

Article

Dimethylglycine as a Potent Modulator of Catalase Stability and Activity in Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss, cognitive decline, and oxidative stress-driven neuronal damage. Catalase, a key antioxidant enzyme, plays a vital role in decomposing hydrogen peroxide (H₂O₂) into water and oxygen, thereby protecting neurons from reactive oxygen species (ROS)-mediated toxicity. In AD, the catalase function is compromised due to reduced enzymatic activity and aggregation, which not only diminishes its protective role but also contributes to amyloid plaque formation through catalase-A β co-oligomers. Hence, therapeutic strategies aimed at simultaneously preventing catalase aggregation and enhancing its enzymatic function are of great interest. In this study, we screened twelve naturally occurring metabolites for their ability to modulate catalase aggregation and activity. Among these, dimethylglycine (DMG) emerged as the most potent candidate. DMG significantly inhibited thermally induced aggregation of catalase and markedly enhanced its enzymatic activity in a concentration-dependent manner. Biophysical analyses revealed that DMG stabilizes catalase by promoting its native folded conformation, as evidenced by increased melting temperature (T_m), higher Gibbs free energy of unfolding (ΔG°), and reduced exposure of hydrophobic residues. TEM imaging and Thioflavin T assays further confirmed that DMG prevented amyloid-like fibril formation. Molecular docking and dynamics simulations indicated that DMG binds to an allosteric site on catalase, providing a structural basis for its dual role in stabilization and activation. These findings highlight DMG as a promising therapeutic molecule for restoring catalase function and mitigating oxidative stress in AD. By maintaining catalase stability and activity, DMG offers potential for slowing AD progression.

Keywords: catalase; oxidative stress; dementia; DMG; thermal stability



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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss, cognitive decline, disorientation, and behavioral disturbances such as aggression and emotional instability, ultimately leading to the loss of functional and adaptive capacities [1–3]. It primarily affects the elderly population, and it is the most prevalent form of dementia worldwide [4,5]. The hallmark pathological features of AD include the accumulation of extracellular insoluble beta-amyloid (A β) plaques and intracellular

neurofibrillary tangles composed of hyperphosphorylated tau protein [6–8]. These abnormal protein aggregates disrupt cellular processes and are closely linked to mitochondrial dysfunction and the overproduction of reactive oxygen species (ROS), leading to oxidative stress [9–12]. Oxidative stress occurs when ROS production overwhelms the cell's antioxidant defense mechanisms, causing damage to lipids, proteins, and DNA. This damage promotes neuronal dysfunction, synaptic loss, and eventual cell death, which together drive the progression of AD [11,13–15].

Cells naturally counter oxidative stress using both enzymatic and non-enzymatic antioxidant systems that neutralize free radicals, prevent their formation, and sequester transition metals that catalyze oxidative reactions [16–18]. Among enzymatic antioxidant enzymes, catalase plays a particularly vital role. Catalase decomposes hydrogen peroxide (H_2O_2), a major ROS, into water and oxygen at an exceptionally high rate, a single catalase molecule can break down millions of H_2O_2 molecules per second [19–24]. This remarkable efficacy highlights its importance in maintaining redox balance and protecting neurons from oxidative damage.

Recent studies suggest that the function of catalase is impaired in AD. Reduced catalase activity has also been reported in the brains of AD patients, which is considered to be a major contributor to elevated ROS levels in the disease [25–27]. Additionally, catalase has been shown to interact directly with A β peptides, forming catalase-A β co-oligomers that have been detected within amyloid plaques, further worsening pathology [28,29]. These observations suggest that catalase dysfunction contributes to disease progression through not only loss of the enzymatic activity but also via toxic aggregation. Therefore, therapeutic strategies aimed at, inhibiting catalase aggregation and enhancing its enzymatic activity could be valuable for mitigating AD-related oxidative stress.

One promising approach involves the use of small molecule metabolites naturally accumulated by organisms under stressful environmental conditions. Small molecules and naturally occurring metabolites are increasingly recognized for their ability to stabilize proteins and reduce misfolding under stress. Well-studied osmolytes such as TMAO, betaine, and proline can shift the folding balance toward the native, functional state and lower the chance of aggregation [30]. Mechanistic interaction toward the pushing folding process, these molecules are often excluded from the immediate surface of the protein, which makes the compact, properly folded state more favorable than the unfolded one [31,32]. By helping proteins keep their natural shape and reducing exposure of hydrophobic regions, they lower the likelihood of forming amyloid-like aggregates. The broader principle of improving stability through structural modulation is also seen in other fields—for example, engineered grooved-porous materials can enhance thermal transport by controlling local micro-environments [33–35]. Although non-biological, this concept mirrors how small molecules can support protein stability. Based on this idea, in the present study, we screened 12 different such small metabolites found in various organisms to assess their effects on catalase aggregation. Using biophysical, biochemical, and in-silico approaches, we identified DMG as the most promising candidate. DMG significantly reduced catalase aggregation while enhancing its enzymatic function, likely by stabilizing catalase in its native conformation. This study highlights the therapeutic potential of DMG and similar molecules in reducing catalase dysfunction and oxidative damage associated with AD.

2. Materials and Methods

Commercially available lyophilized preparation of catalase from bovine liver (Cat. no. C9322) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2 ; 30% *v/v*), potassium chloride, di-potassium hydrogen phosphate, potassium di-hydrogen phosphate, 8-Anilino-naphthalene-1-sulfonic acid (ANS), Thioflavin

T, trimethyl N-oxide (TMAO), proline, trehalose, glycine-betaine, myoinositol, sarcosine, DMG, ectoine, hydroxyectoine, taurine, mannitol, and beta alanine were also obtained from the Sigma.

2.1. Preparation of Stock Solutions

Protein samples of catalase were dialyzed overnight against phosphate buffer (pH 7.0) containing 0.1 M KCl and protein stock solutions were filtered using 0.22 µm syringe filters. The concentration of protein solution was calculated using molar absorption coefficient, ϵ value of 324,000 M⁻¹ cm⁻¹ at 405 nm. The ANS stock solution was prepared using a molar extinction coefficient of 26,620 M⁻¹ cm⁻¹ at 416 nm. All other chemicals were of analytical grade and were used without any additional purification. All solutions for optical measurements were made in a degassed 0.05 M phosphate buffer (pH 7.0) containing 0.1 M KCl, with double-distilled water serving as the aqueous medium. The H₂O₂ stock solution were always freshly prepared in 50 mM phosphate buffer at pH 7.0, and its concentration was determined using a molar extinction coefficient (ϵ_{240}) of 40 M⁻¹ cm⁻¹. And stock solution of DMG and other metabolites were prepared in 50 mM PBS buffer at pH 7.0. All reaction mixtures were monitored for any pH changes upon the addition of metabolites, which shows no significant variation.

2.2. Protein Aggregation Studies for Catalase

Catalase (0.75 mg/mL in 0.05 M potassium phosphate buffer pH 7.0) was incubated overnight in presence of various concentrations (100 µM, 200 µM, 500 µM and 1000 µM) of 12 different osmolyte at room temperature. Then, aggregation profiles were monitored by measuring the change in the light scattering intensity (at 360 nm) at 55 °C for 10 min using a Cary Eclipse fluorescence spectrophotometer (Santa Clara, CA, USA) equipped with a single cell Peltier controller. The measurements were carried out for at least three times. Kinetic parameters (lag time, k_{app} , and I_f) of catalase aggregation were analyzed using the following the equation:

$$I = I_o + \frac{I_f}{1 + e^{-\left(\frac{t-t_o}{b}\right)}} \quad (1)$$

where I is the fluorescence intensity at time t , and t_o is the time at 50% maximal light scattering. I_o represents the fluorescence intensity of the initial baseline, and I_f represents the fluorescence intensity of the final plateau line, respectively. b is a constant. The apparent rate constant, k_{app} for the formation of aggregates is given by $1/b$, and lag time is given by $t_o - 2b$ [36,37]. Each curve was independently analyzed for the respective kinetic parameters. b is the constant that represents slope of the log phase.

2.3. Measurement of Catalase Activity

The catalase activity was measured using a spectrophotometric method with an Agilent Cary UV/Vis Spectrophotometer (Santa Clara, CA, USA). The assay monitored the decrease in absorbance of hydrogen peroxide (H₂O₂) at 240 nm in the presence of catalase, reflecting the catalytic decomposition of hydrogen peroxide [38]. For monitoring the effect of various metabolites on catalase activity, 10 nM concentration of catalase was preincubated at ambient temperature with varying concentration of each metabolite. An enzymatic reaction was initiated upon addition of hydrogen peroxide (H₂O₂) and substrate degradation was monitored by recording the optical density (OD) at 240 nm after 12 min.

2.4. Calculation of Specific Activity

Specific activity of the enzyme was determined by dividing the measured enzymatic activity by the total protein concentration (expressed as mg of protein per unit volume). To

calculate enzyme activity, the final absorbance recorded after 12 min was converted to molar concentration using the molar extinction coefficient of hydrogen peroxide ($43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The resulting enzyme activity (per unit time) was then normalized against the total protein content to yield specific activity.

2.5. Thermal Denaturation Studies

Thermal denaturation studies of catalase were performed using a Jasco V-660 UV/Vis spectrophotometer (Tokyo, Japan) equipped with a Peltier type controller (ETCS-761) at a heating rate of $1 \text{ }^{\circ}\text{C}/\text{min}$ from $20 \text{ }^{\circ}\text{C}$ to $85 \text{ }^{\circ}\text{C}$. The change in absorbance for catalase was followed at 292 nm. Measurements were repeated three times. After each denaturation, reversibility was measured for each sample. The reversibility was checked by comparing the optical property of the native protein before and after denaturation and was found to be identical. All solution blanks were neglected during the data analysis, as they showed negligible change in absorbance with temperature. For obtaining T_m (the midpoint of thermal transition) and ΔH_m (the enthalpy changes in denaturation at T_m) of each transition curve, a non-linear least-squares analysis equation was used:

$$y(T) = \frac{y_N(T) + y_D(T) \exp[-\Delta H_m / R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m / R(1/T - 1/T_m)]} \quad (2)$$

where $y(T)$ is the optical property at temperature T (Kelvin); $y_N(T)$ and $y_D(T)$ are the optical properties of the native and the denatured protein molecules, respectively; and R is the gas constant. During analysis, it was assumed that a parabolic function describes the dependence of the optical properties of native and denatured protein molecules: i.e., $y_N(T) = a_N + b_NT + c_NT^2$, and $y_D(T) = a_D + b_DT + c_DT^2$, where a_N , b_N , c_N , a_D , b_D , and c_D are temperature-independent coefficients. Using a constant value of ΔC_p (the constant pressure heat capacity change), i.e., 1.02 kcal M^{-1} , the value of $\Delta G_D^{\circ}(T)$ (ΔG_D at any temperature T) was estimated with the help of the Gibbs–Helmholtz equation:

$$\Delta G_D(T) = \Delta H_m \left(\frac{T_m - T}{T_m} \right) - \Delta C_p \left[(T_m - T) + T \ln \left(\frac{T}{T_m} \right) \right] \quad (3)$$

2.6. Circular Dichroism (CD) Measurements

Far- and near-UV (CD) spectra of native catalase were measured in the absence and presence of different concentrations of DMG in a Jasco J-810 spectropolarimeter (Tokyo, Japan) equipped with a Peltier-type temperature controller. Each spectrum of the protein was corrected for the contribution of its blank from the entire wavelength range. Far-UV spectra were obtained by recording wavelength scans from 200 to 240 nm, while near-UV spectra were recorded from 250 to 320 nm. The concentration of the protein used was 0.75 mg/mL at pH 7.0. Cells with path lengths of 0.1 and 1.0 cm were used for the measurements of far- and near-UV CD spectra, respectively.

2.7. Fluorescence Spectral Measurements

Fluorescence spectra of the protein samples were collected in Perkin Elmer LS 55 spectrofluorometer (Shelton, CT, USA) using a 3 mm quartz cuvette. Slit widths for both excitation and emission were set at 10 nm with a scanning rate of 100 nm/min at $25 \text{ }^{\circ}\text{C}$. Protein concentration used for all experiments was 0.5 mg/mL . Protein samples were incubated overnight in the absence and presence of various concentrations of DMG. For intrinsic fluorescence measurements, excitation wavelength was set to 295 nm and emission spectra were recorded from 290 to 450 nm. All the measurements were recorded in triplicates.

Extrinsic fluorescence measurements were performed using ANS dye with excitation wavelength of 350 nm and the emission spectra were collected from 400 to 600 nm. ANS concentration was taken 16 times higher than that of protein concentration. Samples were incubated with ANS for 30 min prior to spectrum collection. Each measurement was performed three times and adjusted for the corresponding buffer background.

2.8. Light Scattering Assay

To evaluate the anti-aggregation effect of dimethylglycine (DMG) on catalase, the protein samples were first filtered through a 0.22 μm membrane filter. Catalase was used at a final concentration of 0.75 mg/mL and incubated in the absence or presence of varying concentrations of DMG at 37 °C overnight in a water bath. Following incubation, light scattering measurements were performed at 360 nm (both excitation and emission wavelength of 360 nm) using a Perkin Elmer LS 55 spectrofluorometer (Shelton, CT, USA) using a 3 mm quartz cuvette. All measurements were conducted in triplicates.

2.9. Transmission Electron Microscopy (TEM)

Protein samples incubated at 55 °C in the presence and absence of DMG were pelleted. The pelleted protein samples (10 μL) were placed and kept for drying on a copper grid for 6 min at room temperature. Negative staining was completed with 1.0% uranyl acetate and samples were again allowed to air dry. Finally, samples were examined under FEI Tecnai G2-200 kV HRTA transmission electron microscopy (SAIF, AIIMS, New Delhi, India) operating at 200 kV.

2.10. Fluorescence Microscopy

Catalase (0.75 mg/mL) was incubated overnight at room temperature in the absence or presence of dimethylglycine (DMG). Following incubation, samples were heat-treated at 55 °C for 5 min to induce amyloid-like fibril formation, then allowed to cool to room temperature. The samples were then incubated with ThT solution for 30 min to enable selective binding of the dye to amyloid fibrils. After staining, samples were mounted on microscope slides and examined under a fluorescence microscope. Images were captured using an excitation filter (450–490 nm) and an emission filter (≥ 520 nm). The ThT-fluorescence intensity was quantified from the images to assess the presence and extent of amyloid fibril formation.

2.11. Statistical Analysis

All experiments were performed in triplicates. The mean of the independent measurements was calculated, and standard errors were computed. These standard errors are either represented as error bars in the figures or reported as $\pm$ standard deviation. Statistical significance (p -values) was determined using one-way ANOVA in GraphPad Prism software (version 10).

2.12. Molecular Docking

The crystal structure of human catalase (PDB ID: 7VD9), which includes a heme moiety in the binding site, was selected for the molecular docking studies. Protein structure optimization was carried out using the protein preparation wizard in the GLIDE of Maestro v11. This process involved assigning bond orders, adding hydrogen atoms, optimizing hydrogen bonding networks, and performing restrained energy minimization to refine the protein structure. The ligand, dimethylglycine (PubChem CID: 673), was prepared using the LigPrep module in Maestro. Ligand preparation included the generation of low-energy 3D conformers, ionization state prediction at physiological pH (7.0 ± 2.0), and energy minimization using the OPLS4 force field. To identify potential binding sites on the

protein, SiteMap was employed. Five distinct grid boxes were generated to cover different regions of the protein structure. Docking studies were then performed using Maestro Glide module. Each ligand was docked into the defined grid boxes, and the resulting poses were evaluated based on docking scores and Glide energies. These parameters were used to determine the most favorable binding conformations and interactions between the ligand and the protein.

2.13. Molecular Dynamics (MD) Simulations

Molecular dynamics simulations were performed using GROMACS version 2022.04 (Groningen, The Netherlands) to evaluate the stability and binding behavior of the protein–ligand complexes [39]. The CHARMM27 force field was selected because it has been extensively validated for protein–ligand studies and provides reliable treatment of electrostatic and hydrogen-bonding interactions relevant to allosteric stabilization. Ligand topologies were generated using SwissParam 2023, and protein topologies were prepared using the CHARMM 27 force field modules implemented in GROMACS. Each complex was solvated in a cubic TIP3P water box (edge length 9.6 nm). TIP3P is the recommended water model for CHARMM and ensures consistent hydration behavior around the active and distal binding sites. System neutrality was achieved by adding Na⁺ and Cl[−] ions, and the ionic strength was set to 0.15 M to reproduce physiological salt conditions and realistic electrostatic screening [40]. Energy minimization was performed using the steepest descent algorithm with a convergence criterion of $F_{\text{max}} < 1000 \text{ kJ/mol/nm}$. The system was equilibrated in two steps: (i) NVT ensemble for 1 ns to stabilize temperature at 300 K, followed by (ii) NPT ensemble for 1 ns to achieve correct system density and maintain pressure at 1 bar [41]. This standard equilibration protocol prevents structural artifacts before production runs. Periodic boundary conditions (PBC) were applied in all directions to avoid edge effects and mimic an infinite solvent environment. Long-range electrostatics were calculated using the Particle Mesh Ewald (PME) method, which provides accurate treatment of electrostatic interactions such as hydrogen bonds and salt bridges that contribute to protein stability. The LINCS algorithm was used to constrain all bonds involving hydrogen atoms, allowing a stable 2 fs timestep without numerical instability [42]. The ligand was restrained whenever protein restraints were applied during equilibration to avoid artificial displacement. Production MD simulations were run for 100 ns for each complex. This timescale is commonly used in protein–ligand MD studies and was sufficient for the catalase system, as RMSD and Rg values reached stable plateaus well before 100 ns, indicating that the complexes had entered equilibrated conformational regimes [43,44]. Trajectory analyses included RMSD, RMSF, radius of gyration (Rg), solvent-accessible surface area (SASA), and hydrogen bonding to compare structural stability and dynamic behavior of catalase in the presence and absence of DMG [45,46].

3. Results

3.1. Screening of Metabolites Having Inhibitory Effect on Catalase Aggregation

Since catalase is known to undergo temperature-induced aggregation at its melting temperature (T_m) of 55 °C, we thus examined anti-aggregating potential of 12 different metabolites: proline, trehalose, betaine, sarcosine, dimethylglycine (DMG), myo-inositol, ectoine, hydroxyectoine, taurine, N-acetylaspartate (NAA), mannitol, and β -alanine on catalase. To assess their effect, native catalase was incubated overnight with different concentrations of each osmolyte at room temperature, and its time-dependent oligomerization was monitored by measuring light scattering intensity at 360 nm set at 55 °C. As shown in Figure 1, catalase aggregation followed a typical sigmoidal aggregation–kinetics curve, indicative of a nucleation-dependent aggregation process. In the absence and presence of

metabolites, catalase displayed pronounced aggregation, evidenced by a sharp increase in light scattering over time. These time-dependent aggregation curves obtained in the absence and presence of the metabolites were further analyzed for the effect of metabolites on lag time (nucleation phase), apparent rate constant (K_{app}), and final aggregate intensity (I_f), as presented in Table S1. At least three different effects are observed in the presence of different metabolites. Some metabolites including sorbitol, glycerol, mannitol, trehalose, and β -alanine do not exhibit any significant change in the aggregation behavior. In contrast, metabolites like histidine, sucrose, taurine show a slight increase in the I_f values without impacting lag time and rate of aggregation, K_{app} . Only three metabolites including ectoine, hydroxyectoine, and DMG exhibited moderate to large impact on decreasing the aggregation behavior. To have a feel of the metabolites having modest effect, we have plotted a bar diagram of the percent disaggregates (based on the observed I_f values) obtained in the presence of highest concentration of different metabolites (Figure S1). Out of 12 metabolites, DMG shows a modest effect in inhibiting catalase aggregation. Interestingly, DMG not only impacted the I_f values significantly but also prolonged the lag time indicating that it suppresses catalase aggregation by stabilizing the native state of catalase.

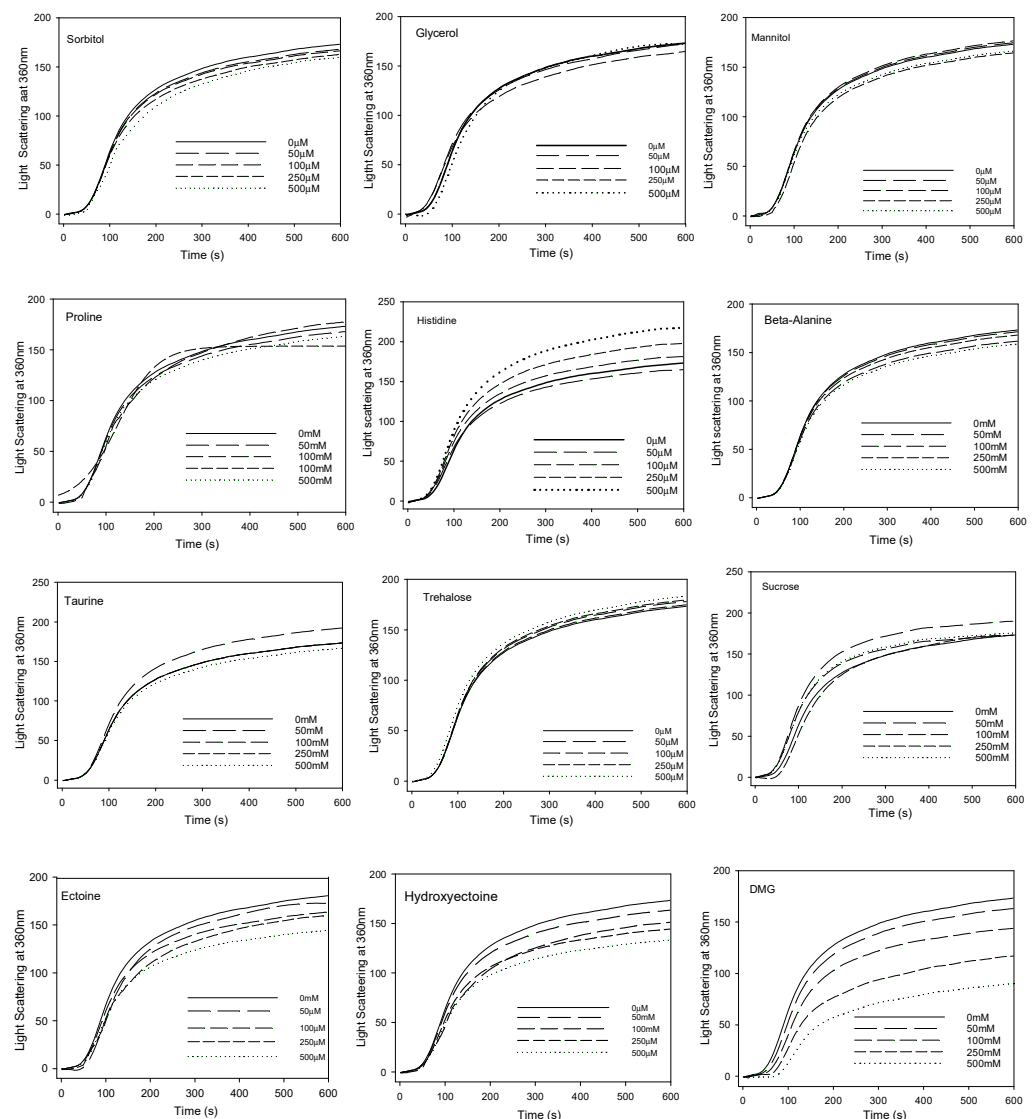


Figure 1. Time-dependent aggregation profiles of catalase with various metabolites. Aggregation kinetics of catalase monitored by observing changes in the light scattering intensity at 360 nm in the presence of increasing concentrations of metabolites.

3.2. DMG Reduces the Amyloid Character of Catalase Aggregates

To further substantiate the anti-aggregating effect of DMG on catalase, we examined the morphology of oligomers formed in the presence of DMG using Transmission electron microscopy (TEM). Figure 2A shows that catalase forms dense, amyloid-like aggregates under thermal stress. However, treatment with DMG led to a marked reduction in both the size and density of these aggregates, suggesting an inhibition of amyloidogenic oligomer formation. We also confirmed these observations using fluorescence microscopy, which showed a clear correlation between the presence of DMG and the disappearance of oligomers, accompanied by a marked decrease in aggregate formation (Figure 2B), which is consistent with the quantitative ThT fluorescence image analysis presented in Figure S2.

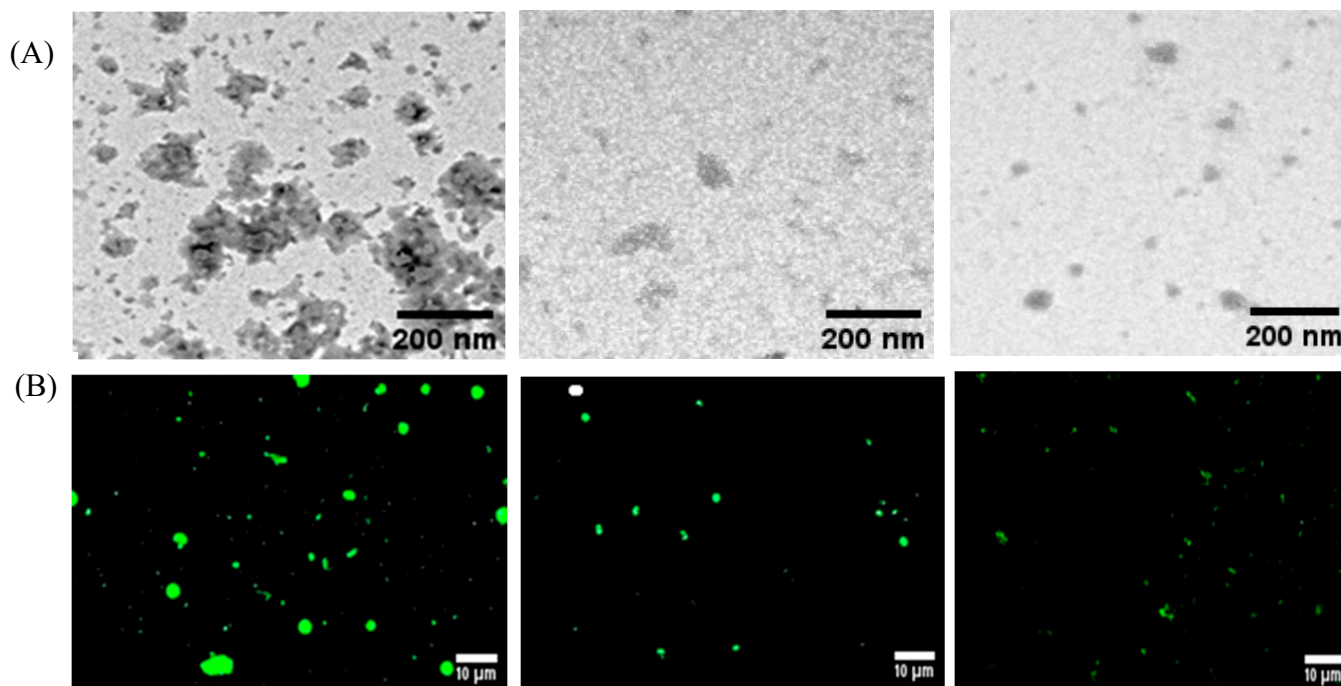


Figure 2. Morphology of catalase aggregates in presence and absence of DMG. (A) TEM images, (B) ThT Fluorescence microscopy images (L-R: Control, in presence of 200 μM and 500 μM DMG, respectively).

3.3. DMG Enhances Catalase-Mediated Decomposition of Hydrogen Peroxide

DMG not only increases the activity of catalase in its native form but also reduces its aggregation, potentially enhancing activity by maintaining a larger native enzyme fraction. To further validate the enhancing effect of dimethylglycine (DMG) on catalase activity, we monitored the time-dependent decomposition of hydrogen peroxide (H_2O_2) in the presence of increasing concentrations of DMG (Figure 3A). As expected, H_2O_2 showed negligible spontaneous degradation in the absence of catalase. Upon the addition of catalase, a gradual decrease in absorbance at 240 nm was observed, producing a typical hyperbolic curve indicative of enzymatic decomposition of H_2O_2 . Notably, in the presence of DMG, the rate of H_2O_2 decomposition increased significantly in a concentration-dependent manner. This enhancement in catalytic efficiency was further supported by a corresponding increase in the specific activity of catalase depicted in a bar diagram (Figure 3B).

3.4. DMG Increases T_m of Catalase

Thermodynamic stability of an enzyme may be enhanced because of reduced aggregation and the consequent increase in protein stability. To examine this relationship for catalase, we investigated its heat-induced denaturation profile in the absence and presence

of varying concentrations of DMG, as shown in Figure 4A. Thermal denaturation curves were analyzed to extract thermodynamic parameters, including the melting temperature (T_m) and the enthalpy of unfolding (ΔH_m), using Equation (2). The calculated values are summarized in Table 1. It is seen in the table that, in the presence of DMG, there is a significant increase in the T_m of catalase, approximately 7 °C higher in 500 μM DMG, as compared to the control. This rise in T_m is accompanied by a corresponding increase in both ΔH_m and the standard Gibbs free energy change of unfolding (ΔG_D°), indicating greater resistance of the enzyme to thermal denaturation. Figure 4B further illustrates in a bar diagram the rise in ΔG_D° values with increasing concentrations of DMG. These results collectively suggest that DMG enhances the thermodynamic stability of catalase, likely by stabilizing its native conformation and reducing its susceptibility to misfolding. Further, we investigated the possible correlation between the I_f values and the lag time calculated using Equation (1), correlation-analysis as shown in Figure 4C, indicated that increase in the I_f values were associated with the corresponding shortening of the lag phase, i.e., reduction in the time required for nucleus formation in catalase aggregation. Overall, these findings demonstrate that DMG not only stabilizes catalase by enhancing its thermodynamic resilience but also suppresses aggregation by delaying nucleation and reducing aggregate formation.

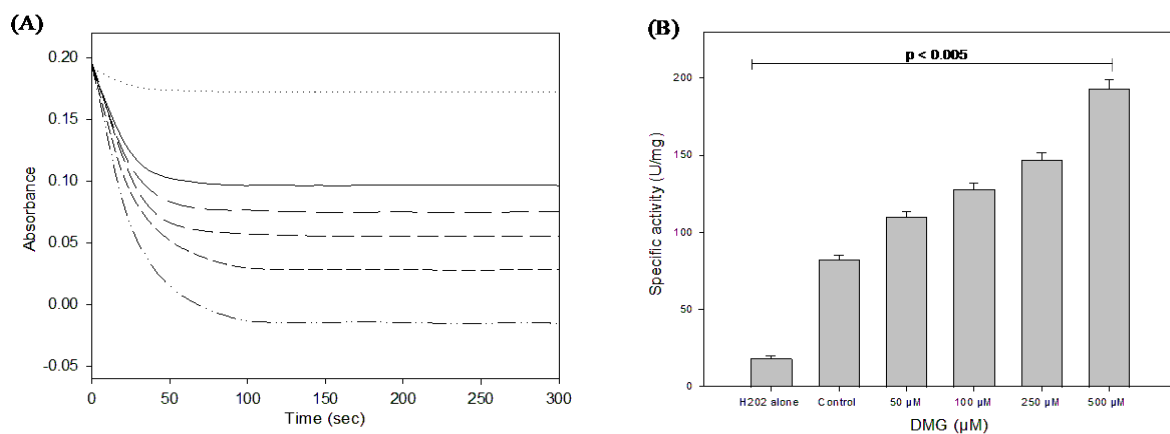


Figure 3. Catalase activity profile in the presence and absence of DMG. (A) The solid line represents the control while the dash lines represent treatment with 50 μM , 100 μM , 250 μM and 500 μM DMG respectively, the dotted line represents H₂O₂ only. (B) Bar graph showing the specific activity of catalase in the absence and in the presence of increasing concentrations of DMG.

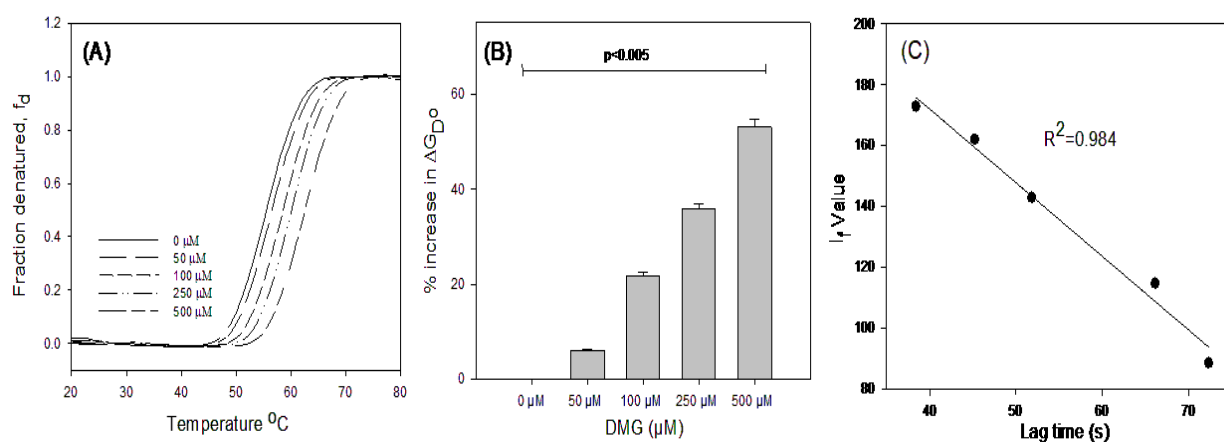


Figure 4. Thermal denaturation profile of catalase in the presence of DMG. (A) Thermal denaturation curve in the presence of increasing concentrations of DMG. (B) Bar diagram indicating percent increase in the ΔG_D° in the presence of increasing concentrations of DMG. (C) I_f versus Lag time correlation plot.

Table 1. Thermodynamic stability parameter (T_m , ΔH_m , and ΔG_D°) of catalase in the absence and presence of DMG.

DMG			
Concentration (μM)	ΔT_m ($^\circ\text{C}$)	ΔH_m (kcal/mol)	ΔG_D° (kcal/mol)
0.00	55.5 ± 1.1	85.4 ± 2.5	6.4 ± 0.25
50	56.5 ± 1.1	88.2 ± 2.6	6.8 ± 0.27
100	58.4 ± 1.2	95.6 ± 2.8	7.8 ± 0.31
250	60.1 ± 1.2	101.4 ± 3.1	8.7 ± 0.34
500	62.6 ± 1.3	107.8 ± 3.2	9.8 ± 0.39

3.5. DMG Induces Significant Structural Alterations in Catalase

Our next curiosity is to investigate if DMG brings about changes in structural conformations of catalase. So, we further examined the conformation of native catalase using multiple spectroscopic probes. Far-UV CD and Near-UV CD spectra (Figure 5A,B) revealed no significant secondary and tertiary structural changes, upon DMG treatment, respectively and this observation is further supported by the MRE values at 222 nm presented in Table S2. We have further performed intrinsic fluorescence analysis to verify the near-UV CD observations. As seen in Figure 5C, there is a little hyperchromicity change in the environment of tryptophan/tyrosine residues upon the addition of DMG, indicating minor increases in the burial of these groups in the hydrophobic environment. To further examine the changes in the exposure of hydrophobic residues to the solvent, we used ANS binding assay because ANS is a hydrophobic fluorescent-dye that selectively binds to exposed hydrophobic regions of a protein, making it a useful probe for detecting changes in the protein's core structure. It is seen in Figure 5D that with an increase in DMG concentration, there is a decrease in ANS fluorescence intensity, which indicates decreased exposure of hydrophobic residues to the solvent and the stabilization of native state packing of hydrophobic amino acid residues of the protein. The calculated λ_{max} values and fluorescence intensities for both intrinsic and ANS fluorescence are summarized in Table S2.

3.6. DMG Binds to an Allosteric Site of Catalase and May Contribute to Enhanced Activity and Stability

An increase in enzyme activity, along with enhanced stability and significant structural changes, was observed upon treatment with DMG, suggesting a potential interaction between DMG and catalase. To explore this possibility, potential ligand-binding sites on catalase were identified using the SiteMap analysis. Five distinct binding pockets were predicted, with site scores mentioned in Table 2. Subsequently, molecular docking of DMG into each of these predicted sites was performed using the Maestro Glide docking tool. The corresponding docking scores and Glide energies are depicted in Table 3. Among the five docking sites, site 1 exhibited the most favorable binding affinity, as reflected by the highest values of docking score and Glide energy. Figure 6A,B illustrate the 2D and 3D binding poses of DMG at this site comprising residues Phe 292, Pro 293, Phe 294, Lys306, Asp307, Tyr308, and Pro304, respectively. This site is found to be distant from the catalytic site (which is made by residues His 78, Asn 148, Phe 153, Phe 161, and Tyr 358), suggesting that it may function as an allosteric modulator of catalase. The binding at site 2 is stabilized through multiple hydrogen bonds with residues Lys 306 and Asp 307 along with additional polar and non-polar interactions. These interactions collectively contribute to the specificity and stabilization of DMG within the binding cleft. To further validate ligand binding affinity and assess conformational variability, six additional potential binding poses

were generated for this site, as shown in Figure S3 and summarized in Table S3. These findings strongly support that DMG interacts with an allosteric site of catalase, potentially underpinning the observed enhancements in enzymatic activity and structural stability.

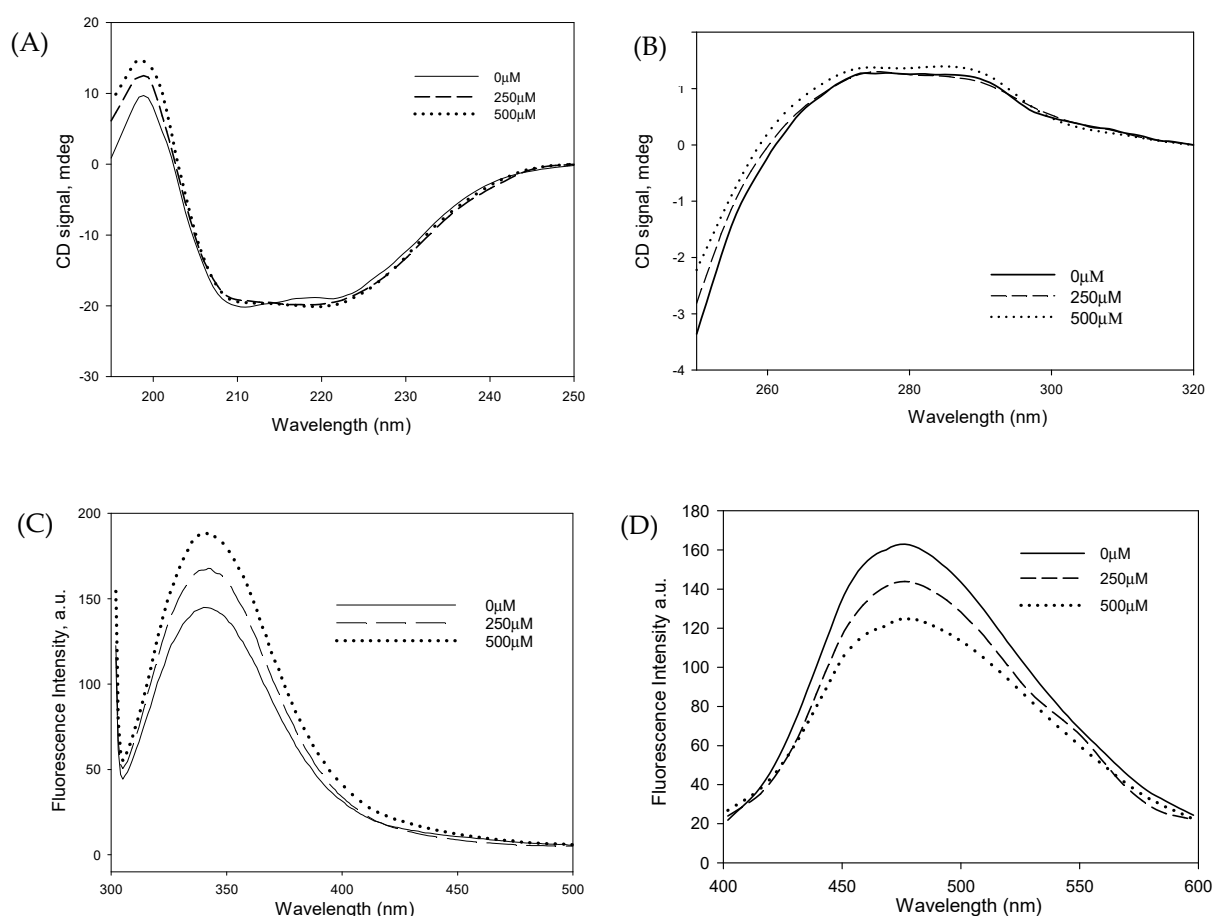


Figure 5. Effect of DMG on structural integrity of catalase. (A) Far-UV CD spectra, (B) near-UV CD spectra, (C) the Trp fluorescence, and (D) the ANS fluorescence spectra of native and DMG-treated catalase.

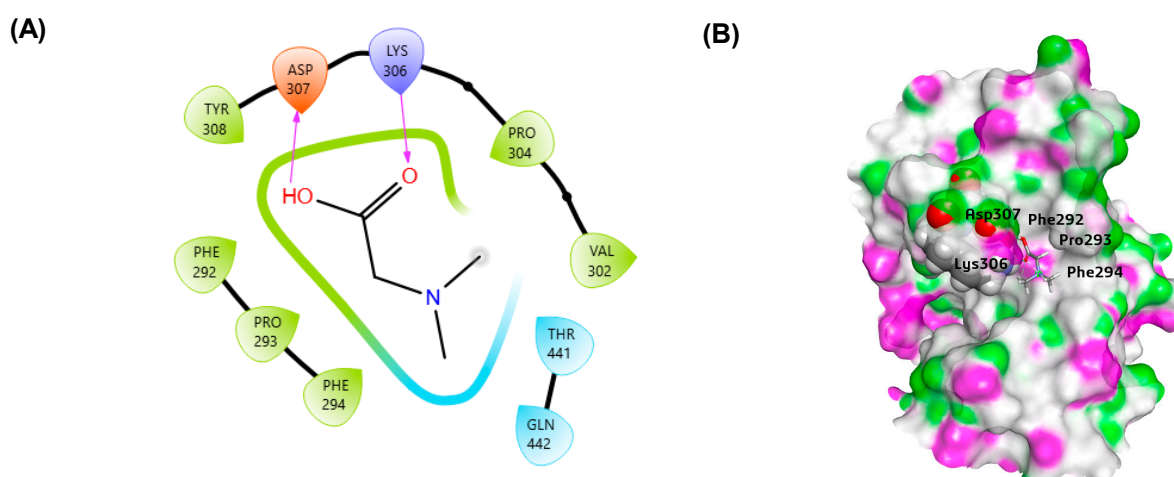


Figure 6. In silico molecular docking studies showing binding of DMG to catalase. (A) 2D ligand interaction diagram of DMG bound near the Lys 306 of catalase, and (B) 3D graphical representation of DMG in the same site.

Table 2. List of different sites in catalase along with corresponding amino acid residues and site scores.

Site Number	Amino Acid	Site Score
Site 1	Lys306, Asp307, Tyr308, Pro304	0.93
Site 2	Ser337, Pro359, Asp363, Pro368	0.81
Site 3	Pro158, Pro162, Arg72, Val73	0.75
Site 4	Gly118, Asp469, Val126, Ala251	0.69
Site 5	Gln18, Ala270, Pro7, Gln11	0.51

Table 3. Values of docking scores and glide energies obtained after docking DMG in all the potent binding sites.

Site Number	Docking Score	Glide Energy
Site 1	−5.6	−30.2
Site 2	−4.5	−25.7
Site 3	−3.8	−20.4
Site 4	−3.4	−18.6
Site 5	−2.3	−13.7

3.7. MD Simulations Highlight DMG-Induced Conformational Shifts

To elucidate the mechanistic insights of the effects of DMG on catalase, a detailed *in silico* MDS analysis was carried out in the presence of DMG co-solvent system. For this, an MD simulation for 100 ns was performed on catalase and catalase-DMG complexes as shown in Figure 7. The MD simulation analysis provides valuable insights into the binding of DMG to catalase and its influence on protein stability and conformational dynamics. The root mean square deviation (RMSD) plot (Figure 7A) shows that the DMG-bound catalase maintains lower fluctuations throughout the 100 ns simulation compared to the unbound catalase, indicating enhanced structural stability upon DMG binding. This stabilization is further supported by the root mean square fluctuation (RMSF) analysis (Figure 7B), which reveals reduced residue-level flexibility across most regions of the protein in the DMG-bound state, particularly in the catalytic domain. The radius of gyration (R_g) profile (Figure 7C) shows that catalase in the presence of DMG remains more compact over time, with slightly lower R_g values, suggesting that DMG promotes a tighter and more stable protein conformation. Consistently, the solvent-accessible surface area (SASA) (Figure 7D) is lower for DMG-bound catalase, indicating reduced solvent exposure and improved packing of hydrophobic residues. The hydrogen bond analysis (Figure 7E) demonstrates stable hydrogen bond formation between DMG and catalase throughout the simulation, highlighting a strong and persistent interaction that contributes to stability. Finally, the bond length analysis (Figure 7F) shows minimal fluctuation, suggesting that the interactions at the binding site are stable and well-maintained during the simulation. Overall, these results indicate that DMG binding confers structural stability to catalase by reducing conformational flexibility, enhancing compactness, and maintaining strong intermolecular interactions. This supports the experimental findings that DMG enhances catalase stability and activity, likely through direct binding that reinforces the native structure and prevents misfolding or aggregation.

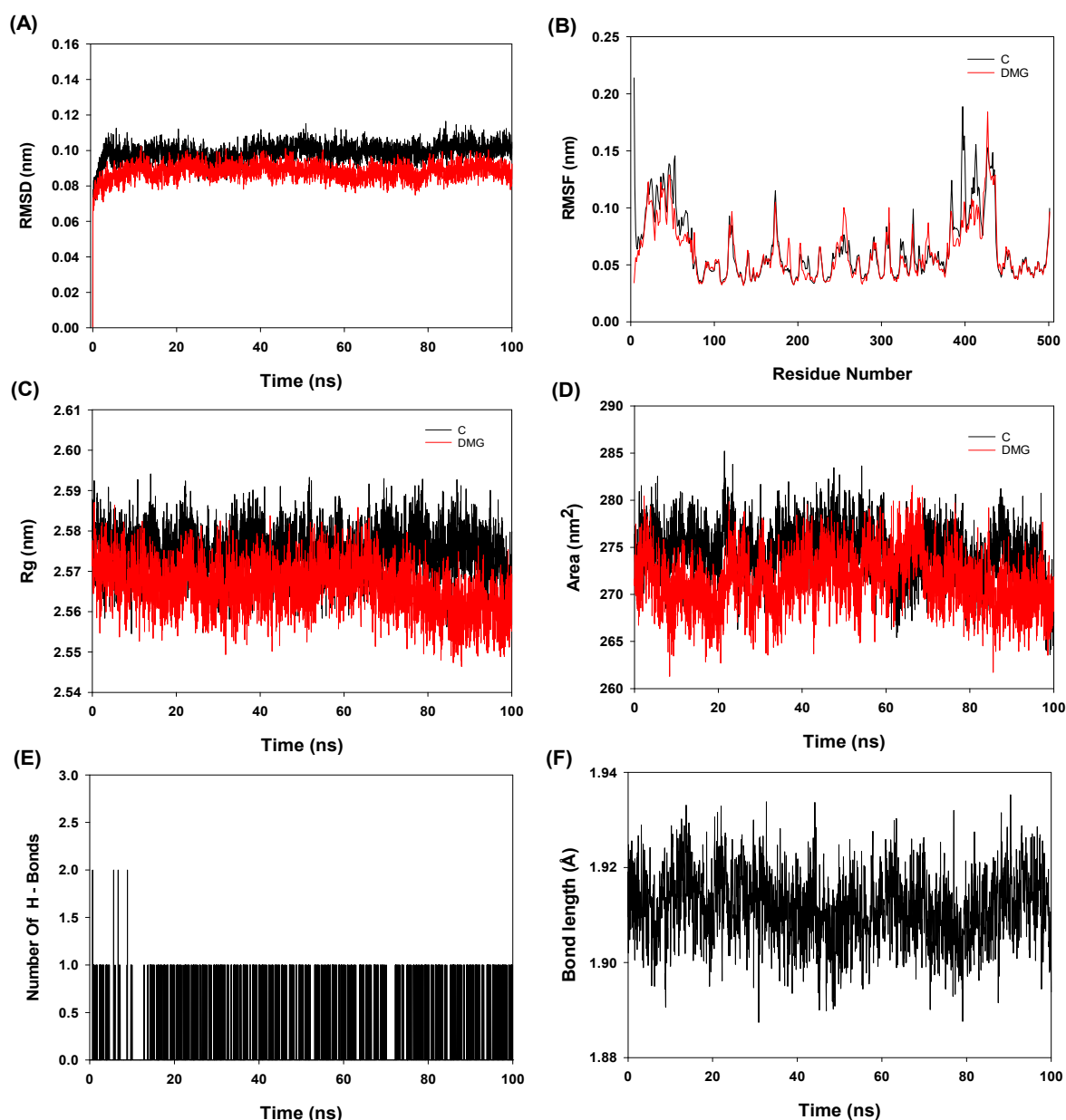


Figure 7. In silico molecular dynamic simulation studies showing binding of DMG to catalase. (A) The RMSD plot showing the changes between the stabilities in the observed systems. (B) The graphical representation of the changes observed in the fluctuation of the constituent residues between the DMG-bound and unbound catalase. (C) The Rg plots showing the difference in the compactness between the DMG-bound and unbound catalase. (D) The graphical representation of the changes observed in the Solvent accessible surface area (SASA) between the DMG-bound and unbound catalase. (E) Hydrogen-bond fluctuations plot highlighting the changes in the observed number. (F) Fluctuations in the hydrogen-bond length throughout the run.

4. Discussion

Alzheimer's disease is characterized by complex pathological processes, including protein aggregation, oxidative stress, and mitochondrial dysfunction, ultimately leading to neuronal degeneration and cognitive decline [2]. Among the numerous molecular factors involved, oxidative stress plays a pivotal role in driving disease progression [47,48]. Our study highlights the central role of catalase, a critical antioxidant enzyme, whose dysfunction can exacerbate ROS accumulation and neuronal damage in AD. Importantly, we identify DMG, a naturally occurring metabolite, as a potent modulator that both

prevents catalase aggregation (Figure 1) and enhances its enzymatic activity (Figure 3). This dual functionality positions DMG as a promising therapeutic candidate for mitigating oxidative stress and related pathologies in AD.

The first step in our study was to evaluate the effect of various naturally occurring metabolites on catalase aggregation. Catalase is known to undergo temperature-induced aggregation at its melting temperature (55 °C) [49], resulting in the loss of function. The screening revealed three important observations: (1) Certain metabolites like sorbitol, mannitol, glycerol, and trehalose do not have a significant effect on the aggregation kinetics of catalase. (2) other metabolites including proline, β -alanine, ectoine, and hydroxyectoine exhibit mild inhibitory effects. (3) DMG shows the strongest inhibitory effect against catalase aggregation (Figures 1 and S1). Kinetic analysis of aggregation curves provided further insights into the mechanism by which DMG exerts its protective action (Table S1). DMG prolonged the lag phase of aggregation and decreased the apparent rate constant (K_{app}), indicating its ability to delay nucleation and slow the growth of aggregates. This suggests that DMG stabilizes the native state of catalase, making fewer aggregation-prone intermediates available for fibril formation.

Catalase has also been implicated in AD pathology through its formation of amyloid-like catalase-A β co-oligomers [29,50]. Our study showed that DMG significantly reduces the formation of such aggregates. Light scattering, TEM imaging and fluorescence microscopy (Figure 2A,B) demonstrated that catalase alone forms dense fibrillar structures, whereas DMG treatment results in fewer, smaller and less ordered aggregates. Catalase activity assays further confirmed that DMG not only prevents loss of activity but increases the enzyme's ability to decompose H₂O₂ (Figure 3A,B). To understand whether this protection is associated with changes in thermodynamic stability, we investigated heat-induced denaturation of catalase in the presence of DMG. A substantial increase in the melting temperature (T_m) of catalase, along with higher ΔH_m and ΔG° values, indicated greater resistance to unfolding (Table 2). A strong correlation between reduction in aggregation (I_f values) and increase in lag phase suggested that DMG shifts the equilibrium toward the native state. Spectroscopic analyses (CD, intrinsic fluorescence and ANS binding) supported this conclusion by showing reduced exposure of hydrophobic residues and stabilization of the folded state (Figure 5).

In addition to our findings, previous studies show that catalase can be influenced by small molecules through allosteric-like mechanisms. The most established example is NADPH, which binds at a surface cleft away from the heme site and maintains catalase in its active form by preventing accumulation of inactive intermediates [51–53]. Disease-relevant ligands such as amyloid- β have also been reported to bind catalase with high affinity and inhibit H₂O₂ breakdown, although a precise binding pocket is not clearly defined [54–56]. These observations support the idea that catalase can be modulated at sites other than the catalytic center. Since our docking and MD simulations indicate that DMG binds in a distal pocket rather than at the heme moiety, its effect is likely similar—stabilizing the native structure and helping maintain enzymatic function (Figure 7). While our computational analysis suggests that DMG may bind at a site capable of influencing catalase conformational dynamics, the present study does not include kinetic measurements (K_m or k_{cat}) needed to establish true allosteric activation. Accordingly, the enhanced enzymatic activity observed under thermal stress is more cautiously attributed to DMG's ability to preserve the native, functional conformation of catalase by preventing its conversion into aggregation-prone intermediates. This interpretation is consistent with the behavior of many small-molecule allosteric ligands that function primarily as protein stabilizers. For example, tafamidis stabilizes the transthyretin tetramer and prevents its dissociation into monomers—the rate-limiting step in amyloid formation [57,58]—while lenalidomide stabi-

lizes a non-native complex between cereblon and its substrates, promoting their targeted degradation [59,60]. By analogy, DMG may not induce large active-site rearrangements but rather helps catalase maintain a greater population of its native, active state, thereby resisting stress-induced inactivation.

AD specific stressors, including change of pH, H₂O₂ overload, increase in temperature, and even mutations are considered to be the pivotal components leading to a decreased catalase stability and hence an increase in aggregation propensity [19,61,62]. Increased levels of reactive sugar intermediates, carbonyl groups, homocysteine, reactive nitrogen species, and alterations in levels of various metal ions also significantly add onto the catalase deficiency or aggregation via direct covalent modification or binding to the catalase [63,64]. Several studies have shown that catalase has a melting temperature close to 55 °C, and at this transition point the enzyme forms partially folded intermediates that are highly prone to aggregation [49]. Importantly, these intermediates share structural features with catalase that has undergone oxidative damage, such as loss of tertiary structure, increased exposure of hydrophobic residues, and reduced enzymatic activity. Since DMG stabilizes catalase structure, reduces exposure of hydrophobic patches and preserves enzyme activity, it is reasonable to expect similar protection under other disease-relevant oxidative stress conditions. Recent metabolomic studies also suggest a connection between DMG and Alzheimer's disease [65,66]. DMG has been identified as one of six plasma metabolites that can distinguish AD patients from healthy individuals, indicating that changes in DMG levels may reflect underlying metabolic shifts in the disease [67,68]. DMG is also a partial agonist at the NMDA receptor glycine site and has been explored for neuroprotective effects in other neurological models, although results remain mixed [69,70]. While these findings do not establish a direct role of DMG in AD progression, they support its biological relevance and further justify investigating whether stabilizing catalase could be one of the mechanisms through which DMG may act in a disease context. Finally, although our model uses temperature-induced unfolding as a controllable trigger, AD-relevant stressors such as A β oligomers, H₂O₂ overload and nitrosative stress also destabilize catalase in vivo [50,71]. These biochemical insults produce partially unfolded intermediates and co-aggregation with A β , events that resemble those captured in our assays. Future work will test DMG in cellular models of oxidative and nitrosative stress to evaluate whether this stabilizing effect extends beyond in vitro analysis.

5. Conclusions

In summary, our study shows that dimethylglycine (DMG) can protect catalase from misfolding and aggregation, increase its thermodynamic stability, and improve its ability to break down hydrogen peroxide. Together, these results suggest that DMG helps keep catalase in its healthy, active form. Docking and molecular dynamics studies further support the idea that DMG may act through an allosteric-like site away from the heme center, although more work is needed to confirm the exact mechanism. Protein misfolding and aggregation are common features of many neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease [72–74]. Recent research shows that naturally occurring metabolites can influence protein stability and prevent aggregation, making them attractive low-toxicity candidates for therapeutic development [75–77]. Our findings place DMG within this growing group of metabolite-based protein stabilizers. Since, catalase dysfunction contributes to oxidative stress in AD, these results suggest that DMG could help restore antioxidant defense in the brain. However, this work was carried out under controlled in-vitro conditions. Future studies in cell or animal models, especially under Alzheimer's-related stress such as A β exposure or nitrosative damage,

will be important to determine whether the stabilizing effects of DMG translate to disease-relevant environments.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biophysica6010002/s1>, Figure S1: Percent disaggregation profile of catalase with various metabolites; Figure S2: Quantified ThT fluorescence intensity profile showing osmolyte-induced reduction of catalase aggregates in the presence and absence of DMG; Figure S3: In silico molecular docking studies showing alternate binding conformations of DMG to Catalase. Table S1: Aggregation kinetics parameters of Catalase in the absence and presence of various metabolites; Table S2: Spectroscopic analysis of protein structural changes upon treatment with DMG; Table S3: Docking scores and Glide energies for multiple binding poses of DMG at the allosteric binding site of catalase.

Author Contributions: A.P.D., S.Y. and K.S. (Kuldeep Singh) performed the experiments. A.G. and L.R.S. conceived the idea and analyzed the data. A.G., K.S. (Kananbala Sarangthem), and L.R.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data produced or examined in this study are available within this published article and its supplementary information files.

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