Role of Conserved Glycine in Zinc-dependent Medium Chain Dehydrogenase/Reductase Superfamily

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The medium-chain dehydrogenase/reductase (MDR) superfamily consists of a large group of enzymes with a broad range of activities. Members of this superfamily are currently the subject of intensive investigation, but many aspects, including the zinc dependence of MDR superfamily proteins, have not yet been adequately investigated. Using a density functional theory-based screening strategy, we have identified a strictly conserved glycine residue (Gly) in the zinc-dependent MDR superfamily. To elucidate the role of this conserved Gly in MDR, we carried out a comprehensive structural, functional, and computational analysis of four MDR enzymes through a series of studies including site-directed mutagenesis, isothermal titration calorimetry, electron paramagnetic resonance (EPR), quantum mechanics, and molecular mechanics analysis. Gly substitution by other amino acids posed a significant threat to the metal binding affinity and activity of MDR superfamily enzymes. Mutagenesis at the conserved Gly resulted in alterations in the coordination of the catalytic zinc ion, with concomitant changes in metal ligand bond length, bond angle, and the affinity ($K_d$) toward the zinc ion. The Gly mutants also showed different spectroscopic properties in EPR compared with those of the wild type, indicating that the binding geometries of the zinc to the zinc binding ligands were changed by the mutation. The present results demonstrate that the conserved Gly in the GHE motif plays a role in maintaining the metal binding affinity and the electronic state of the catalytic zinc ion during catalysis of the MDR superfamily enzymes.

The medium chain dehydrogenases/reductase (MDR) superfamily with ~350-residue subunits contains the classical liver alcohol dehydrogenase, quinone reductase, leukotriene B4 dehydrogenase, and many other forms (1, 2). The MDR types of alcohol dehydrogenases (EC 1.1.1.1) are zinc metalloenzymes and frequently possess two tetrahedrally coordinated zinc ions per subunit; one has a catalytic role at the active site, and the other plays a structural role at a site thought to influence subunit interactions. In other zinc metalloenzymes, the catalytic zinc interacts with three ligands, one His, and two Cys residues and a water molecule, whereas the structural zinc interacts with four protein ligands (3, 4). One of the best characterized zinc-containing enzyme families is the zinc-dependent MDR family containing both a structural zinc and a catalytic Zn, which catalyzes the oxidation of primary or secondary alcohols to the corresponding aldehydes or ketones using NAD(P)H as a cofactor (1, 5).

Zinc participates in enzymatic catalysis, structural organization, and regulation of function in thousands of proteins. The chemical properties of zinc in enzymes are largely attributed to its function as a relatively strong Lewis acid (6). In general, fast ligand exchange, stereochemical flexibility, and redox-inertness are additional characteristics for the selection of zinc ions in the function of many proteins (7). Nevertheless, its physical properties render zinc invisible to most spectroscopic methods of investigation, precluding the application of many techniques that have been instrumental in understanding the functions of other transition metal ions (6).

In the last decade studies on metal binding in proteins have focused mainly on the metal/first-shell interactions (7, 8). The role played by first-shell ligands is crucial as they contribute to the stability of the metal complex and determine the selectivity of the binding site (9, 10). These first-shell ligands may also alter
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the metal coordination geometry or the ligand binding mode (mono/bidentate) (9, 11). Recent studies have highlighted the role of the second shell in protein-metal recognition and metalloprotein function (12–18). Specifically, a study of zinc finger structures has shown the significance of the second layer packing in shielding the negatively charged zinc finger cores (12). Subsequently an ab initio/continuum dielectric method calculation confirmed this finding and showed that second-shell ligands contribute to the energetics stabilization of the metal complex (13). Several studies on human carbonic anhydrase II (14, 15), metallo-phosphatase calcineurin (16), human serum transferrin (17), EcoRV restriction endonuclease (18), and designed metal binding sites (19–21) suggest that the second-shell ligands serve to orient the first-shell residues at proper positions to enhance the affinity of the binding site for the metal and fine-tune the reactivity of the first-shell catalytic water. Although surveys of protein zinc sites and several studies have been designed to decipher the function of the second-shell ligands in protein-metal recognition, no systematic studies for zinc-dependent MDRs have been conducted, and many questions remain. In general, the specific function of second-shell residues is not well understood.

L-Arabinitol 4-dehydrogenase (LAD, EC 1.1.1.12), a common enzyme found in yeasts and filamentous fungi, catalyzes the second step of the recently elucidated fungal l-arabinose metabolic pathway by oxidizing l-arabinitol to l-xylulose, with concomitant NAD⁺ reduction (22). The LAD enzyme belongs to the family of zinc-containing alcohol dehydrogenases (23). Previously, we characterized an LAD from Neurospora crassa (NcLAD) (24). The protein is a homotetrameric with a subunit molecular mass of 41 kDa and requires zinc for catalysis. The structures of the apoenzyme complexed with NAD and zinc have been determined (25) and reveal that the subunit, like that of the other MDR family members, is organized into two domains separated by a deep cleft, with the active site lying at its base. Each monomer contains a bi-domain architecture; domain 1 (residues 5–167 and 308–362) contains the residues of the other MDR family members, is organized into two domains separated by a deep cleft, with the active site lying at its base. Each monomer contains a bi-domain architecture; domain 1 (residues 5–167 and 308–362) contains the residues involved in substrate binding, catalysis, and coordinating the active site Zn, whereas domain 2 (residues 168–307), consisting of a dinucleotide-binding Rossmann fold (26), is responsible for binding NAD⁺.

As an outcome of density functional theory (DFT)-based screening, a strictly conserved glycine residue, Gly-77, was identified in the GHE motif of MDR superfamily, but its role remains unclear. Its strict conservation through evolution implicates an absolute requirement for a small amino acid residue at this position. We were compelled to address the structural and functional role of this evolutionarily preserved Gly in the zinc-dependent MDR superfamily. In this study we used in vitro, in silico, and spectroscopic methods to reveal the function of this Gly and to discern its contribution to metal binding affinities and active site charge distribution. By a combination of site-directed mutagenesis, DFT calculation, isothermal titration calorimetry (ITC), and spectroscopic analysis, we demonstrate that Gly is part of a conserved motif. It contributes to both the metal binding affinity and the active site charge distribution in MDR superfamily proteins and also quite probably in other structurally related proteins.

EXPERIMENTAL PROCEDURES

Materials, Bacterial Strains, and Culture Condition—Reagents for PCR and Ex-TaqDNA polymerase were purchased from Takara (Takara Shuzo Co., Japan). Restriction enzymes were obtained from New England Biolabs. pET-28a expression vector, plasmid isolation kit, and nickel-nitrilotriacetic acid Superflow column for purification were from Qiagen (Hilden, Germany). Oligonucleotide primers were obtained from Bioneer (Daejeon, South Korea). Electrophoresis reagents were from Bio-Rad, and all chemicals were from Sigma. All the computational calculations were performed on 600 core CPU Intel Xeon E5640 LINUX cluster.

Structure and DFT-based Screening—The crystal structure of NcLAD was solved by x-ray diffraction at 2.6 Å resolution (PDB entry 3M6I) (25). Analysis of the first layer of packing with donor atoms (e.g. N, S, or O) were identified with bond distances within 2.9 Å from metal ion (8). The heavy atoms of the first-shell residues were then selected as centers to search the second-shell ligands using a cutoff of 3.5 Å (27, 28). A maximum distance of 6.4 Å from the metal center was used to locate residues interacting directly or indirectly to the first shell ligands. Three residues, Cys-53, His-78, and Glu-79, lie within 2.9 Å of the first coordinating shell of zinc, and Glu-163 has been proposed for its role in product release during the catalysis (25). Excluding four amino acid residues, the second layer of catalytic zinc site embodied a total of 11 amino acid residues. All 11 amino acids residues were replaced with alanine computationally. The mutant structures were achieved by in silico mutating the wild-type structure using the protein design extension in Discovery Studio 3.0 (DS3.0, Accelrys Inc., San Diego, CA). The original coordinates of zinc were extracted during the mutant modeling (29), and hydrogen atoms were added using CHARMM forcefield. In silico side chain replacement and modeling were carried out with the routine “Build mutant” of MODELLER 9.9 (30). The resulting model was then minimized with DS3.0 (molecular dynamics cascade) using 5,000 minimization steps at 0 K, then 10,000 molecular dynamics steps (1 fs per step) via an NVT ensemble (where the number of particles N, the volume V, and the temperature T of the system are kept constant) at 310 K.

The local density approximation within the DFT formalism, as implemented in the DMol³ module of the Materials Studio 5.5 (Accelrys) was used in this work (31, 32). The cluster including first and second shell amino acid residues along with zinc were subjected to quantum mechanical calculation. To estimate the solvation effects of the rest of the enzyme that is not included in the model cluster, a homogeneous polarizable medium was considered with a continuum solvation model known as COSMO (conductor-like screening model), which is implemented in the DMol³ module (33). Geometry optimization calculations were performed using local density approximation with the usage of the Perdew-Wang parameterization (34) with the double numerical polarization (DNP) basis set (35). These second-shell ligands were verified for their thermodynamic contribution and zinc binding affinity with the first-shell residues using the Perdew-Wang functionals in DMol³ (35). Obtained thermodynamic parameters from DMol³ were.
construction and expression of four mdr family enzymes—
the plasmid containing the wild-type NcLAD gene was used for the production of the wild-type NcLAD protein. 
Escherichia coli strains harboring wild-type and mutated genes of NcLAD for protein expression were grown in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/ml) at 37 °C. Isopropyl-β-D-thiogalactopyranoside was added to the culture medium with a final concentration of 0.3 mM, and incubation was continued with shaking at 25 °C. The proteins were expressed in E. coli BL21 (DE3) using pET-28a (Novagen). To investigate the general role of the conserved Gly in zinc-dependent MDR family, we have also studied three other MDR enzymes. The three enzymes, Halofex fex mediterranei glucose dehydrogenase (HmGDH) (37, 38), Hypocrea jecora LAD (HjLAD), and Rhizobium etli CFN42 xylitol dehydrogenase (ReXDH) (39), were also cloned and expressed.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA), and confirmed by DNA sequencing. The recombinant plasmids containing the wild-type NcLAD, HmGDH, HjLAD, or ReXDH gene were used as the DNA templates. The plasmids containing the correct mutant genes were then used to transform E. coli BL21 (DE3), and colonies selected by kanamycin resistance were used for protein expression.

Purification and Protein Quantification—Purification of the wild-type and mutant enzyme was carried out following the same procedure as described previously (24). Cell pellets were resuspended in 20 mM sodium phosphate buffer, pH 7.5. The cell suspension was incubated on ice for 30 min in the presence of 1 mg/ml lysozyme. Cell disruption was carried out by sonication at 4 °C for 5 min, and the lysate was centrifuged at 4,000 × g for 5 min, and the lysate was centrifuged at 14,000 × g for 20 min at 4 °C to remove the cell debris. The resulting crude extract was retained for purification. The cell-free extract was applied onto a nickel-nitritolriacetic acid Super flow column (3.4 × 13.5 cm, Qiagen) previously equilibrated with a binding buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). Unbounded proteins were washed out from the column with a washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0). Then, the protein was eluted from the column with an elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Purification of HjLAD, HmGDH, and ReXDH was obtained as described previously (38, 39). The purity and identity of purified enzymes were confirmed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie Blue R250 using the prestained ladder marker (InVitrogen) as reference proteins. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard protein (40).

Enzyme Assay and Determination of Kinetic Parameters—
Initial rates were determined by measuring the absorbance change at 340 nm using a UV-visible spectrophotometer (Thermo Scientific, Waltham, MA) at 25 °C in 50 mM Tris, pH 8.0. Kinetic measurements with substrate and cofactor concentrations varied from below their K_m to 10-fold higher than their K_m. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol NAD(P)H/min under the assay condition. Kinetic parameters were determined in at least three independent measurements. The kinetic parameters, such as K_m and V_max, were determined from non-linear regression fitting of the Michaelis-Menten equation using Prism 5 (Graphpad Software, Inc., CA). Enzyme assay for three other MDR family enzymes was carried out as described in previous studies (38, 39).

Preparation and Reconstitution of Apoenzymes—
NcLAD wild-type and its variants G77A and G77E were purified and dialyzed to remove loosely bound or unbound metal ions. Furthermore, catalytically inactive apoLAD enzymes were prepared as described previously for an alcohol dehydrogenase (41–43). The resulting apoLAD enzymes (1–2 mg/ml) were reconstituted with 0.5, 1.0, 1.5, 2.0, 5.0, and 10 eq of ZnSO4 or ZnCl2 at room temperature (44) and assayed for catalytic activity. All glassware used for these procedures was washed with 1 M HNO3 and 1 M HCl for 16 h followed by rinsing with deionized distilled water and Chelex buffer.

Circular Dichroism—
Circular dichroism (CD) experiments were performed with a Jasco J-815 (Jasco Corp., Tokyo, Japan) spectrophotometer at 20 °C. Each spectrum was recorded in the 190–300-nm region at the rate of 100 nm/min. CD spectra were corrected with respect to the base line, and the measured ellipticity for each sample was expressed in millidegrees.

Inductively Coupled Plasma Mass Spectrometry for Analysis of Metal Concentrations—
Purified preparations of NcLAD wild-type and G77A and G77E mutant proteins were buffer-exchanged with 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, pH 8.0 (24). NcLAD enzymes were concentrated by ultrafiltration using Amicon-Ultra15 (Amicon Corp., Danvers, MA) to 5–6 mg/ml. The concentration of metals (zinc, Mn2+, Mg2+, Cu2+, Fe2+, Ni2+, Cd2+, Co2+, and Ca2+) was determined by high resolution inductively coupled plasma-Ms on a (OES Optima 2000 DV, PerkinElmer Life Sciences) at the Korea Basic Science Institute (Seoul, South Korea). The final buffer was used as a blank. Samples of the final buffer (from the last change) were also analyzed for metal content, and the observed concentrations were subtracted from those obtained in the enzymes samples. Three trials of each sample were taken and averaged.

Isothermal Titration Calorimetry—
The binding of zinc to the wide-type NcLAD and its mutants was monitored by ITC. The titration experiments were performed on Nano ITC low volume titration calorimeter (TA instruments, New Castle, DE). Apoproteins for ITC were prepared as previously reported (45). In a typical ITC experiment, ZnSO4 (10 μM) was titrated with the wild-type NcLAD (0.125 mM), G77A (0.1 mM), or G77E (0.09 mM) mutants. All solutions were filtered, degassed to avoid bubble formation, and equilibrated to the corresponding temperature before each experiment. The syringe was inserted into reaction cell, stirring (300 rpm) was started, and the instrument was equilibrated at 25 °C until the base line was flat and stable. The parameters used are described in the supplemental data. The titration data were analyzed using NanoAnalyzer software (TA Instruments) and an independent model to obtain the

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fitting graph and thermodynamic data of the experiments. The intrinsic molar enthalpy change (ΔH), the binding stoichiometry (n), and binding constant (K) for the binding process were obtained from the best fit of the calorimetric data. The Gibbs free energy change (ΔG) and the entropy change (ΔS) of binding were calculated from K and ΔH using the fundamental equations of thermodynamics, ΔG = −RT lnK; ΔS = (ΔH − ΔG)/T.

DFT Calculation on Zinc Coordination at Active Site—The crystal structure of NcLAD was obtained from the Protein Data bank (PDB code 3M61). The G77E mutant model was obtained by computational method as described in the previous section. G77E mutant structure was solvated with TIP3P explicit water as implemented in DS 3.0. To predict the structure and coordination geometry of zinc-bound wild-type NcLAD and G77E mutant, a cluster of active site residues (Cys-53, His-78, Glu-79, and Glu-163), target amino acid residues (Gly-77), and universal water molecule (4) was used in DFT calculation.

The generalized gradient approximation within DFT formalism, as implemented in the DMol3 module of the Materials Studio 5.5 (Accelrys Inc.), was used in this work (31, 32). All geometry optimization calculations were performed using the Becke-Lee-Yang-Parr (BLYP) exchange correlation functional (46) and the DNP basis set, as this was the best available set in DMol3 (47). This basis set considers a polarization d function on heavy atoms and a polarization p function on hydrogen atoms. With the BLYP/DNP theory, level errors are expected to be in the second decimal place of calculated bond lengths (angstroms) and on the order of 2–5 (kcal/mol) in energies (48). Consequently, full geometry optimization for each complex was carried out at the BLYP/DNP level using the DMol3 program. Vibrational frequencies were then computed using the same theory to verify that each complex was at the minimum of its potential energy surface. No imaginary frequency was found in any of the complexes. All quantum chemical calculations were performed in a water environment with the electronic structure package DMol3. To achieve self-consistent field energy convergence for each optimization cycle, the thermal treatment of electron occupancy with an electronic temperature of 0.005 Hartrees was implemented to aid convergence when needed (49). To incorporate solvent effects, the COSMO module employed by DMol3 was also implemented (50). For the solvent used in this study, water, the dielectric constant adopted was ε = 78.54. After the full optimization of structures, the metal ion site was analyzed by measuring bond distances and bond angles from the zinc ion to the first shell coordinating residues. The resulting structures were compared with the initial models, and the metal-ligand bond lengths and bond angles were compared with average values reported in the literatures. Furthermore, these fully optimized structures were used to estimate the electronegativity (χ) and hardness (η) (51, 52).

Electronegativity and Hardness of NcLAD-Zinc Complexes—One atomic parameter has long been known to be of great use in chemistry, the average of the ionization potential and electron affinity: the electronegativity. According to Koopmans’ theorem, the highest occupied molecular orbital (HOMO) energy (−εHOMO) is related to the ionization potential (I), and lowest unoccupied molecular orbital (LUMO) energy (−εLUMO) is related to electron affinity (A). Then average value of the HOMO and LUMO is related to the electronegativity (χ) defined by Mulliken (53). In addition, the HOMO-LUMO gap is related to the hardness (54). The HOMO and LUMO energies have been used to estimate the electronegativity (χ) and hardness (η) from the expressions

\[ \chi = \frac{\epsilon_{\text{HOMO}} + \epsilon_{\text{LUMO}}}{2} \]

and

\[ \eta = \epsilon_{\text{LUMO}} - \epsilon_{\text{HOMO}} \]

EPR Spectroscopy—X-band EPR spectra for NcLAD wild type and G77E were measured on a Bruker BioSpin spectrometer (GMBH) using a standard rectangular Bruker EPR cavity equipped with an Oxford helium flow cryostat (ESR 900). The microwave frequency (ν = 9.775 GHz) was measured using a Hewlett-Packard (HP5350B) frequency counter. The magnetic field was measured using a Bruker gaussmeter (ER035M). The EPR samples were prepared in solutions buffered to pH 8.0 with 0.05 M Tris-HCl-containing enzyme (55).

RESULTS

DFT-based Mapping of Critical Residues for MDR Protein Function—Alanine scanning mutagenesis was successfully used to study molecular determinants of ligand binding and protein function (56–58). However, the large number of mutants that are obtained from the replacement of all of the residues of a functional site makes the experimental procedure laborious and time consuming. Therefore, we sought to devise a computational screening approach that would allow rapid probing of residues lying in the second layer of the metal binding site. The accurate prediction of ligand binding affinities to a protein remains a desirable goal of computational biochemistry. Many available methods use molecular mechanics to describe the system. However, molecular mechanics force fields cannot fully describe the complex interactions involved in ligand binding, electron transfer, and polarization (59). To account for these interactions, in this study a DFT-based quantum mechanical code DMol3 was applied. Our recently determined crystal structure of NcLAD (25) was used to identify functionally critical amino acid residues in zinc-dependent MDR family enzymes. Three residues, Cys-53, His-78, and Glu-79, lie within 2.9 Å of the first coordinating shell of zinc. A 3.5 Å distance from the heavy atom of first shell-coordinating amino acid residues, was then used to locate the second-shell amino acid residues (Fig. 1). In total, a cutoff of 6.4 Å was used to identify the second-shell residues, with the zinc as the center for the search. The second layer of packing embodied a total of 11 amino acid residues, excluding the first shell-coordinating residues (Fig. 1). All 11 amino acids residues were replaced with alanine computationally, and then the quantum mechanical calculations were carried out to predict the energetic consequences (ΔG and Kd toward zinc) of mutations at all 11 residues. After vibration frequencies were analyzed for the optimized geometry of all the structures, the zero point vibrational energies, the standard molar enthalpies (ΔH), and standard molar Gibbs free energies (ΔG) were obtained. DFT calculations for in silico screening based on Kd indicated that out of 11 alanine mutants in the second shell of catalytic zinc, G77A substitution significantly altered the zinc binding affinity in the catalytic pocket of NcLAD (Table 1). The protein sequence of NcLAD was subjected to a BLAST search against PDB and a NCBI non-redundant protein data base. Multiple sequence
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Site-directed Mutagenesis of Gly-77—A number of Gly-77 mutants were constructed to confirm the importance of the Gly-77 residue by measuring their enzyme activity using the substrate L-arabinitol. The mutants including G77A, G77S, G77E, G77H, G77C, G77R, G77F, and G77W, and G77D were expressed in E. coli and purified to homogeneity as judged by SDS-PAGE (supplemental Fig. S3A). The overall expression of each mutant was similar to that of the wild-type enzyme and the purified protein from each mutant exhibited similar CD spectra, with ellipticity minima of comparable amplitude in the range of 220–240 nm (supplemental Fig. S3B). This similarity was in accordance with the structural superimposition of wild type and mutants (supplemental Fig. S3C). These results indicate that the global folding of mutant and wild-type NcLADs is similar. However, analysis of activity and comparison with the wild-type NcLAD revealed markedly decreased activity or abolished catalysis in NcLAD mutants. When compared with the wild-type enzyme at saturating substrate concentrations, the Gly-77 mutants showed almost no measurable activity.

The apoNcLAD wild-type enzyme exhibited a very low specific activity (0.3 ± 0.05 units mg-protein⁻¹) and could be activated by the addition of ZnSO₄. The specific activity of the zinc-reconstituted NcLAD wild-type enzyme was ∼90% that of the activity of holo-NcLAD wild-type. Although the wild-type NcLAD could be reactivated using exogenously supplied Zn, no such activation of G77A or G77E was observed. On the basis of their apparently weaker coordination environment, the G77A and G77E mutants are not likely to be activated by zinc reconstitution.

To further understand the basis of variations in specific activity between mutant enzymes, the kinetic constants $k_{cat}$ and $K_m$ were determined. However, detailed kinetic parameters were impossible to determine due to extremely low activity of the Gly-77 variants (Table 2), suggesting that Gly-77 plays a critical role in catalysis of LAD. Most notably, similar results were observed when other MDR enzymes including HjLAD (G90A, G90E), ReXDH (G65A, G65E), and HmGDH (G61E) were subjected to mutagenesis at the position equivalent to Gly-77 in NcLAD (Table 2, Fig. 2). Presumably, this conserved Gly has a common role in all MDR superfamily enzymes.

**Metal Content Analysis**—Purified NcLAD wild-type and mutants were prepared to determine metal content. The stoichiometry analysis of zinc compared with wild-type NcLAD revealed a significant reduction of total zinc content in mutants with a stoichiometry of 2.08 ± 0.1 Zn²⁺/subunit for the wild-type, 1.43 ± 0.12 Zn²⁺/subunit for G77A, and 1.31 ± 0.15 Zn²⁺/subunit for G77E. Mn²⁺, Ca²⁺, Ni²⁺, Cd²⁺, and Co²⁺ were below the detection limit, and no appreciable amounts of Mg²⁺, Fe²⁺ or Cu²⁺ were detected (Supplemental Table S1). The wild-type NcLAD contained two zinc atoms per monomer as inferred from the inductively coupled plasma-MS analysis, consistent with the presence of both a structural metal ion and a catalytic metal ion.

ITC was used to characterize the thermodynamics of NcLAD and its variants (G77A and G77E) binding to zinc. The heat output due to enzyme-metal interaction was fitted using Nano-Analyze software (TA Instruments) to give the dissociation output due to enzyme-metal interaction was fitted using Nano-Analyze software (TA Instruments) to give the dissociation constant $K_d$, the binding enthalpy $\Delta H$, and the binding order (stoichiometry). The ITC representative thermograms for the titration of apoNcLAD wild-type, G77A, and G77E with ZnSO₄ are shown, respectively, in Fig. 3. The binding curves were best fitted by a two-site binding equation, assuming a model of independent, i.e. non-interacting, sites. A zinc titration demonstrates that the binding affinity ($K_d$) of zinc to the wild-type NcLAD is 100- and 110-fold higher than that of G77A and G77E, respectively (supplemental Table S2). When compared with the wild-type (∼32.55 kJ mol⁻¹), the G77A and G77E mutants showed an increase in $\Delta G^\circ$ of 11.4 and 11.6 kJ mol⁻¹, respectively. The thermodynamic parameters summarized in supplemental Table S2 indicated that the mutation led to an increase in both negative enthalpy and negative

alignment was then performed between the protein query sequence and observed hits. Gly-77 was strictly conserved among the 68 MDR sequences from the PDB (supplemental Fig. S1) and the 250 MDR sequences from NCBI non-redundant database (supplemental Fig. S2). The role of the Gly-77 position was, therefore, further investigated by thorough site-directed mutagenesis.

**DFT-based quantitative in silico screening of critical residues in NcLAD**

| NcLAD variants | $\Delta G^\circ$ of binding | $K_d$ |
|----------------|---------------------------|-------|
| WT             | -43.8                     | 2.13 x 10⁻² |
| S2A            | -33.6                     | 1.30 |
| S55A           | -27.2                     | 0.170 |
| D56A           | -22.7                     | 125 |
| G77A           | -15.1                     | 2.50 x 10¹ |
| F127A          | -20.6                     | 316 |
| P164A          | -31.1                     | 3.50 |
| V167A          | -18.0                     | 794 |
| I191A          | -21.3                     | 182 |
| Y309A          | -34.4                     | 0.920 |
| K356A          | -19.5                     | 388 |

**TABLE 1**

Thermodynamic parameters including $K_d$ toward zinc ion were determined for NcLAD wild type and its mutants are given.
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**TABLE 2**

Kinetic parameters of Gly mutants

| Enzymes         | Specific activity | Coenzyme | Substrate | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------------|------------------|----------|-----------|-----------|--------------|
|                 | Units mg-protein$^{-1}$ | $K_m$ | $v_{max}$ | $k_{cat}$ | $K_m$ | $v_{max}$ |
| NcLAD$_{WT}$    | 41.2 ± 1.4        | 0.90 ± 0.02 | 16.3 ± 1.2 | 1600 ± 39 | 100 ± 9    |
| G77A            | 0.400 ± 0.02      | 0.020 ± 0.01 | 31.1 ± 2.3 | 15.8 ± 2.2 | 0.500 ± 0.09 |
| G77S            | 0.300 ± 0.02      | 0.020 ± 0.01 | 72.4 ± 6.2 | 12.1 ± 2.1 | 0.160 ± 0.04 |
| G77E            | ND                | ND        | ND        | ND        | ND        |
| G77D            | ND                | ND        | ND        | ND        | ND        |
| G77C            | ND                | ND        | ND        | ND        | ND        |
| G77H            | ND                | ND        | ND        | ND        | ND        |
| HjLAD$_{WT}$    | 89.2 ± 10.3       | 0.70 ± 0.01 | 16.6 ± 1.3 | 3560 ± 70 | 222 ± 18   |
| G90A            | 0.300 ± 0.02      | 1.5 ± 0.1  | 38.1 ± 3.1 | 12.3 ± 2.1 | 0.750 ± 0.18 |
| G90E            | ND                | ND        | ND        | ND        | ND        |
| ReXDH$_{WT}$    | 32.1 ± 5.7        | 0.50 ± 0.01 | 15.5 ± 3.1 | 1220 ± 28 | 78.0 ± 16.9 |
| G65E            | ND                | ND        | ND        | ND        | ND        |
| HmGDH$_{WT}$    | 38.4 ± 7.6        | 0.50 ± 0.01 | 12.3 ± 1.6 | 1900 ± 54 | 158 ± 30   |
| G61E            | ND                | ND        | ND        | ND        | ND        |

Entropy, whereas the negative Gibbs free energy was decreased as a consequence of the mutation. These results suggest that although the interaction between the G77A/G77E and the zinc is enthalpically more favorable than that for the wild-type NcLAD, the entropic loss is also increased, indicating that the unfavorable entropic change outweighs the enthalpic advantage.

**DFT Calculation on Zinc Coordination at Active Site**—The influence of Gly-77 replacements on the structural and electronic properties of the enzyme/metal ternary complex was further investigated by a DFT-based computational study. Each of the initial structures was energy-minimized using BLYP/DNP basis sets. The zinc coordination angles and coordination distances in the zinc primary coordination sphere were compared between the wild-type and mutant structures. The optimized zinc-ligand bond lengths for a tetrahedral binding site in the wild-type NcLAD are Zn-N (His) 2.09 Å, Zn-S (Cys) 2.29 Å, Zn-O (Glu) 2.0 Å, and Zn-O (water) 2.06 Å (Fig. 4A), which are in good agreement with the values reported by others (60, 61): Zn-N (His) 2.07–2.09 Å, Zn-S (Cys) 2.21–2.35 Å, Zn-O (Asp/Glu) 1.95–2.04 Å, and Zn-O (water) 2.12–2.15. The angles for the tetrahedral binding site in the NcLAD wild-type are reasonably close to the ideal average values for the tetrahedral coordination (Fig. 4B). Overall, the calculated bond length parameters are in agreement with the PDB surveys of similar zinc coordination. However, a significant perturbation of zinc binding was observed with a sulfur of Cys-53 and oxygen of the water molecule at the primary coordination shell of G77E. The distances of Zn-S (Cys), Zn-O (water) for the wild-type and the G77E mutant are 2.29/2.06 and 2.7/6.8, respectively (supplemental Table S3, Fig. 4A and C). Most strikingly, the G77E mutant has acquired trigonal planar electron pair geometry, where the fourth ligand of zinc coordination, oxygen of water (Zn-O (water)) was replaced by Glu-163, and Zn-S coordination was disrupted. The protein-metal affinity was significantly compromised by Gly-77 substitutions.

**Electronegativity of NcLAD-Zinc Complexes**—The electronegativity ($\chi$) and hardness ($\eta$) of NcLAD wild-type and G77E mutant zinc complexes (Fig. 4) were evaluated. The $\chi$ values in Table 3 provide a measure of zinc ability to act as a Lewis acid. To provide a physical basis for the greater $\chi$ values of the G77E zinc site as compared with the wild-type zinc site, the net charge transferred by all the ligands to zinc was computed from the zinc charge. As the $\chi$ of the complex increases, the net charge transferred from the ligands to zinc decreases (Table 3).
The concepts of the electronegativity incorporated with oxidation state and coordination number suggest that the electronegativity increases with increasing oxidation state and decreases with increasing coordination number (62). Indeed, our DFT calculations on NcLAD variants provide the same trend, where the increased electronegativity of G77E zinc core gave a decrease in the coordination number (Table 3).

**DISCUSSION**

The first step in our study was to identify critical amino acid residues that could contribute to understanding the relationship between function, structure, and dynamics of MDR family enzymes including LAD. We focused primarily on the second-shell residues for mutational studies because these residues often have indirect but significant effects on both structure and function of enzymes and are not well understood. In NcLAD, we screened critical residues by calculating their affinities toward zinc using DFT and identified Gly-77 as a residue that is critical in LAD catalysis and strictly conserved in the zinc-dependent MDR family. More specifically, Gly-77 is part of the conserved GHE motif, which is conserved in all zinc-dependent MDR family members, and its role has not previously been investigated.

Unusual zinc binding behaviors in Gly-77 NcLAD mutants were observed. The stoichiometry analysis of zinc revealed a significant reduction of the total zinc content in mutant NcLADs compared with the wild-type NcLAD. The observations from the inductively coupled plasma-MS analysis encouraged us to carry out theoretical calculations on the wild-type and mutant structures and to optimize the geometry and find the geometrical parameters of the zinc primary coordination sphere. The catalytic zinc sites in MDR including NcLAD are generally composed of three amino acid ligands and a water molecule. The catalytic zinc in NcLAD is coordinated by the active site residues (Cys-53, His-78, and Glu-79) and a water molecule completing a near-tetrahedral coordination sphere. The zinc coordination geometries are governed by electrostatics particularly repulsions between the zinc-coordinating atom-bonded pairs. Therefore, all T4 zinc sites are found to have tetrahedral coordination geometries (60). The trigonal planar geometry of zinc in G77E mutant is rare, and only a few such examples are known (63, 64). In a trigonal planar zinc coordination geometry of the G77E mutant, zinc is approximately in the same plane of the three ligands with the absence of zinc-thiolate coordination. It is evident from the bond lengths and bond angles obtained by DFT calculations that the mutation at position Gly-77 forces a distortion of the metal complexes from the ideal geometry, weakening the corresponding bonds and resulting in distorted bond angles and an increase in the bond lengths (Fig. 4).

Zinc complexes show facile four-to five-coordinate interconversion during catalysis, where zinc acts as a Lewis acid and facilitates zinc-bound water to ionize to a nucleophilic hydroxide in enzyme. Ionization of the activated water or its polarization brought about by a base form of an active-site amino acid provides hydroxide ions at neutral pH, and displacement of water or expansion of the coordination sphere results in Lewis acid catalysis by the catalytic zinc atom (65). Notably, Lee and
Lim (66) reported that the zinc Lewis acidity can be modulated by its bound ligands; a net change of 0.2–1.6 Å in bond lengths can modulate zinc Lewis acidity. Whether a given molecule is a Lewis acid or a base is determined by its \( \chi \) value (53). Using the operational definition, we quantitatively estimated the electronegativity of the NcLAD wild-type and G77E mutant zinc cores. G77E mutant showed higher electronegativity compared with NcLAD wild type. However, the increased electronegativity would also increase the hardness of G77E mutant zinc core in a linear correlation (52). Based on the hard-soft acid-base theory, zinc is regarded as a borderline acid, showing properties intermediate between hard and soft (67). Indeed, zinc has the ability to polarize its bound ligands and can interact with ligands such as sulfur from Cys-53, nitrogen, and oxygen from His-78, Glu-79, and water, respectively, in the wild-type NcLAD. A high \( \eta \) value means high hardness. In this respect, G77E mutant zinc core has a much “harder” character (Table 3), resulting in a strong preference for ligand such as the oxygen from glutamate (68). Therefore, the hard G77E mutant zinc core will be less polarizable than that of the soft NcLAD wild-type zinc core. Soft molecules are more reactive than the hard molecules if electron transfer or rearrangement is necessary for the reaction (51). The soft nature of NcLAD wild-type catalytic zinc core accounts for its catalytic activity, where abstraction of the proton followed by hydride transfer is involved (55). Comparatively, the hard G77E mutant zinc core can resist changes in its electronic state (supplemental Fig. S4). Taken together, the zinc ion and the residues bound directly to the zinc ion represent a functional charge/dipole complex. Polarization of this complex, which translates to coordination distortion, may tune the electronegativity/hardness of zinc and, hence, its reactivity.

### TABLE 3
Electronegativity (\( \chi \)) and hardness (\( \eta \)) values of NcLAD wild-type, G77A, and G77E mutants zinc cores

| Enzyme   | Zinc core                  | \( \chi \) | \( \eta \) | CT | CN |
|----------|----------------------------|------------|------------|----|----|
| NcLAD\(_{WT}\) | [Zn\(^{2+}\), Cys Glu His Gly Glu H\(_2\)O] | 3.67       | 1.12       | 1.36 | 4 |
| NcLAD\(_{G77A}\) | [Zn\(^{2+}\), Cys Glu His Ala Glu H\(_2\)O] | 4.02       | 2.48       | 1.27 | 3 |
| NcLAD\(_{G77E}\) | [Zn\(^{2+}\), Cys Glu His Glu Glu H\(_2\)O] | 4.49       | 3.07       | 1.23 | 3 |

\( ^a \) Evaluated at the local density approximation-Vosko-Wilk-Nusair/DNP level.

\( ^b \) Net charge transfer (CT) from all the ligands to Zn\(^{2+}\), which is equal to \( (2 - \text{the local density approximation/Vosko-Wilk-Nusair Natural bond orbital charge on Zn}^{2+}) \).

\( ^c \) Coordination number.

FIGURE 4. Active site coordination. Optimized geometries of the wild-type NcLAD and G77E mutant were obtained from BLYP/DNP level of calculation. Active site coordinating bond lengths (Å) of the wild type (A) and G77E (C) with the catalytic zinc are shown. Similarly, the active site bond angles (\( \angle \)) for the wild-type (B) and G77E (D) were mapped using MS 5.5. For a clear image, hydrogens were removed, and figures were generated using MS 5.5.
EPR measurements were carried out to provide additional evidence to the nature of the zinc coordination in the wild-type NcLAD and the G77E mutant. Zinc containing a filled orbital \(3d^{10}\) can participate in enzymatic redox reactions. The reactivity of zinc is governed by its potential as a Lewis acid and by its amphoteric properties (4). In addition, the filled states, respectively, which are not EPR active species due to its orbital of \(d\) orbital of zinc results in ligand-field stabilization energy that is zero in all directions, rendering zinc invisible to most spectroscopic methods of investigation (6, 69). However, the EPR studies revealed marked differences between the wild-type NcLAD and the G77E mutant (Fig. 5). Similar spectral changes in other metal ions reported previously support that single point mutation may result in differences in EPR spectra (70–72). No signal was observed in the EPR spectra of the wild-type NcLAD and G77E mutant. EPR experiments were performed as described under “Experimental Procedures.”

Collectively, these studies report the discovery and detailed characterization of a strictly evolutionarily conserved Gly-77 residue in the zinc-dependent MDR superfamily and the unexpected change of the zinc-coordination geometry in the mutant MDR. The systematic DFT investigation followed by mutagenesis, thermodynamic analysis, and EPR studies on the catalytic zinc site in the MDR enzymes identified the conserved Gly amino acid residue that maintains electronic and geometrical flexibility at the primary coordination shell of the zinc ion during catalysis of the MDR family enzymes. The conserved Gly in the GHE motif is involved in the metal binding affinity and the electronic state of the catalytic zinc ion in the MDR family. The reported observations should be helpful in understanding the protein/metal interaction in the zinc-dependent MDR family.

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FIGURE 5. X-band EPR spectra (shown in units of gauss) of the wild-type NcLAD and G77E mutant. EPR experiments were performed as described under “Experimental Procedures.”
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