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Original Research Article

Methylglyoxal Binding to Bovine Liver Catalase Results in Loss of Activity and Heme Dislocation

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HIGHLIGHTS

- Increase in concentration of methylglyoxal caused a progressive increase in electrophoretic mobility and detachment of heme from the monomer.
- MG-modified enzyme showed reduced affinity towards the substrate hydrogen peroxide.
- Molecular modeling studies showed that MG can access the heme and arginine residues close to it.

ABSTRACT

Glycation, the non-enzymatic attachment of glucose to protein, is one of the important events in the pathophysiology of diabetes mellitus, Alzheimer's, Parkinson's and other diseases. Methylglyoxal (MG), a dicarbonyl compound formed during glycation, monosaccharide autoxidation, and metabolism is elevated during diabetes mellitus. Among other antioxidant enzymes, catalase is important for the defense against oxidative damage. However, antioxidant enzymes including catalase can themselves become targets of non-enzymatic modification by methylglyoxal. In this study, catalase was incubated with increasing concentrations of MG for different time intervals. Structural and functional alterations to catalase were monitored by a variety of approaches, namely, assay of enzyme activity, staining of gels for activity as well as heme, measurement of protein carbonyls and Arg pyrimidine, which is a specific MG modification product. A progressive increase in electrophoretic mobility and detachment of heme from the monomer were observed with increasing concentrations of methylglyoxal. The MG-modified enzyme showed reduced affinity towards the substrate hydrogen peroxide. Molecular modeling studies revealed that MG can access the heme and arginine residues close to it. Thus, the decrease in activity of methylglyoxal-modified catalase may be important in aggravating the severity of secondary complications seen in diabetes mellitus.

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Introduction

A number of posttranslational modifications take place in normal cells and this process regulates cellular events. To name a few, acetylation, phosphorylation, and polyubiquitination are required for normal functioning of cells and are finely regulated processes. Besides these, there are post translational modifications which are not regulated by cells. This nonenzymatic post translational modification brings about structural and functional changes in biomacromolecules (Suji and Sivakami, 2004). Among these are non- enzymatic modifications of proteins, DNA, and aminolipids by low molecular compounds like methylglyoxal (MG), glyoxal, malondialdehyde, and glucose (Suji and Sivakami, 2004). Carbonyl group of these compounds interacts with amino groups of protein to form advanced glycation end products (AGEs).

MG is a toxic compound produced as a byproduct of glycolysis, a product of metabolism of acetol and an intermediate in catabolism of the amino acid threonine, the ketone body, and acetone (Kalapos, 1999). MG is present in various foodstuffs to the extent of 100-700 microgram (1.4-10.1 µM /l) (Kasai et al., 1982). Although the cellular level of this compound is low in normal cells (approximately 1 µM), it is increased to critical levels (5-6 fold) during insulin dependent diabetes and 2-3 fold in non-insulin dependent diabetes patients (McLellan et al., 1993).

The major target of MG in protein is arginine and to a lesser extent lysine. Arginine in proteins interacts with MG to form hydroimidazolone MG-H1 and dihydroxyimidazolidine that further rearranges to form AGEs (Oya et al., 1999). Besides causing damage to proteins directly, MG is also known to contribute to oxidative stress and affect the ability of the cells to cope with oxidative damage by modifying antioxidant enzymes like catalase (Yan and Harding, 1997; Bakala et al., 2012; Scheckhuber, 2015), glutathione peroxidase (Park et al., 2003), superoxide dismutase (Yan and Harding, 1997), ascorbate peroxidase (Hoque et al., 2012) as well as serum albumin (Faure et al., 2005). Non-enzymatic glycation and modification by reactive carbonyl compounds including MG could be one of the important underlying causes for the decline in the enzyme activity in the liver (Toleikis and Godin, 1995), heart (Yadav et al., 1997), and kidney (Kakkar et al., 1995) of diabetic rats.

The tetrameric catalase (240 kDa) isolated from bovine liver contains a heme prosthetic group and an NADPH cofactor. The iron present in the heme exists in different oxidation state generating electrostatic field which is stabilized by the presence of Arginine354, His218, and Asp348 (Putnam et al., 2000).

Oxidative stress in a system can result from excessive accumulation of free radicals or an inadequacy of antioxidant enzymes to scavenge the free radicals. The increased MG levels combined with the decreased activity of the detoxifying enzyme, glyoxalase I during diabetes increases the possibility of chemical modification of the cellular proteins including those of the antioxidant enzymes (McLellan et al., 1993; Philips et al., 1993). It is thus necessary to understand the mechanism by which the antioxidant enzymes are altered by MG modification. Catalase, a Heme containing antioxidant enzyme was chosen as the model protein in this study in view of its importance in the context of diabetes.

Materials and Methods

Chemicals

Catalase (bovine liver), methylglyoxal, 2, 4-Dinitrophenylhydrazine (DNPH), and guanidine hydrochloride were purchased from Sigma Aldrich, USA. DAB (3, 3, diaminobenzidine), HRP (Horse Radish Peroxidase), hydrogen peroxide, HEPES, potassium phosphate salts, EDTA, acrylamide, N' N' methylene bisacrylamide, TEMED, ammonium persulphate, tricholoroacetic acid, and hydrochloric acid were purchased from Sisco Research Laboratory, Mumbai, India.

Spectrophotometric assay for enzyme activity

The catalase assay was performed according to the methods of Aebi (1983). A decrease in the absorbance at 240 nm was observed for 60 sec. The reaction mixture contained 1 ml of 10 mM H_2O_2 and 10 µg/ml catalase in 50 mM phosphate buffer, pH 7.0 containing 0.1 mM EDTA.

Catalase (100 µg/ml) was incubated with a constant concentration of 5 mM MG at 37 °C in 5 ml of 0.1 M HEPES buffer, pH 7.0, for varying time intervals. Individual vials with and without MG were removed at 0, 0.5, 1, 2, 4, 6, 24, 72, and 168 hr. In another set of experiments, catalase (100 µg/ml) was incubated with varying concentrations of MG (0.5, 1, 2.5, 5, 7.5, 10, and 20 mM) at 37°C for a fixed time period of 24 hours. In all cases at the end of the incubation period, the samples were dialyzed for 3 h at 4°C with several changes of buffer. Modification of the enzyme was studied thereafter.

The activities were expressed relative to the control activity at each incubation time, which was set at 100% for the time dependent studies. For dose dependent studies, the activities were expressed relative to the enzyme activity in the absence of MG

Measurement of AGE formation

Fluorescence measurements at Ex_{λ} 370 nm Em_{λ} 440 nm were carried out in order to monitor the formation of

total AGEs. In addition, the property of the glycoxidation product Arg pyrimidine to fluoresce at Ex_{λ} 335 nm Em_{λ} 385 nm was used for its estimation.

Determination of protein carbonyl content

The measurement of protein carbonyl content was used to determine the extent of protein oxidation (Levine et al. 1990). Briefly, the samples (100 µg/ml) were precipitated by using 0.5 ml trichloroacetic acid (20 %) and centrifuged at (11,000 g for 3 min). The pellet was mixed with 0.5 ml of 10 mM DNPH in 2 M HCl and incubated at room temperature for 1 hour with vortexing every 10 min. 0.5 ml of 20% trichloroacetic acid was added to the mixture and again centrifuged. The pellet was washed thrice with 1ml of ethanol: ethyl acetate (1:1), dissolved in 1ml of 6 M guanidine hydrochloride in 20 mM potassium phosphate, pH adjusted to 2.3 with trifluoroacetic acid, and absorbance measured at 366 nm. The carbonyl content was calculated using a Molar Extinction Coefficient (ε) value of 22,000 M^{-1} $cm^{-1}$and the results expressed as nmole/mg protein.

Catalase activity stain using Horse Radish peroxidase (HRP)

5.0% Acrylamide gels were used for native PAGE performed according to the method of Laemmli (1970). The stacking (4%) and resolving (5%) were polymerized in a 10 cm x 10 cm x 1 mm slab gel unit. The amount of the sample in each well was 50 µg. The gel was first run under non-denaturing conditions in the cold. The samples were allowed to stack at 50 V and then run at 100 V. The gel was then rinsed with distilled water 2-3 times for 5 minutes each and then stained in 10ml potassium phosphate buffer (50 mM, pH, 7.0) containing 0.5 mg/ml DAB and 150 µl of 1 mg/ml HRP. After 90 minutes of incubation, the gel was washed in 0.02 M H_2O_2 until achromatic bands with dark edges against a dark brown background were visible (Gregory and Fridovich, 1974).

Heme staining using diaminobenzidine

To check if modification causes any change to the heme moiety, heme specific staining was carried out. For this, 5% acrylamide gels were run under non-denaturing conditions in the cold. The samples were allowed to stack at 50 V and run at 100 V. The gel was then washed with distilled water for five minutes and stained with 0.1 M HEPES, pH, 7.0 containing 0.5 mg/ml diaminobenzidine and 200 µl of 40% H_2O_2. The gel was incubated in the dark at 25 °C till achromatic bands appeared over a brown background. Diaminobenzidine reacts with H_2O_2 to give a dark background which is decolorized wherever heme decomposes H_2O_2.

Molecular docking

The structure of catalase tetramer (PDB code 1TGU) was obtained from Protein data bank (http://www.rcsb.org/). Ready to dock conformation of ligand methylglyoxal was obtained from ZINC database (http://zinc.docking.org/). Catalase, being a tetramer protein for docking studies monomer catalase (A chain) was obtained using the program Chimera (http://www.cgl.ucsf.edu/chimera). H_2O was removed from catalase monomer and polar hydrogen was added and finally Gasteiger charges were added. With the aid of AutoDock tools, the ligand root of Methylglyoxal was detected and the rotatable bonds were defined. AutoDock Tools version 1.5.6 (Morris et al., 2009) was used to convert the receptor and ligand to PDBQT format.

The molecular docking program Autodock VINA (Trott, 2010) was used to study the binding of methylglyoxal to catalase (full rigid protein). The search space was a box with XYZ dimensions 74 Å × 82 Å × 74 Å respectively, and was centered on 34.4, 34.0, and 31.7. Both search space dimensions encompass the entirety of the proteins, as is required for blind docking. The molecular docking results presented in figures were prepared in Chimera and BIOVIA/Discovery studio 2016.

Statistical analysis

All the results of non-computational experiments are expressed as mean ± S.D of three different experiments. Statistical differences were analyzed using one-way analysis of variance (ANOVA). $P<0.05$ was considered significant. K_m and V_{max} values were obtained from Lineweaver-Burk plots drawn using Sigma plot.

Results

Effect of time at a constant concentration of MG

At a constant concentration of 5mM, a marked decline in catalase activity was observed at the end of 1 hr after which there was no further inactivation up to 6 hr. A steep decrease in activity occurred between 6 hr and 168 hr, when the enzyme retained 33.24 ± 4.73 % of the control activity (Fig. 1A). The inactivation of the enzyme was accompanied by significant elevations in the levels of total AGE, Arg pyrimidine, and protein carbonyl contents (Fig. 2A), which are indicators of damage by methylglyoxal. It was observed that up to an incubation time of 4 hours, protein carbonyl content did not significantly increase, beyond which there was a steep 5-fold increase till 168 hours. In the case of total AGEs as well as Arg pyrimidine there was a steady increase up to 24 hours after which there was significant increase at the end of 168 hours.

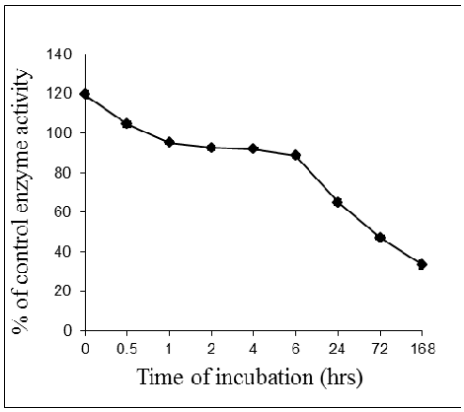

Figure 1A. Effect of incubation time on catalase activity. Effect of 5mM MG for varying time periods on catalase activity expressed as % of control activity (i.e. activity in the absence of MG at the corresponding time period). Activity was significantly lower than control ($p<0.001$ by ANOVA) between 0.5-168 hr. All values are expressed as mean ± S.D. of three separate estimations.

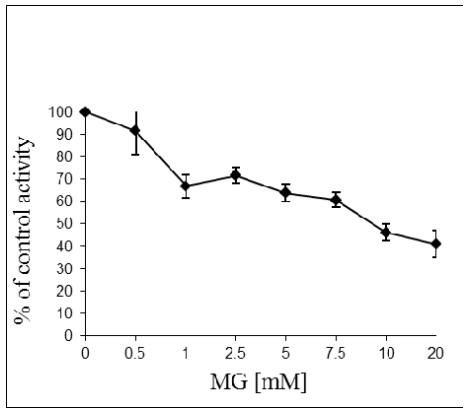

Figure 1B. Effect of concentration of MG on catalase activity. Effect of different concentrations of MG at the end of 24 h on catalase activity expressed as % of control activity (i.e. activity in the absence of MG). Activity was significantly lower than control ($p<0.001$ by ANOVA) in the presence of 1-20 mM MG. All values are expressed as mean ± S.D. of three separate estimations.

Effect of concentration of MG at the end of 24 hr

With increasing concentrations of MG, it was observed that catalase activity fell by 33.27% at the end of first hour after which there was a gradual decline till 20mM MG when 53.94% decrease in activity was observed. (Fig. 1B) Increasing MG concentrations resulted in increased levels of AGE, Arg pyrimidine, and protein carbonyls (Fig. 2B). Protein carbonyl content steadily increased and reached 10 times the control levels at 20mM MG. Up to 5mM MG, both total AGE and Arg pyrimidine levels increased by similar amounts, after which the levels of total AGE increased more rapidly than Arg pyrimidine**.**

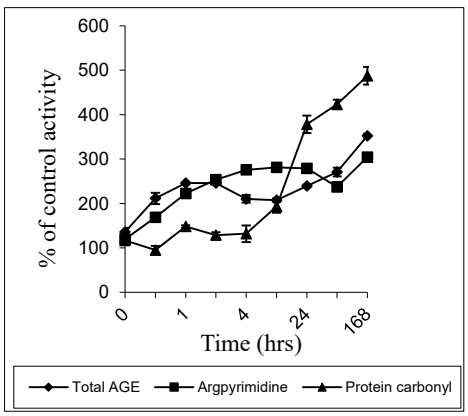

Figure 2A. Measurement total AGEs, Arg pyrimidine and protein carbonyls (incubation time). The levels of AGEs, Arg pyrimidine and protein carbonyls expressed as % of control levels. Total AGE and Arg pyrimidine levels were significantly elevated ($p < 0.001$ by ANOVA) between 0.5-168 h whereas significant elevations in protein carbonyls ($p < 0.001$ by ANOVA) occurred between 6-168 hr. All values are expressed as mean ± S.D. of three separate estimations.

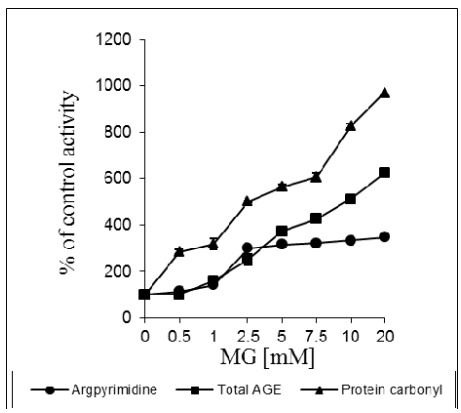

Figure 2B. Measurement total AGEs, Arg pyrimidine and protein carbonyls (concentration of MG). The levels of AGE, Arg pyrimidine and protein carbonyls expressed as % of control levels. Arg pyrimidine and protein carbonyls were significantly elevated ($p<0.001$ by ANOVA) with 0.5-20 mM MG whereas significant elevations in AGE levels ($p<0.001$ by ANOVA) occurred with 1-20 mM MG. All values are expressed as mean ± S.D. of three separate estimations.

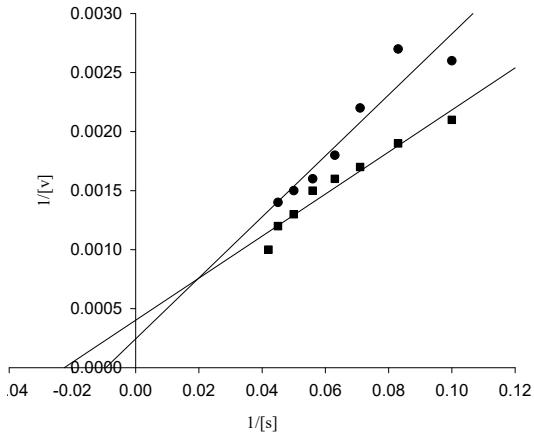

Figure 3. Line-weaver Burke plots for native (■---■) and modified catalase (•---•).

Effect of MG modification on kinetic constants

The Lineweaver Burk plot gave a K_m of 108 mM for the modified enzyme, while that for the unmodified catalase was 43 mM with no significant change in V_{max} values (Fig. 3).

Gel stains

The activity stain using Horse Radish Peroxidase is indicative of true catalase activity. Using this stain, achromatic zones with dark brown edges corresponding to enzyme activity were seen against a light brown background (Fig. 4). When stained for the heme moiety using diaminobenzidine and H_2O_2, achromatic bands against a brown background were seen (Fig. 5). Besides, as the concentration of MG increased to 10 and 20mM, the intensity of heme band decreased (Fig. 5).

Molecular docking

The functional catalase is composed of tetramers and each monomer is divided into four domains (Fig. 6). An eight-stranded antiparallel β-barrel forms the hydrophobic core region of each subunit. The threading arm connects two subunits by hooking through a long wrapping loop

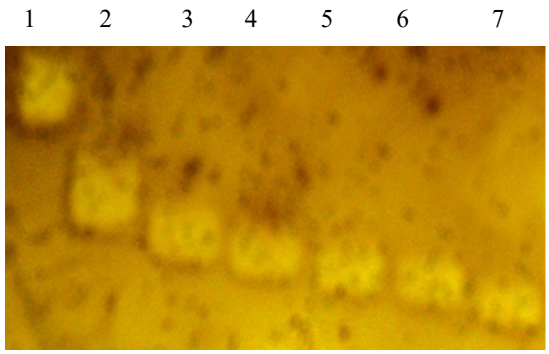

Figure 4. Activity staining of catalase modified with varying concentration of MG. Catalase was modified with various concentration of MG for 3 days and stained for enzyme activity using Horse Radish peroxidase on non-denaturing (5%) PAGE. From left: lane 1: control catalase, lane 2-7: 1, 2.5, 5, 7.5, 10, 20 mM MG.

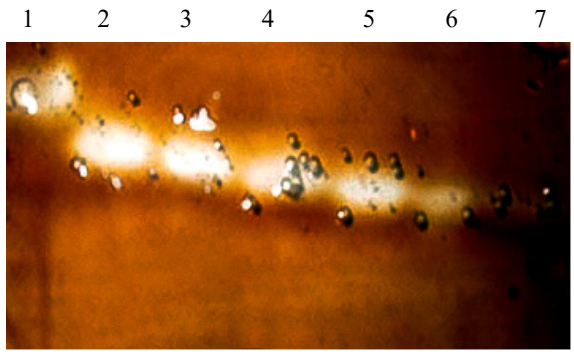

Figure 5. Electrophoretic mobility of catalase. Changes in electrophoretic mobility of catalase on non-denaturing PAGE after treatment with different concentrations of MG for 3 days at 37°C in 0.1 M potassium phosphate buffer (pH, 7.0) after heme staining. From left: lane 1: catalase control, lane 2-7: 1, 2.5, 5, 7.5, 10, 20 mM MG.

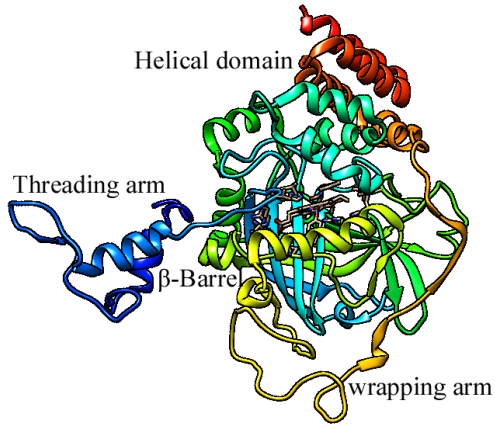

Figure 6. Structure of bovine catalase monomer.

around another subunit. Each monomer has a heme ring forming the bottom of the 25 Å long channel extending from the surface of the molecule. The pentacoordinate iron is accessible at the distal end to the substrate H_2O_2. The reactivity of iron is finely controlled by Arginine residue around heme. There are in all 4 Arginine residues interacting with heme ring (Fig. 7A). Docking of Methylglyoxal to catalase resulted in 10 different conformations. All the conformations were analyzed to investigate if MG can freely access the heme ring similar to substrate H_2O_2. The lowest energy conformation was -3.3 kcal/mol in which MG was bound to the surface of catalase far away from active size. However, pose number 6 with binding energy -3.1 kcal/mol MG sits in the distal side of heme at a distance of 3.6 Å from the Fe atom of heme (Fig. 7B).

Discussion

Oxidative stress and consequent damages have been considered as important causes, effects or both in many pathological processes. Catalase plays a crucial role in reducing damage by scavenging intracellular H_2O_2, which can cause damage directly or indirectly.

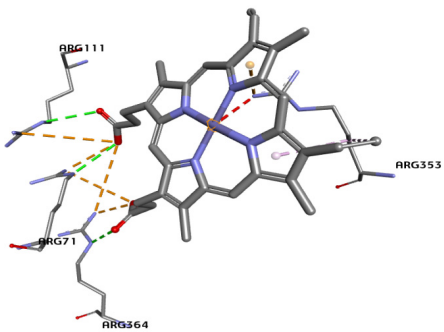

Figure 7A. Heme and arginine docking structure. Heme and interacting arginine close to it. Residues other than arginine interacting with heme are not shown for clarity.

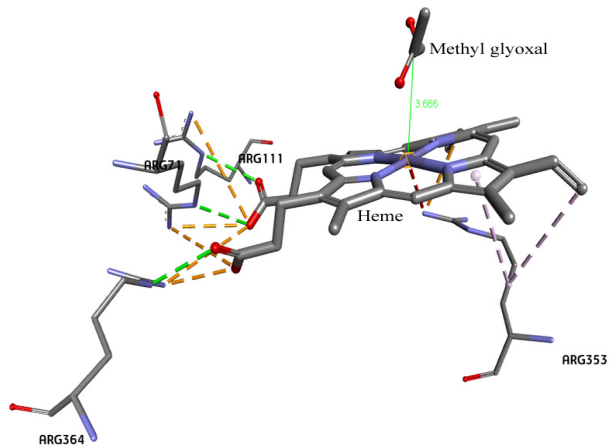

Figure 7B. Methyl glyoxal and catalase docking structure. Methyl glyoxal bound to catalase. The carbon atom of methyl glyoxal is at a distance of 3.6 Å from the Fe atom of heme.

Catalase inactivation leads to oxidative damage through hydrogen peroxide generation or due to increased levels of superoxide radical (Yan and Harding, 1997).

Increased sugar and methylglyoxal levels associated with diabetes could also lead to inhibition of catalase resulting in impaired defence against toxic oxygen radicals.

MG predominantly modifies arginine under minimal modification conditions. However, at higher concentrations, lysine is also susceptible to modification by MG. Our observation of AGE formation within an hour is in accordance with reports of rapid MG-derived formation of AGEs in case of BSA (Uchida et al., 1997) and RNase (Ahmad et al., 1997). As low as 0.3 mM MG has been shown to modify 5-10% of cellular proteins (Chaplen et al., 1998).

In this study, as the concentration of MG increased, total AGE also increased linearly but Arg pyrimidine failed to show linearity. This could be due to the fact that Arg pyrimidine is a direct product of Arginine modification only. In contrast, the levels of total AGEs are formed from further rearrangement of various initial products and can increase linearly**.** The interaction of MG with lysine groups in proteins is known to produce free radicals like superoxide and hydroxyl (Suji and Sivakami, 2007). The kinetics of AGE formation parallels the decrease in enzyme activity suggesting that the inactivation of the enzyme is caused by the formation of AGEs. The fall in enzyme activity after 6 hours coincides with the elevation in protein carbonyl content indicating that the oxidation of amino acid residues by free radicals formed during AGE formation could have contributed significantly to the inactivation of the enzyme at this stage. Thus, the inactivation of the enzyme appears to be caused by both direct adduct formation by MG as well as the indirect oxidative damage induced by free radicals generated from such adducts.

Further, the effect of modification by MG is evident in the electrophoretic mobility of catalase. Increasing the concentration of methylglyoxal was accompanied by increased mobility during electrophoresis indicating progressive loss of positive charges. This results in increased net negative charge and electrostatic potential on the surface leading to enhanced mobility (Uchida et al., 1997). The electrostatic potential on the surface of protein is an important factor in enzyme substrate interaction.

The increased K_m of the modified catalase reveals that the affinity of the enzyme for hydrogen peroxide decreases, resulting in decreased ability to scavenge hydrogen peroxide. The oxidation of amino acids within and outside the active site like those involved in heme binding may have contributed to the observed change in substrate affinity.

It was observed that glycation induces dimer formation in catalase. Dimers could be detected in CBB staining as well as by heme staining (data not shown). However, they could not be detected by activity stain indicating that the dimer is devoid of catalase activity. Samejima et al. (1962) reported that the dimers of mammalian catalase are enzymatically inactive. In contrast, one recent observation indicates that the dimer is the smallest unit of catalase that can exist in an enzymatically active form (Prakash et al., 2002).

The presence of heme as a cofactor in catalase offers an important tool for studying the structural integrity of the protein. Molecular docking studies revealed the dislodging of the heme moiety upon modification by MG. This was supported by the absence of heme staining as well as catalase activity stains on the gels of the modified enzyme. In a recent report by Scheckhuber (2015) it was observed that Arg93 and Arg444 showed the highest levels of MG modification. Under the same conditions Arg354, at the catalytic center of the enzyme was not modified. It was therefore proposed MG cannot access the active center where Arg354 is situated. The docking simulation reported here showed that MG can traverse through the 25 ºA channel and access the heme ring. Thus, it appears that the initial loss of activity is due to heme dislocation rather than Arg modification. The lag in the formation of Arg pyrimidine appears to agree with this. Besides, the iron from the dislocated heme can also promote oxidative damage by the Fenton reaction, resulting in loss of not only catalase but other activities too.

In the present study, the observed changes appear to take place after a lengthy incubation with low concentrations of MG. These experimental conditions are closer to those encountered during diabetes *in vivo*. It is therefore possible that during long term exposure to MG *in vivo*, the heme can get dislocated triggering further oxidative stress. Thus, a combination of enzyme assays, heme as well as activity staining along with molecular docking studies appears to reveal the probable pathway for the loss of catalase activity during MG modification.

Conclusion

Catalase plays an important role in the maintenance of the cellular integrity by reducing the levels of free radicals generated during the physiological processes. Glycation products cause damage to biomolecules by altering their structures and free radical generation. It can be concluded from the results presented in the manuscript that MG caused the loss of activity of catalase by altering its structure (dimer formation and heme dislocation) and oxidative damage induced during the process of glycation. The molecular docking study also suggested that the heme was dislocated in the presence of methylglyoxal. Recent reports in literature suggest that the complications of diabetes are both due to the formation of AGEs as well

as oxidative stress.

Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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