.99. The data shown are the average of two independent experiments done in duplicate.

Table 2, the EhΔTMPiG-L H43A mutant showed no difference in affinity for the substrate (or Kₘ) compared with EhΔTMPiG-L, monitored in a parallel assay, in the absence as well as presence of externally added metal. But the mutant did have lower Vₘₐₓ values in the absence of added metal, which explains the lower activity observed in Fig. 1C as well. The addition of metal stimulates the activity by enhancing the Vₘₐₓ of the reaction by approximately 2-fold and was statistically significant (p value vis-à-vis the assay in the absence of added metal was 0.0021). In the parallel assay for the wild type EhΔTMPiG-L also, an approximately 2-fold enhancement in Vₘₐₓ values in the presence of metal was observed, which was statistically significant (p = 0.0012 when calculated vis-à-vis the assay done in the absence of added metal) (Table 2).

The mutants EhΔTMPiG-L D46A and EhΔTMPiG-L H140A on the other hand, had low catalytic activity in the absence of metal. The Vₘₐₓ values of both mutants were approximately half that of the wild type EhΔTMPiG-L. Additionally, both mutants were poorly stimulated by metal. As can be seen from Table 2, the Vₘₐₓ values were only marginally improved by the addition of metal. The stimulation observed upon addition of metal for EhΔTMPiG-L D46A and EhΔTMPiG-L H140A was not statistically significant (p = 0.26 and 0.46, respectively, when calculated vis-à-vis the assay carried out in the absence of added metal in each case). The Kₘ values, however, were not significantly affected in either of these mutants compared with the wild type, both in the absence or presence of externally added metal, indicating that the binding of the substrate was likely to be largely unaffected by the mutations. Thus, taken together, our results suggest that both Asp-46 and His-140 are catalytic residues in EhΔTMPiG-L.

The Proposed Model—There are at least two possible models that could be proposed to explain the above results. For example, it is possible to speculate that Asp-46 and His-140 are critical for attainment of the catalytically efficient conformation of the active site. In the absence of either Asp-46 or His-140, despite metal binding and induction of the requisite global conformational change, it is possible that the optimum geometry of the active site remains unattained. Alternatively, it is possible to speculate that Asp-46 and His-140 participate as a general acid-base pair (GABP) (Fig. 3), somewhat like that suggested for LpxC, a Zn²⁺-dependent UDP-3-O-((R)-3-hydroxymyristoyl)-N-acetylgalcosamine deacetylase but without the polarization of a water molecule by the metal (13).

In such a general acid-base pair model, the deprotonated Asp-46 polarizes a molecule of water, generating the nucleophile for attack on the carbonyl group of the substrate. The intermediate that is thus formed is stabilized by the protonated His-140. In the second step, the protonation of His-140 by another molecule of H₂O promotes the subsequent bond rearrangements that in turn assist the removal of the acetyl group from the substrate.

Such a model would also explain the low level of activity observed in the mutants EhPIG-LΔTM D46A and EhPIG-LΔTM H140A. Because substrate binding is unaffected, we may assume that the substrate is sitting correctly in the pocket. Even in the absence of His-140, in the EhPIG-LΔTM H140A mutant, the nucleophile for attack on the amide bond is created by Asp-46; but, in the absence of stabilization of the intermediate and
Catalytic Mechanism of E. histolytica PIG-L

TABLE 2

Steady-state parameters of de-N-acetylase activity of the wild type EhΔTMPIG-L versus the mutants

| Sample                  | No added metal | With added metal |
|-------------------------|----------------|------------------|
|                         | $K_m$ (μM)    | $V_{max}$ (pmol·h⁻¹ per μg protein) | $K_m$ (μM)    | $V_{max}$ (pmol·h⁻¹ per μg protein) |
| EhΔTMPIG-L*             | 1.95 ± 0.20   | 64.4 ± 2.7       | 2.09 ± 0.23   | 121.6 ± 4.9 |
| EhΔTMPIG-L H43A         | 1.99 ± 0.18   | 42.6 ± 1.3 ($p = 0.018$) | 2.63 ± 0.59   | 74.2 ± 3.8 ($p = 0.017$) |
| EhΔTMPIG-L D46A         | 1.82 ± 0.18   | 33.3 ± 0.0 ($p = 0.007$) | 1.95 ± 0.22   | 37.9 ± 3.0 ($p = 0.005$) |
| EhΔTMPIG-L H140A        | 1.6 ± 0.25    | 30.6 ± 0.8 ($p = 0.007$) | 1.37 ± 0.21   | 40.3 ± 4.6 ($p = 0.007$) |

* Previously reported EhΔTMPIG-L to have an apparent $K_m$ of 1.95 ± 0.24 μM and a $V_{max}$ of 55.15 ± 2.96 pmol·h⁻¹ per μg of protein in the absence of externally added metal; in the presence of externally added metal the apparent $K_m$ was 2.05 ± 0.18 μM, and $V_{max}$ was 130.08 ± 5.62 pmol·h⁻¹ per μg of protein (9). We attribute these differences to experimental variations and variations in amount of endogenous unlabeled GlcNAc-PI/GlcN-PI in the substrate preparations.

FIGURE 3. The general acid-base pair (GABP) mechanism proposed for the catalytic activity of E. histolytica PIG-L. The deprotonated Asp-46 polarizes a molecule of water, generating the nucleophile for attack at the carbonyl moiety of the substrate. The protonated His-140 stabilizes the intermediate formed in this process, making a hydrogen bond with the substrate. The subsequent bond rearrangements result in cleavage of the acetyl group from the substrate and simultaneous reprotonation of His-140 through the participation of a second molecule of H₂O.

FIGURE 4. pH dependence of EhΔTMPIG-L D46A mutant versus the wild type. The activity of the two protein variants was studied as a function of pH in 50 mM acetate (pH 3.5, 4.5, 5.5, 6.5) or in 50 mM HEPES (pH 7.5, 8.5) buffers as reported previously (9). The data shown are for 2 h in the absence of externally added divalent metal. The activity for each protein at different pH is shown relative to the maximum activity (100%) exhibited by it.

assistance by His-140, the probability of the acetyl group leaving from the substrate is low, resulting in much lower catalytic efficiency. Similarly, H₂O is a weak nucleophile in the absence of the polarizing Asp-46 in the EhPIG-LTM D46A mutant. Hence, the attack by a H₂O molecule occurs with much lower probability in the absence of Asp-46. The protonated His-140 would continue to stabilize the catalytic intermediate and participate in the elimination of the acetyl group. The low probability of attack by a water molecule in the absence of the polarizing Asp-46 would explain the much lower activity seen in the EhPIG-LTM D46A mutant. Thus, the absence of either Asp-46 or His-140 in such a model would result in crippled, but not completely abrogated, catalytic activity, and metal binding would be unable to compensate for the absence of the key residue.

The major support for such a model also comes from the fact that the optimum pH for the activity of EhΔTMPIG-L is 5.5. A deprotonated aspartate with $pK_a$ ~ 4.5 and a protonated histidine with $pK_a$ of ~ 6.5 could participate to provide an optimum pH of 5.5 for the catalysis. In such a case, one would expect the optimum pH of the mutant, EhΔTMPIG-L D46A, to shift to a higher pH.

We tested this hypothesis by studying the pH profile of the de-N-acetylase activity of the EhΔTMPIG-L D46A mutant. Indeed, the pH optimum for this mutant was 6.5 as against 5.5 for the wild type mutant (Fig. 4), lending credence to a catalytic model that involves a general acid-base pair mechanism. As a corollary to this, we also expected that the optimum pH of the EhΔTMPIG-L H140 mutant would shift to pH 4.5. However, due to issues of stability of the EhΔTMPIG-L H140 mutant at low pH we were unable to test whether the optimum pH for this mutant had indeed shifted to the lower pH.

DISCUSSION

Based on homology and the presence of the conserved AHADD as well as HXXH motifs, the eukaryotic PIG-L protein has been classified as a member of the larger family of metal-dependent deacetylases. This family of enzymes includes, for example, MshB, a deacetylase involved in mycolith biosynthesis of Mycobacterium tuberculosis. The crystal structure of MshB provided the first evidence for the role of histidine and aspartate residues of the AHADD and HXXH motifs in metal ion co-ordination. Baker and co-workers showed that His-13 and Asp-16 of the AHADD motif, along with the C-terminal His-147 of the HPDH motif, were involved in co-ordinating the central Zn²⁺ in MshB (11). The authors also suggested that Asp-15 of the AHADD motif was ideally placed in the catalytic pocket to act as a catalytic base. They proposed a model in which the carbonyl bond of the substrate was polarized by the central metal, making it susceptible to nucleophilic
attack by a water molecule which, in turn, had been polarized by Asp-15. Building on this model, Ferguson and co-workers used semi-quantitative complementation assays in conjunction with homology modeling to study the metal-binding and catalytic residues of rat PIG-L (12). From this data, they proposed a role for His-49 and Asp-52 of the AHADD motif, along with the C-terminal His-157 of a HSNH motif, in metal binding. Based on its positioning within the catalytic pocket and the fact that this was the only mutant that showed no activity in their assays, they also hypothesized that Asp-51 of the AHPDD motif could act as a catalytic base and proposed a catalytic model very similar to the one proposed for MshB by Baker and co-workers.

PIG-L from *E. histolytica* too has the homologous conserved motifs described above (9). However, presence of the metal ion is not critical for the function of EhΔTMPiG-L, and we have shown previously that the metal ion alters the $V_{\text{max}}$ but not the $K_m$ of the enzyme for its substrate (9). We show here that this is also the case with the mutants of this enzyme, EhΔTMPiG-L H43A, EhΔTMPiG-L D46A, and EhΔTMPiG-L H140A. In other words, the metal ion plays no role in substrate binding by EhΔTMPiG-L. The metal ion also does not appear to polarize a water molecule, as has been proposed for other deacetylases; if it did, it would have lowered the $pK_a$ of water and hence altered the optimum pH at which the enzyme would work (9). Thus, the mechanism of catalysis does not appear to be conserved. Indeed, our results suggest that although the AHADD and HXXH motifs continue to be important for the functioning of the PIG-L enzyme from *E. histolytica*, the conserved residues appear to have taken on new functions. Specifically, Asp-46 and His-140, instead of binding to metal, as in other deacetylases, now appear to participate directly in the catalysis itself, as a general acid-base pair. That conserved residues can take on new functions in the course of evolution is certainly very interesting. But more interesting, perhaps, from the clinical biochemistry point of view is the fact that this suggests the possibility of selectively targeting the pathogen *vis-à-vis* the host by identifying specific inhibitors to the *E. histolytica* PIG-L.

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