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REGULATION OF CATALASE: INHIBITION BY PEROXYNITRITE AND REACTIVATION BY REDUCED GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE

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1. ABSTRACT

The regulation of stable catalase from *Aspergillus niger* was investigated. The preincubation of catalase with peroxynitrite (PN) resulted in a significant decrease in the production of O_2, while the subsequent incubation with reduced glutathione (GSH, 1mM) led to restoration of the enzymatic activity. Western blot analysis revealed not only the increased immunoreactivities of 3-nitrotyrosine and S-nitrosocysteine in a PN-dose-dependent manner, but also conversely decreased immunoreactivity of 3-nitrotyrosine by the subsequent preincubation of catalase with GSH (1mM)/glutathione S-transferase (GST). The inhibition of the catalase after PN-treatment may be due to conformational changes of the enzyme via tyrosine-nitration/cysteine-nitrosation and the binding of active nitrogen/oxygen species to the Fe^{3+}-protoporphyrin groups of the enzyme. The reverse of these processes to restore enzymatic activity by GSH/GST may be a vital antioxidative mechanism.

2. INTRODUCTION

Catalases are ubiquitous heme-bound enzymes that function in the control of oxygen-derived free radicals and are found in various aerobic organisms including bacteria, higher plants and animals (1, 2). These enzymes have a high molecular weight ranging from 220-350kDa. They are tetrameric proteins containing Fe^{3+}-protoporphyrin groups bound to their active sites (1-4). However, probably due to the binding of a molecule of NADPH to each subunit (5), *A. niger* catalase exhibits a much greater stability to high concentrations of H_2O_2 in comparison to its mammalian counterpart (2). It is believed that catalases are extremely important in cellular defense from oxidative stress via the protective destruction of H_2O_2 into H_2O and O_2 (1, 4, 6). Additionally, these enzymes may play a vital role in cellular detoxification with their ability to oxidize various compounds such as alcohols, alkyl peroxides, formic acid and azide (2, 7, 8).

Peroxynitrite (PN), a derivative of nitric oxide (NO) (9-12), is a strong oxidant (11, 12). Nitric oxide/PN-mediated oxidative stress has been implicated in the pathogenesis of Parkinson's disease (13, 14), Alzheimer's disease (10, 13-15), artherosclerotic lesions (16, 17), Huntington's disease (13, 18), multiple sclerosis (19) and autoimmune myocarditis (17). Moreover, PN can inactivate various enzymes including $alpha_1$-antiproteinase (20), glutamine synthase (21), gylceraldehyde-3-phosphate dehydrogenase (22), acotinase (23), cytochrome P-450 (24), Ca^{2+}- ATPase of the sarcoplasmic reticulum (25), mitochondrial creatine kinase (26), tyrosine hydroxylase (27), Mn^{2+}-superoxide dismutase (28), prostacyclin synthase (29), tissue inhibitor of metalloprotease-1 (30), glutathione reductase (31), nicotinamide nucleotide transhydrogenase (32), cyclic AMP-dependent protein kinase (33), glutathione peroxidase (34) and protein kinase C (35). The nitration of proteins, via the formation of 3-nitrotyrosine, could be a hallmark of NO/PN-mediated oxidative damage (16).

Although catalases are antioxidant enzymes (36-38), it remains unclear whether these enzymes could be conversely inhibited/damaged by excessive exposure to PN, and whether their activities could subsequently be restored via enzymatic reduction/modification. Hence, *A. niger* catalase was chosen in this investigation, due to its exceptional stability, to clarify these processes.

3. MATERIALS AND METHODS

3.1. Materials

A. niger catalase was obtained from OXIS International, Inc., Portland, OR, USA. Hydrogen peroxide (3%) was from Diamond Products, Seffner, FL, USA. Sodium dodecyl sulfate (SDS), reduced glutathione (GSH), glutathione S-transferase (GST), acrylamide and EDTA were supplied by Sigma Chemical Company, St. Louis, MO, USA. Western blotting reagents, nitrocellulose membranes, and anti-rabbit IgG alkaline phosphatase conjugate were obtained from Bio-Rad Laboratories, Hercules, CA, USA. Peroxynitrite and rabbit polyclonal anti-nitrotyrosine were purchased from Upstate Biotechnology, Lake Placid, NY, USA. Rabbit polyclonal anti-S-nitrosocysteine was provided by Calbiochem-Novabiochem Corporation, San Diego, CA, USA. Microcon centrifugal filter tubes (30,000Da-cut off) were from Millipore Corporation, Bedford, MA, USA.

3.2. Preparation of peroxynitrite (PN)-treated catalase (PN-catalase) and subsequent reduced glutathione (GSH)-treated PN-catalase (PN-catalase$_{GSH}$)

Three milligrams of *A. niger* catalase was incubated with varied amounts of PN for 15 minutes at 25°C in a final volume of 400µl of 50mM potassium phosphate buffer (pH 7.4) containing 1mM $FeCl_3$ and 1mM EDTA (39). The incubation mixture was then transferred to centrifugal filter tubes (30kDa-cut off) and centrifuged at 13,500 rpm at 4°C to remove excess PN, $FeCl_3$ and EDTA. Peroxynitrite-treated (PN)-catalase was recovered with an appropriate amount of 50mM potassium phosphate buffer (pH 7.4). Alternatively, the recovered PN-catalase was further incubated with 1mM GSH for 30 minutes or 3mM GSH for 15 minutes at 25°C, and excessive GSH was removed with centrifugal filtration (30kDa-cut off) at 13,500 rpm at 4°C. The resulting samples (PN-catalase$_{GSH}$) were dissolved with an appropriate amount of 50mM potassium phosphate buffer (pH 7.4). The above treated catalase was used for the following experiments involving the production of O_2 and Western blot.

3.3. Production of O_2 catalyzed by catalase

The incubation mixture was in a final volume of 25ml of 1M potassium phosphate buffer (pH 7.4) containing 3%(v/v) H_2O_2, and 1.0mg of catalase preincubated with, or without, 200µM PN. In another parallel experiment, PN-catalase was further treated with 3mM GSH (PN-catalase$_{GSH}$) prior to the incubation in H_2O_2 solution. The reaction was carried out for 20 minutes at 25°C. The emission of O_2 was monitored while recording its volume at 2-minute intervals.

3.4. Enzymatic modification of PN-catalase$_{GSH}$ by glutathione S-transferase (GST)

With minor modifications, the experimental procedures basically followed those as described in past studies (33, 40, 41). The incubation mixture was in a final volume of 33µl of 50mM potassium buffer (pH 7.4) containing 60µg of PN-catalase or PN-catalase$_{GSH}$, 1mM of various protease inhibitors (pepstatin-A, bestatin, leupeptin, and transepoxysuccinyl-L-leucylamido(4-guanido)-butane), and varied amounts of GST. The reaction was carried out for 30 minutes at 37°C, and then terminated by the addition of 6µl of 10% SDS (without beta-mercaptoethanol or diothiothreitol), excluding sample boiling (33). The samples were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (42) and subsequent Western blot analysis. Anti-nitrotyrosine and anti-S-nitrosocysteine were used separately as primary antibodies (0.05µg/ml) (42), and anti-rabbit IgG-AP conjugate (43) as the secondary antibody. Finally, the enzymatic modification of PN-catalase$_{GSH}$ was monitored by their altered immunoreactivities.

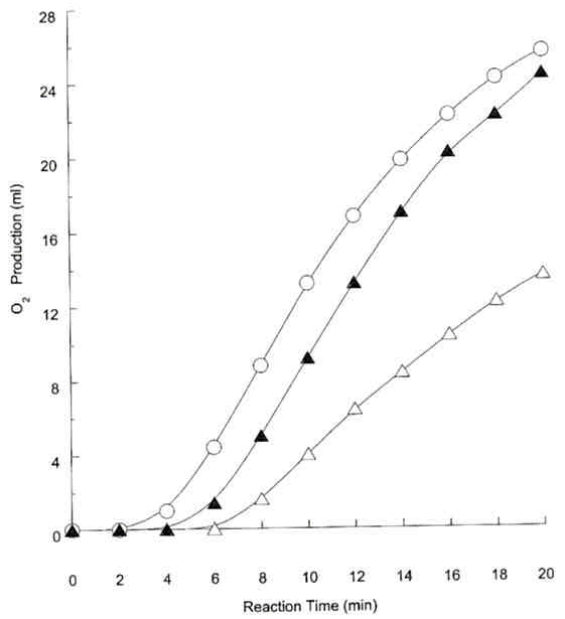

Figure 1. Inhibition of catalase by peroxynitrite (PN), and subsequent recovery of enzymatic activity by reduced glutathione (GSH). Catalase was preincubated with (△) and without (○) PN. Alternatively, PN-incubated catalase was further incubated with GSH (▲). All samples were finally incubated with 3% H_2O_2 as described under *Materials and Methods.*

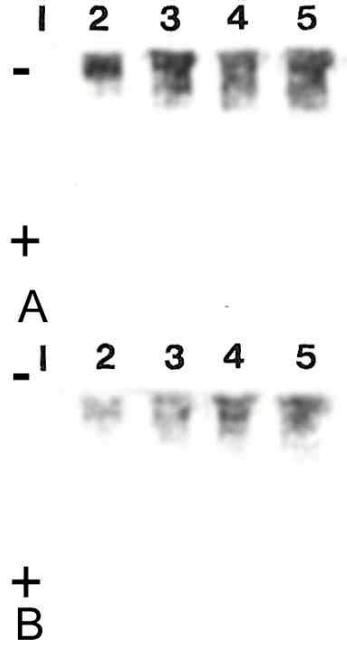

Figure 2. Dose-dependency of peroxynitrite-treatment on (A) tyrosine-nitration, and (B) cysteine-S-nitrosation of catalase. Samples were subject to SDS-PAGE (12%). Immunoreactivities were detected with anti-nitrotyrosine or anti-S-nitrosocysteine in Western blot. Lanes 1-5: catalase (60μg) treated with PN at 0, 50, 100, 200 and 400μM, respectively.

3.5. SDS-PAGE and Western blot

Bio-Rad's MiniPROTEIN 3 Cell was employed for both SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (42) and protein blotting (to the nitrocellulose membrane). Experiments required pre-cooled tank buffer and prefrozen Towbin transfer buffer. Ten milliamps for 30 minutes and 350mA for 50 minutes were required for SDS-PAGE and protein blotting, respectively. The entire process of Western blot was accomplished in 6 hours.

4. RESULTS AND DISCUSSION

At the beginning of this investigation, both *A. niger* and bovine liver catalase were tested. The instability of catalase from bovine liver, especially after PN-treatment, led to inadequate results (data not shown). Accordingly, the more stable *A. niger* catalase was exclusively used throughout this entire study. The time-dependent production of O_2, up to 20 minutes, is illustrated in figure 1. At the early phase of incubation, less than 8 minutes, the PN (200μM)-treatment of catalase resulted in more than 90% inhibition of O_2-production. However, as the reaction time progressed, the percentage of inhibition decreased, and at the end of 20 minutes approximately 50% of inhibition remained. In comparison, the further treatment of PN-catalase with GSH (1mM) (PN-catalase$_{GSH}$) restored 57% of catalase activity at the end of 8-minute incubation; thereafter, the percentage of restored activity significantly increased. Particularly, at the end of 20-minute incubation, the inhibition due to PN-treatment was largely abolished and 96% of catalase activity was restored (figure 1).

Among the 730 amino acid residues of *A. niger* catalase, 26 are tyrosine and 3 are cysteine (44). Upon PN-treatment of catalase, 3-nitrotyrosine and S-nitrosocysteine may be formed as demonstrated by their respective immunoreactive bands (figure 2, A and B). Both the smaller number of cysteine residues (44) and the instability of S-nitrosocysteine (45, 46) may account for the much less intensity of its immunoreactive bands (figure 2B). In contrast, the much greater number of tyrosine residues and the greater stability of 3-nitrotyrosine resulted in the stronger intensity of immunoreactive bands (figure 2A). The stability of PN-modified amino acid residues was sustained by omitting the reducing agent (beta-mercaptoethanol or diothiothreitol) and the step of sample boiling, together with a 4°C- cooling process in SDS-PAGE and protein blotting (47). As a result, the accurate observation of PN-dose dependency, up to 400μM, on the progressively increased immunoreactivity was accessed (figure 2, lanes 1-5).

The nitration of tyrosine and the S-nitrosation of cysteine may partially account for the inhibition of catalase via PN-treatment, because these modifications may lead to conformational changes, which may be unfavorable for substrate binding and catalytic processes. It has been speculated that the binding of NO to the Fe^{3+}-protoporphyrin groups of catalase to form a transient complex can lead to a moderate enzymatic inhibition (4). Likewise, the binding of PN and its active derivatives, including NO_2, •OH and other nitrogen/oxygen species

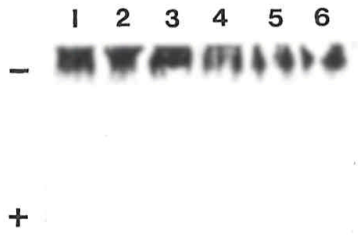

Figure 3. Denitration/modification of peroxynitrite-treated (PN)- catalase by incubation with reduced glutathione (GSH) and subsequently with glutathione S-transferase (GST). Samples were subject to SDS-PAGE (15%). Immunoreactivities were detected with anti-nitrotyrosine in Western blot. Lanes 1-3 (controls, without preincubation of GSH): PN (200μM)-catalase (60μg) directly incubated with GST at 6, 12 and 24μg, respectively; lanes 4-6: PN-catalase preincubated with GSH (1mM) prior to incubation with GST at 6, 12 and 24μg, respectively.

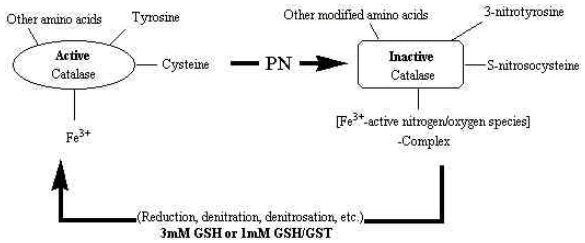

Figure 4. The mechanism of modulation on catalase activity**.**

(48), to the heme groups (1, 3, 4), may drastically reduce the accessibility of the active site on the enzyme, thus synergistically inhibiting the enzyme. Conversely, incubation of the catalase with 3mM GSH may lead to transnitrosation (denitrosation), reduction of the nitro-group into an amino-group and/or removal of the active nitrogen/oxygen species from the heme group, thereby reactivating the enzyme. Moreover, the presence of 3mM GSH may stabilize the binding of NADPH to the subunits of the catalase (5). Typically, the intracellular concentration of GSH is found between 1 and 5mM (49). Preincubation of PN-catalase with 1mM GSH, at its lower end of intracellular concentration, may form an intermediate complex, which facilitates subsequent GST-catalyzed restoration to its native activity. The incubation of PN-catalase with 1mM GSH (PN-catalase$_{GSH}$) did not significantly alter its 3-nitrotyrosine immunoreactivity (data not shown). However, the subsequent incubation of PN-catalase$_{GSH}$ with GST may modify its 3-nitrotyrosine residues and remove the active nitrogen species bound to Fe_3^+ (figure 3, lanes 4-6). The decreased immunoreactivity is most likely due to conversion of 3-nitrotyrosine to 3-aminotyrosine residue of the enzyme (figure 3), rather than proteolysis, because sufficient protease inhibitors had been added to the incubation mixture. The probable mechanisms of inactivation and reactivation of catalase is summarized in figure 4. A much less intensity in the decrease of S-nitrosation by incubation with GSH/GST was also observed (data not shown), and this may be due to the insufficient number, 3, of cysteine residues and/or the pre-removal of the nitroso-group via transnitrosation to form S-nitrosoglutathione. Other amino acids such as lysine, arginine and glutamine may also be modified upon PN-treatment. In conclusion, either full or partial processes to reverse PN-mediated modifications of catalase, especially catalyzed by GSH/GST, may be pivotal to restore the enzymatic activity (figure 4) and maintain cellular homeostasis.

5. ACKNOWLEDGEMENT

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