The de-N-acetylation of N-acetyl-d-glucosaminylphosphatidylinositol De-N-acetylase of Glycosylphosphatidylinositol Biosynthesis Is a Zinc Metalloenzyme

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The de-N-acetylation of N-acetyl-d-glucosaminylphosphatidylinositol (GlcNAc-PI) is the second step of mammalian and trypanosomal GlcNAc-PI biosynthesis. Glycosylphosphatidylinositol biosynthesis is essential for Trypanosoma brucei, the causative agent of African sleeping sickness, and GlcNAc-PI de-N-acetylase has previously been validated as a drug target. Inhibition of the trypanosome cell-free system and recombinant rat GlcNAc-PI de-N-acetylase by divalent metal cation chelators demonstrates that a tightly bound divalent metal cation is essential for activity. Reconstitution of metal-free GlcNAc-PI de-N-acetylase with divalent metal cations restores activity in the order Zn<sup>2+</sup> > Cu<sup>2+</sup> > Ni<sup>2+</sup> > Co<sup>2+</sup> > Mg<sup>2+</sup>. Site-directed mutagenesis and homology modeling were used to identify active site residues and postulate a mechanism of action. The characterization of GlcNAc-PI de-N-acetylase as a zinc metalloenzyme will facilitate the rational design of anti-trypanosome parasite drugs.

Glycosylphosphatidylinositol (GPI)<sup>1</sup> acts as a membrane anchor for a significant proportion of eukaryotic cell surface glycoproteins. The structure, biosynthesis, and function of GPI anchors and related molecules have been extensively reviewed (1–6). The basic conserved GPI core of NH₂CH₂CH₂PO₄⁻H<sub>2</sub>O [N-acetyl-D-glucosaminylphosphatidylinositol (GlcNAc-PI)] has been described in detail in recent reviews and books (7, 8). De-N-acetylated GPI anchors, or GPI-anchored proteins, are particularly abundant in protozoan parasites such as Trypanosoma brucei, the causative agent of African sleeping sickness in humans and the related disease Nagana in cattle (1). Sleeping sickness is invariably fatal if untreated and affects upwards of 300,000 people each year. Current drugs are highly toxic and difficult to administer, leaving an urgent need for new therapeutic agents. Bloodstream form T. brucei cells express ~5 × 10⁶ GPI-anchored variant surface glycoprotein homodimers that form a dense surface coat, protecting the parasite from the complement pathway of the host and undergoing antigenic variation to evade specific immune responses (9, 10). Disruption of GPI biosynthesis is fatal to the bloodstream form parasite in culture (11–13), and a conditional null mutant of the gene encoding the GlcNAc-PI de-N-acetylase in T. brucei (TbGPI12) has validated this enzyme as a drug target (11).

Mammalian and trypanosomal GlcNAc-PI de-N-acetylases have a single pass N-terminal transmembrane domain with the majority of the protein found on the cytoplasmic face of the endoplasmic reticulum (14). T. brucei GPI12 has been partially purified from T. brucei membranes after detergent solubilization (15), whereas rat GlcNAc-PI de-N-acetylase (rPIG-L) has been expressed in Escherichia coli and isolated as a complex with GroEL (16). The specificities of GlcNAc-PI de-N-acetylases from T. brucei and HeLa cells have been examined in vitro using substrate analogues (8, 17). The T. brucei enzyme has less stringent substrate specificity than the mammalian enzyme, allowing the design of substrate-based species-specific inhibitors (18–20). However, the GlcNAc-PI de-N-acetylases are not well characterized. No structural data are available, and the active site and mechanism of action have yet to be determined.

The crystal structures of two proteins with limited homology to the GlcNAc-PI de-N-acetylases have recently been reported (21–23). The crystal structure of MshB, a zinc-dependent N-acetylglucosamine α1-O-1-d-myo-inositol de-N-acetylase involved in mycothiol synthesis in Mycobacterium tuberculosis,
revealed an unusual HPDZ binding motif in the proposed active site (22, 23). This motif is conserved in the GlcNAc-Pi de-N-acetylases, despite the low level of overall sequence identity with MshB. The protein TT1542 from *Thermus thermophilus* of unknown function has 23% sequence identity to MshB and also contains the HPDZ motif. The crystal structure of TT1542 revealed a tertiary structure similar to MshB, but no metal ion was observed in the putative active site (21).

Here, we report that mammalian and trypanosomal GlcNAc-Pi de-N-acetylases are zinc metalloenzymes and identify the active site residues by site-directed mutagenesis and molecular modeling. A putative mechanism of action is proposed.

**EXPERIMENTAL PROCEDURES**

**Materials**—[9,10-3H]Myristic acid (40 μCi/mmol), UDP-[6-3H]GlcNAc (UDP-[6-3H]GlcNAc, 20 Ci/mmol), and En'Hance™ were purchased from PerkinElmer Life Sciences. High performance liquid chromatography-grade solvents were purchased from BDH, and all of the other reagents were purchased from Sigma.

**Semi-synthetic and Synthetic Substrates**—The synthesis of rGlcNac-1-6-N-myo-inositol-1-HP-octadecyl (GlcNIP) has been described by Crossman et al. (24). The formation of semi-synthetic 1-6-N-acetyl GlcNAC-Pi was conducted as described by Milne et al. (15). N-Acetyl derivatives of the above compounds were prepared as described previously (25). The identity and purity of the synthetic substrates were assessed by negative ion electrospray-mass spectrometry (ES-MS), and the concentration of stock solutions was determined by measurement of the inositol content by selected ion-monitoring gas chromatography-mass spectrometry (26).

**High Performance Liquid Chromatography**—Glycolipid standards and samples were applied to 10-cm aluminum-backed Silica Gel 60 and developed with chloroform/methanol/13 α ammonium/1 m ammonium acetate/water (180/140/9/9/23, v/v). Dried HPTLC plates were sprayed with En'Hance, and radiolabeled components were visualized by fluorography. To avoid contamination of the inositol content by selected ion-monitoring gas chromatography-mass spectrometry (26).

**Typanosome Cell-free System Assays**—Bloodstream form T. brucei (variant MITat1.4) were isolated, and membranes (cell-free system) for site-directed Mutagenesis—Rat Liver were grown in Luria-Bertani medium with 50 μg/ml carbenicillin at 37 °C until A500 was 0.5, induced with 200 μM isopropl β-D-thigalactopyranoside, and cultured for an additional 16 h at 21 °C. Cells were harvested by centrifugation at 4500 × g for 20 min at 4 °C, resuspended in phosphate-buffered saline, washed three times in phosphate-buffered saline containing 0.25% w/v bovine serum albumin, and then incubated with an anti-mouse-phycocerythrin (PE) conjugate (STAR12A, Serotec) for 30 min. Cells were washed additionally three times in phosphate-buffered saline with 0.25% w/v bovine serum albumin before flow cytometry analysis in FACS-Calibur instrument (BD Biosciences).

**Expression of rPIG-L**—The QuikChange procedure (Stratagene) was used to create a mutation that removed an ethionine codon. The 5′-CCACCTCTCACGCCTTTGCAAGATGTTGCTTTG-3′ and 5′-ACGCTCTAGATCTCAGAAGAAGGCTTAGAGTTGG-3′ (mutation in boldface) were used to plasmid pMEBEB- rPIG-L-FLAG-sXho. The DNA encoding residues 24–252 of rPIG-L was amplified by PCR from pMEBEB-PIP-L-FLAG-sXho using a 5′-AAACTCCGCAAATGAGGTGCAGAAGGAAATG-3′ that is targeted to an Nool site (italics) and a six-residue N-terminal sequence (MDKVLDD, underlined) and a 3′-primer (5′-TTTTCCTCCGACTTGTACCAGGACTGAGAGTGGG-3′) that incorporated a C-terminal HA tag (GYPYPDPDYDA, underlined) and a Xho site (italics). The PCR fragment was subcloned into pET-Blue-2 (Novagen) with Ncol and XhoI to give the plasmid pETB- rPIG-L-HA, which incorporated a hexahistidine tag to the C terminus of the N-terminal sequence framing.

**E. coli Tn5**—Novotnay transformed pPET-nrPIG-L-HA were grown in Luria-Bertani medium with 50 μg/ml carbenicillin at 37 °C until A500 was 0.5, induced with 200 μM isopropyl β-D-thigalactopyranoside, and cultured for an additional 16 h at 21 °C. Cells were harvested by centrifugation at 4500 × g for 20 min at 4 °C, resuspended in phosphate-buffered saline, washed three times in phosphate-buffered saline containing 0.25% w/v bovine serum albumin, and then incubated with an anti-mouse-phycocerythrin (PE) conjugate (STAR12A, Serotec) for 30 min. Cells were washed additionally three times in phosphate-buffered saline with 0.25% w/v bovine serum albumin before flow cytometry analysis in FACS-Calibur instrument (BD Biosciences).

**ES-MS/MS De-N-acetylase Assay**—All of the buffers and solutions

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2 Details of the mutagenic primers are contained in Supplemental Tables S1 and S2.
were treated with Chelex100 (sodium form) to remove divalent cations prior to use. The recombinant protein eN-rPIG-L-HA (500 ng/μl) was incubated with 1.0 mM 1,10-phenanthroline (1,10-PO) in buffer A for 0.5 h at 37 °C. Treated eN-rPIG-L-HA (1 μl) was added to GlcN-I-P-C18 (1 nmol) in incorporation buffer (39 μl) with or without 10 μM divalent metal cations. The solution was briefly vortexed, sonicated for 5 s, and incubated at 37 °C for 0.5 h. The mixture was diluted with 5% propan-1-ol (1 ml) and analyzed by HPTLC and fluorography along-side [3H]myristate-labeled GlcNc-PI and GlcN-I-PI standards (Std). The lower band visible in some lanes is Manα1-4GlcNc-PI. B, the effect of preincubation with increasing concentrations of 1,10-PO prior to labeling with UDP-[6-3H]GlcNAc in the trypanosome cell-free system (0 mM (lane 1), 0.1 mM (lane 2), 0.5 mM (lane 3), 1.0 mM (lane 4), and 2.5 mM (lane 5)).

Expression of Soluble rPIG-L—Truncation of the N-terminal transmembrane domain of rPIG-L at residues 7, 14, and 21–27 failed to produce soluble recombinant rPIG-L in E. coli. A BLAST search with rPIG-L revealed a hypothetical E. coli protein YAIS with reasonable homology to rPIG-L but lacking the transmembrane domain. A pET vector pETB-eN-rPIG-L-HA was constructed, coding for a 28-kDa fusion protein eN-rPIG-L-HA comprising residues 1–6 of E. coli YAIS followed by residues 26–252 of rPIG-L and C-terminal HA and hexahistidine tags. The expression in E. coli and purification by metal ion affinity chromatography produced soluble eN-rPIG-L-HA (Fig. 2A). The identity of the protein was confirmed by Western blotting with anti-HA (Fig. 2B) and by tryptic peptide mass fingerprinting.

Metal Ion Dependence of Soluble rPIG-L—Initial assessment of the activity of eN-rPIG-L-HA by measuring the de-N-acetylation of [3H]myristate-labeled GlcNc-PI confirmed that the fusion protein retained activity for its natural substrate (Fig. 2C, lane 3). To provide quantitative data and enable higher substrate concentrations to be used, a ES-MS-MS technique was devised to monitor the de-N-acetylation of the synthetic substrate GlcNc-I-P-C18 (see “Experimental Procedures”). Although GlcNc-I-P-C18 has been reported to be a poorer substrate than GlcNc-PI in the HeLa cell-free system where the enzyme is membrane-bound (34), a specific activity of 2.3 nmol/mg/h was recorded. This figure compares favorably with the previously reported specific activity of 390 pmol/mg/h for the de-N-acetylation of GlcNc-PI by partially purified TbGPI12 from T. brucei (15).

The activity of eN-rPIG-L-HA was inhibited by the presence of micromolar concentrations of Zn²⁺ (IC₅₀ 35 μM), a feature previously observed for several Zn²⁺ metalloenzymes (35, 36). Incubation of eN-rPIG-L-HA with 1.10-PO produced a loss of de-N-acetylation activity in a time- and concentration-dependent manner, such that treatment with 2.5 mM 1,10-PO for 1 h at 37 °C was sufficient to completely remove all activity. Attempts were made to restore activity to the metal-free inactive protein (apo-eN-rPIG-L-HA) by reconstitution with a variety of metal cations (33). Prewashing the cell-free system to remove any loosely associated divalent metal ion did not affect the de-N-acetylation activity. However, preincubation of the cell-free system with 1,10-PO or pyridinedicarboxylic acid inhibits de-N-acetylation, whereas the non-chelating 1,7-PO has no effect (Fig. 1A). Inhibition by 1,10-PO occurs in a concentration-dependent manner (Fig. 1B), suggesting the presence of a strongly bound metal ion that is essential for TbGPI12 activity.

Expression of soluble rPIG-L—A structural model of rPIG-L was constructed using homology modeling with WHAT IF (29). The structure of M. tuberculosis MshB (Protein Data Bank entry 1Q7T (23)) was used as the template structure. No attempt was made to model the large insertions in the rPIG-L sequence (Fig. 8). Rotamers of conserved residues were left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged. All of the other residues were initially mutated to alanines. Rotamers were then modeled using the WHAT IF backbone-dependent rotamer libraries. At each position, rotamer quality was left unchanged.
ions without success. However, under less harsh conditions where partially active apo-eN-rPIG-L-HA was obtained, the activity could be increased by the addition of divalent metal cations. Furthermore, the lower the activity of the apo-eN-rPIG-L-HA sample, the less the activity could be restored. As an example, treatment of eN-rPIG-L-HA with 1.0 mM 1,10-PO for 30 min at 37 °C produced a 83% loss in activity that could be partially restored (to a maximum of 28%) by the subsequent addition of metal ions (10 μM) with restoration occurring in the order Zn2⁺, Cu2⁺ > Ni2⁺ > Co2⁺ > Mg2⁺ (Fig. 3). The addition of 10 μM of the same metal ions to untreated eN-rPIG-L-HA had a negligible effect on its de-N-acetylase activity (with the exception of zinc, which showed 40% inhibition). These results indicate that apo-eN-rPIG-L-HA is unstable, probably undergoing irreversible misfolding. The amount of de-N-acetylase activity that can be reconstituted appears to be a balance between the rate of metal ion stripping and the instability of apo-eN-rPIG-L-HA.

Site-directed Mutagenesis—Sequence alignment of the GlcNAc-PI de-N-acetylase enzymes from *T. brucei* and *Rattus norvegicus* with MshB from *M. tuberculosis* revealed a number of conserved residues, despite their low level of sequence identity (Fig. 4). Many, but not all, of these residues are also completely conserved in putative GlcNAc-PI de-N-acetylase homologues that are identified in a number of sequenced eukaryotic genomes (see Fig. 4, *consensus line*). The sequence alignment was used to construct a homology model of the rPIG-L active site (Fig. 8). The sequence alignment and model show that a large proportion of the conserved residues are found in the proposed active site, including the putative zinc-binding residues and catalytic base (22, 23). Site-directed mutagenesis was used to mutate the three putative zinc-binding residues and other residues proposed to participate in catalysis or substrate binding (Fig. 4). The rPIG-L mutants were cloned into pEGFP mammalian expression vector, which fused GFP to the C terminus of the expressed protein. CHO-K1 M2S2 cells were transiently transfected with various recombinant plasmids, and the level of GFP expression and cell surface restoration of CD55 was analyzed by flow cytometry (Fig. 5).

Transfection with pcDNA-rPIG-L expressing wild type rPIG-L restored cell surface expression of CD55 (defined as a PE signal ≥ 10² relative fluorescence units (r.f.u.), CD55-positive) with no GFP signal (Fig. 5A), whereas transfection with empty pEGFP expressing EGFP produced a GFP signal (defined as a GFP signal ≥ 10¹ r.f.u., GFP-positive) with no CD55 restoration (Fig. 5B). Transfection with pEGFP-rPIG-L-WT expressing the rPIG-L-WT-GFP fusion restored cell surface expression of CD55 with CD55-positive cells showing varying levels of GFP signal (Fig. 5C). These results indicate that, for wild type rPIG-L enzyme at least, cell surface restoration of CD55 is effectively complete in transfected cells, whereas the GFP signal varies continuously depending on the level of protein expression in individual cells. The fact that full CD55 restoration can be achieved in transfectants expressing undetectable amounts of GFP suggests that the amount of rPIG-L required to complement the M2S2 mutant cell line is extremely small.

Analysis of rPIG-L Mutants by Functional Complementation—To compare the activity of the rPIG-L alanine mutants, M2S2 cells were transiently transfected with plasmids expressing various rPIG-L-GFP fusion proteins and the level of GFP expression and cell surface restoration of CD55 was analyzed by flow cytometry (Table I). The activity of the rPIG-L mutants was compared by measuring the mean GFP signal of CD55-positive cells, allowing the rPIG-L mutants to be grouped into three distinct classes defined as follows: (i) those with activities similar to wild type rPIG-L with a mean GFP signal of 3–17 r.f.u. (+ class, Fig. 6A); (ii) those with negligible activities compared with wild type rPIG-L with a mean GFP signal of > 10² r.f.u. (− class, Fig. 6B); and (iii) those that display activity only when a GFP signal is detected with a mean GFP signal of 20–35 r.f.u. (± class, Fig. 6C).

Two mutants in the + class, D147A and H226A, displayed lower GFP signals than the wild type enzyme (Fig. 6D), which may reflect poor folding of these mutants, because the level of fluorescence of GFP fusions has been related to the stability of the upstream protein (37). However, the level of rPIG-L activity of these mutants is still sufficient to completely restore cell surface expression of CD55. The – class mutants produce incomplete cell surface restoration of CD55, even at very high levels of rPIG-L-GFP expression, indicating that the mutation has severely reduced the activity of the enzyme. In contrast, the ± class mutants are able to completely restore cell surface expression of CD55, but only as the levels of rPIG-L-GFP expression increases, indicating that the mutation has only a minor detrimental effect on the activity of the enzyme.

Analysis of TbGPI12 Wild Type and Alanine Mutants by Functional Complementation—TbGPI12 wild type was cloned into the pEGFP to give the plasmid pEGFP-TbGPI12-WT and transiently transfected into CHO-K1 M2S2 cells. Flow cytometry analysis showed that the TbGPI12-GFP fusion fully restored cell surface expression of CD55 but failed to produce the range of GFP signals observed for the rPIG-L-GFP fusion (data not shown). The low GFP signal for TbGPI12-GFP, similar to that seen for rPIG-L-GFP mutants D147A and H226A (Fig. 6D), presumably reflects a lower level of protein stability and/or expression than rPIG-L-GFP wild type (37).

Site-directed mutagenesis was used to mutate the three putative zinc-binding residues of TbGPI12 to alanine (Fig. 4). The TbGPI12 mutants were cloned into pEGFP, transiently trans-
fected into CHO-K1 M2S2 cells, and analyzed by flow cytometry. To allow the comparison of the activities of the TbGPI12 mutants, successfully transfected cells were selected for analysis by considering only those cells with a GFP signal (Fig. 7). As expected, transient transfection with pEGFP produced no CD55-positive cells (Fig. 7A), pDNA-rPIG-L, having no GFP signal, gave no cell count at all (Fig. 7B), and pEGFP-TbGPI12-WT gave 93.2% CD55-positive cells. The TbGPI12 mutants gave significantly decreased the levels of de-N-acetylase activity, pEGFP-TbGPI12-H41A gave 9.2% CD55-positive cells (Fig. 7D), pEGFP-TbGPI12-D44A gave 0.8% CD55-positive cells (Fig. 7E), and pEGFP-TbGPI12-H150A gave 34.5% CD55-positive cells (Fig. 7F). The relative severity of the mutations on the apparent de-N-acetylase activity was the same as that for the corresponding putative zinc-binding residues in rPIG-L (Table I).
Flow cytometry analysis of M2S2 CHO-K1 cells transiently transfected with plasmids expressing GFP fusions of rPIG-L alanine mutants.

Experimental details are as outlined in Fig. 5. + Class mutations produce cell surface restoration of the GPI-anchored protein, human CD55, and a GFP signal proportional to the level of protein expression. − Class mutations produce incomplete cell surface restoration of CD55, even at very high levels of protein expression. ± Class mutations produce complete cell surface restoration of CD55 when a GFP signal ≥10 r.f.u is detected.

**Table I**

| Mutant | Class | GFP signal* | Proposed role in MshB (22, 23) |
|--------|-------|-------------|-------------------------------|
| WT     |       | 9.4         |                                |
| D147A  | −     | 154.6       | Zinc coordination              |
| D51A   | −     | >10,000b    | Catalytic base                 |
| D52A   | +     | 879.5       | Zinc coordination              |
| E53A   | ±     | 21.3        | H-bonds to αNH of Asp31        |
| N80A   | +     | 14.1        | Maintaining active site geometry|
| H88A   | ±     | 27.0        | Substrate binding              |
| E91A   | +     | 15.4        | Maintaining active site geometry|
| D116A  | +     | 13.6        | Substrate binding              |
| D147A  | +c    | 3.2         | Maintaining active site geometry|
| H154A  | +     | 10.7        | Proton donor (direct)          |
| N156A  | ±     | 16.5        | Proton donor (indirect)        |
| H157A  | ±     | 32.1        | Zinc coordination              |
| H226A  | ±c    | 3.4         | Maintaining active site geometry|
| Q229A  | +     | 11.4        | Substrate binding              |

* Average GFP signal of cells showing cell surface restoration of CD55. CD55-positive cells are defined as having a PE signal ≥10 r.f.u.

+ No CD55-positive cells were detected at any level of GFP expression.
+ GFP signal observed was lower than wild type.

**DISCUSSION**

We have demonstrated that the mammalian and trypanosomal GlcNAc-PI de-N-acetylases require the presence of a strongly bound divalent metal cation for activity. We propose that native GlcNAc-PI de-N-acetylases contain zinc at the active site, because they contain a conserved zinc binding motif (Fig. 4) (22, 23) and are inhibited by micromolar concentrations of Zn²⁺ (characteristic of zinc metalloproteins (35)) and because Zn²⁺ is most effective in reconstituting enzymatic activity in the apoprotein (Fig. 3).

Analysis of the mutagenesis data in the context of the rPIG-L model reveals that there are essentially four types of conserved residues in the GlcNAc-PI de-N-acetylases. The first group of residues directly coordinates the zinc ion, His⁴⁹, Asp⁵², and His¹⁵⁷, which form a classic zinc-binding triad. Although the H49A and D52A have large effects on activity (Table I), the
H157A mutation only has an intermediate effect. The model shows that His49 and Asp52 are close to the proposed catalytic base, Asp51 (Fig. 8), whereas His57 occupies a position on the far side of the active site. Furthermore, His157 may be positioned sufficiently closely to be able to replace the zinc-binding role of His157 with minimal structural rearrangement.

The second group of residues are those involved in catalysis and/or substrate binding and directly line the active site cleft. Asp51 occupies a position near the zinc ion but does not interact with it directly. Rather, as seen in one of the MshB crystal structures (Protein Data Bank entry 1Q74 (22)), it appears to coordinate a water molecule bound to zinc (Fig. 8). The fact that this is the only mutation that produces no detectable activity but is not involved in zinc coordination suggests that this may be the catalytic base, activating a water molecule for attack on the acetamidocarbonyl carbon. A conserved histidine, His154, is positioned opposite this water molecule (Fig. 8) and could be involved in the coordination and polarization of the acetamido group as observed in several other de-N-acetylases (22, 38). However, the mutation of this histidine has no significant effect (Table I). There are two further conserved residues lining the active site, Arg227 and Gln230. These are too distant from the catalytic center to be involved in the reaction mechanism and have previously been proposed to participate in substrate binding (23). Mutation of these residues show weak or intermediate effects (Table I).

The third group of residues are those in a second shell of conservation buried behind active site residues. Glu53, Glu91, and His226 are all buried and coordinate residues from the active site (beyond the area shown in Fig. 8). Being further away from the active site, mutations of these residues have almost no detectable effect on activity.

The fourth group consists of conserved residues far away from the active site (beyond the area shown in Fig. 8). These are mostly conserved hydrophobic residues involved in packing the core of the enzyme with the exception of Asn186, Asp146, and Asp147, which are solvent-exposed and overall do not show significant effects on mutation.

Given the apparent similarity of the active site of the GlcNAc-PI de-N-acetylase with that of MshB and zinc metalloproteases, we are able to postulate a putative catalytic mechanism consistent with our experimental observations (Fig. 9). In this mechanism, the acetamidocarbonyl oxygen of GlcNAc-PI binds directly to zinc ion, polarizing the carbonyl bond and leaving a partial positive charge on the carbon atom. Nucleophilic attack of a zinc-bound water molecule activated by the general base Asp51 forms a tetrahedral intermediate stabilized by coordination to zinc. Subsequent protonation by Asp51 or His154 in a non-rate-limiting step allows the loss of GlcN-PI with displacement of the zinc-bound acetate by an incoming water molecule completing the catalytic cycle.

In summary, we have identified the active site residues of the GlcNAc-PI de-N-acetylases and postulated a catalytic mechanism. The GlcNAc-PI de-N-acetylase of T. brucei, the causative agent of African sleeping sickness, has been genetically validated as a drug target (11), and parasite-specific inhibitors of the enzyme have been designed and synthesized (19). Our characterization of the GlcNAc-PI de-N-acetylases as zinc metalloenzymes will facilitate further rational design of inhibitors and potential drug leads, because the pharmacology of inhibiting zinc metalloenzymes is well established (39).

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GlcNAc-PI De-N-acetylase Is a Zinc Metalloenzyme

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