Importance of catalase in adaptive response and resistance to hydrogen peroxide in Haloferax IRU1

H Amirkhani, E Asgarani, M Khodabanode

Abstract

Haloferax IRU1 is an extremely halophilic Archaeon, which is highly resistant to the lethal effects of hydrogen peroxide. To investigate the role of catalase in the tolerance of Haloferax IRU1 to hydrogen peroxide stress, the expression and activity of catalase by this archaeon was studied. When cells were treated with several levels of hydrogen peroxide, activity and expression of catalase increased, in comparison to that of untreated cells. This suggests that catalase is an important factor in the tolerance of Haloferax IRU1 to hydrogen peroxide stress. To investigate the adaptive response to the lethal effects of hydrogen peroxide in Haloferax IRU1, cultures when pretreated with sub-lethal levels of hydrogen peroxide, became more resistant to its lethal effects and exhibited higher levels of catalase than those of un-pretreated cultures. This indicates that catalase is induced during the adaptive response of this strain to hydrogen peroxide stress.

INTRODUCTION

Woese and Fox have proposed that all organisms can be classified into three domains: Archaea, Bacteria and Eucarya (Woese and Fox1994). Members of the domain Archaea are phylogenetically divided into three kingdoms, namely, Euryarchaeota, Crenarchaeota and Korarchaeota. Most members of the kingdom Euryarchaeota are predominantly methanogens, but there are two other phenotypes found in this group, sulfur metabolizing thermophiles and extreme halophiles (Danson et. al. 1992).

The extremely halophilic Archaea require at least 2M NaCl or equivalent ionic strength for growth and the most growth is in saturated or near-saturated brines (Madigan et. al. 2000). Haloferax IRU1 is an extreme halophilic member of the kingdom Euryarchaeota, that was obtained by filtration of the Uromia salt lake water located in the north-west of Iran. After microscopic, morphological and biochemical studies and determination of the optimal concentration of salts required for growth, this microorganism was identified as Haloferax IRU1. It shows high resistance to ionizing radiation (gamma rays), non – ionizing radiation (Ultraviolet light) and to the oxidative agent hydrogen peroxide (H$_2$O$_2$) (Asgarani et. al. 2007). Ionizing radiation and many other oxidative agents such as hydrogen peroxide produce different types of reactive oxygen species (ROS) including the superoxide anion (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (.OH) will be generated (Halliwell 1994). These species are capable of damaging DNA, protein and lipid membranes, sometimes causing lethal damage to cells (Cadenas 1989). They can be produced by both environmental and endogenous sources (Halliwell 1994).

Considering the high resistance of Haloferax IRU1 against oxidative stress, it is reasonable to assume the presence of developed defensive mechanisms in this organism that allow cells to survive after exposure to high levels of ionizing radiation and hydrogen peroxide. Enzymatic defenses include superoxide dismutase, catalase and peroxidases (Gaetani et. al. 1989). Superoxide dismutase, converts superoxide radicals to H$_2$O$_2$, and catalase converts H$_2$O$_2$ to water and oxygen (Brionkhanov and Netrusor 2004). Under normal physiological conditions there is a balance between ROS generation and their elimination by different antioxidants. If the production of oxidants is enhanced or the power of the antioxidant system is decreased, oxidative stress arises (Fridowich 1978).

Cells pre-exposed to comparatively mild and sub lethal stress situations activate adaptive responses and acquire tolerance to subsequent more lethal stress. Such responses, named adaptation, are observed not only in bacterial cells
but also in eukaryotic organisms (Crawford and Davics 1994). Detailed mechanisms of adaptation to oxidative stress have been most extensively characterized in bacterial cells (Engelmann and Hecker 1996) and have also been studied in yeast (Mutoh et. al. 1995, Mutoh et. al. 1999).

An advantage of using an archaeon as a model system for studying adaptive responses is identifying the mediators of this response, as compared with eukaryotes (Crawford and Davics 1994). In the present study, the role of catalase in the adaptive response and resistance of Haloferax IRU1 to oxidative stress has been investigated.

**MATERIAL AND METHODS**

Organism and culture condition. Haloferax IRU1 was provided by Mahdeye Shirzad (Shirzad 2005) and was grown in the complex medium containing 20% (w/v) NaCl, 0.5% (w/v) yeast extract, 3.46% (w/v) MgCl₂, 6 H₂O, 4.94 % (w/v) MgSO₄, 7H₂O, 0.092% (w/v) CaCl₂, 2 H₂O, 0.058% (w/v) NaBr, 0.05 % (w/v) KCl, 0.017% (w/v) NaH₂CO₃, adjusted to pH 7. Cultures were grown at 47 °C with shaking at 200 rpm.

Preparation of cell extracts. Cells were harvested by centrifugation at 7,500 g for 15 min and then resuspended in dH₂O. Cell suspensions were frozen (-70 °C, 5 min) and thawed (37 °C, 2min) five times and then sonicated at 4 °C (30s - pulse on, 60s - pulse off) for a total of 9 min. Cell debris was removed by centrifugation (10,000 g for 5 min), and the crude cell extracts were stored on ice.

Catalase assay. Catalase activity was measured by the method of Aebi (Aebi, H. 1984), in which H₂O₂ was used as the substrate. The initial rate of disappearance of H₂O₂ (0 to 60 sec) was recorded with a Beckman spectrophotometer at a wavelength of 240 nm. The catalase activity was expressed as units per milligram of protein, using a standard curve obtained with commercially available catalase.

Protein analysis. The protein concentration was measured with BCA protein assay reagent kit (Ray Biotech) and bovine serum albumin (BSA) as the standard (Bradford 1976). The protein bands were visualized by Coomassie blue staining. The catalase content of cell extract was determined by SDS-PAGE analysis using of bovine catalase (Rosch) as the standard, and catalase band intensity was analyzed by gel scanner (Pharmacia LKB).

Western blot analysis and quantification. Protein extract was separated by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was transblotted onto a nitrocellulose membrane, blocked with 5% milk powder in TBS (TRIS-buffered saline pH 7.4) for 2 hours, and then incubated with rabbit polyclonal anti-bovine catalase antibody (1:100 dilution) for 90 min, washed with TBS, and incubated with secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase, (1:1,000 dilution) for 90 min. After 3 washes in TBS, the paper strip was treated with substrate solution (2 ml of 0.3% 4-chloro-1-naphtol in methanol was mixed with 2 ml TBS and 15µl H₂O₂) for 10 min at room temperature, and the signal was detected with a gel scanner (Pharmacia LKB).

Antiserum production. Antibody was elicited against purified bovine liver catalase in a laboratory rabbit. In the first injection the 20 mg of catalase was emulsified with an equal volume of complete Freund,s adjvant (Sigma), and in the second injection incomplete adjuvant was used, and applied subcutaneously to multiple sites of the rabbit at weekly intervals. In the third injection only 20 mg of catalase was applied intramuscular. Final injection was carried out intramuscular with 7 mg of catalase one week later. After bleeding of rabbit, serum was collected and antiserum was isolated and frozen at -20 °C.

Treatment with H₂O₂. To determine the lethal effect of H₂O₂, cells in the mid – logarithmic phase of growth were treated with various doses of H₂O₂ (5 – 35 mM) for 60 min. Following treatment, cultures were sampled every 10 min and appropriate dilutions were plated onto solid media. Colony counting was performed after incubation at 47°C for 5-7 days.

Determination of minimum inhibitory concentration (MIC) for H₂O₂. To determine the inhibitory growth effect of H₂O₂ on Haloferax IRU1, cells in the mid – logarithmic phase of growth were treated with various doses of H₂O₂ (3–15 mM) for 4 hours, and cell growth was monitored by measuring the change in turbidity of the cultures at 600 nm, using a Beckman spectrophotometer. The dose of H₂O₂ that allowed the cells to grow at a rate of 90% of that of untreated cells was represented as the MIC.

Adaptive response analysis. To examine adaptive response of Haloferax IRU1 to H₂O₂, cells were pretreated with MIC, 0.1 MIC and 0.01 MIC of H₂O₂ for 0.5, 1, 4 and 16 hours, followed by treatment with a lethal dose of H₂O₂ for 60 min. Cultures were then sampled every 10 min, diluted and plated onto solid media (Praul and Taylor 1997). Bacterial cultures were then grown for 5-7 days at 47 °C. The survival of
pretreated cells was then compared to that of un-pretreated cells. It should be noted that cell survival was compared after several pretreatments.

Effect of H$_2$O$_2$ on catalase expression Exponentially growing cultures of Haloferax IRU1 were treated with various doses of H$_2$O$_2$ (5 –35 mM) for 60 min and then harvested by centrifugation (7500 g, 15 min). Cell pellets were separated electrophoretically on 13 % (w/v) by SDS-PAGE. The gel was then stained with Coomassie blue. The catalase band of every sample was analyzed with a gel scanner. The percentage yield of the catalase band that was determined for every sample was then compared.

**RESULTS**

Determination of the resistance and adaptive response of Haloferax IRU1 to H$_2$O$_2$ Cells in the mid – logarithmic phase of growth were treated with various doses of H$_2$O$_2$ for 60 min and plated to evaluate percentage survival. The resulting H$_2$O$_2$ survival curve (Fig.1) showed that Haloferax IRU1 was highly resistant to the lethal effects of H$_2$O$_2$. The lethal dose of H$_2$O$_2$ for Haloferax IRU1 was determined as 34.91 mM. The minimum inhibitory dose of H$_2$O$_2$ (MIC) that allowed the cells to grow at a rate of 90 % of that untreated cells was found to be 3.11 mM.

Figure 1 shows that pretreated Haloferax IRU1 exhibited an adaptive response to H$_2$O$_2$ demonstrating a large shoulder in the survival curve in comparison to that of untreated samples. The results also demonstrated that as H$_2$O$_2$ concentrations increased during pretreatment, cellular survival also increased. However the durations of pretreatment did not have any effect on cellular survival (data not shown).
Importance of catalase in adaptive response and resistance to hydrogen peroxide in Haloferax IRU1

Figure 2
Figure 2. Survival curves of cells pretreated with 0.03 (green), 0.3 (yellow blue), 3 (pink) mM HO and unpretreated cells (dark blue) for (a) 0.5 h (b) 1 h (c) 4 h (d) 16 h, followed by treatment with 40 mM HO for 60 min.

Figure 3
Antiserum Production Serum was examined by dot blotting in order to producing antibody before third injection. Results showed that antibody was produced against to purified bovine liver catalase in body of rabbit before third injection. Serum was also examined by dot blotting in order to titrating antibody in final step of injection. Results showed that antibody with concentrations 1/50 and 1/100 can detect catalase enzyme in both purified bovine liver catalase and Haloferax extract.

Western blot analysis demonstrated that Haloferax catalase band is in the near position with purified bovine liver catalase band on SDS – PAGE gel electrophoresis (Fig. 3).

Figure 4
Figure 3. to detecting catalase band protein in Haloferax extract with western blotting and SDS-PAGE gel electrophoresis. 1 – molecular weight marker 2 – standard catalase 3 – extract. Results were showed catalase band in Haloferax extract was setting in the same position to standard catalase.

Analysis of catalase expression after H₂O₂ treatment Protein extracts of pretreated cells with MIC, 0.1 MIC, 0.01 MIC for 16 h, were analyzed both electrophoretically on 13 % (w/v) SDS-PAGE and with western blotting. Analysis of the catalase content of the cells, using a gel scanner showed that percentage catalase expression rose from 5.2 % in untreated cells to 5.4 %, 5.9 % and 6 %, for cells treated with 0.03, 0.3 and 3 mM of H₂O₂ for 16 h, respectively. The catalase activity was measured spectrophotometerically. The obtained results indicated that catalase expression and activity increased with rising levels of pretreatment, in comparison to those of non – pretreated cells (Fig. 4, 5).

Figure 5
Fig.4. The role of catalase in the adaptive response. 13 % (w/v) SDS-PAGE of pretreated cells, showing (lane 1) Protein molecular weight marker, (lane 2) standard enzyme (Roche), (lane 3) untreated cells, (lane 4) cells pretreated with 0.03 mM of HO, (lane 5) 0.3 mM HO and (lane 6) 3 mM of HO
Importance of catalase in adaptive response and resistance to hydrogen peroxide in Haloferax IRU1

To elucidate the effect of catalase on the resistance of Haloferax IRU1, exponentially growing cultures were exposed to increasing doses of H$_2$O$_2$ for 60 min, catalase expression and activity increased accordingly, as compared to that of untreated cells (Fig. 6, 7). Maximum of percentage catalase expression (12 %) was obtained when cells were treated with 20 mM H$_2$O$_2$ for 60 min, compared to that of untreated cells which showed a percentage catalase expression of 7.5 %. This emphasized the positive effect on catalase expression and activity.

**Figure 6**

Figure 5. Catalase activity indicating a) activity and b) specific activity (U/mg) of untreated cells (sample number 1) and cells pretreated with 0.01 MIC (sample number 2), 0.1 MIC (sample number 3) and MIC (sample number 4), for 16 hours.

**Figure 7**

Figure 6. Effects of HO on catalase expression. 13 % (w / v) SDS–PAGE of exponentially growing cells, showing (lane 1) protein molecular weight marker, (lane 2) enzyme catalase standard (roche), (lane 3) untreated cells, (lane 4) cells treated with 5 mM HO, (lane 5) 10 mM HO, (lane 6) 15 mM HO (lane 7) 20 mM HO, (lane 8) 25 mM HO and (lane 9) 30 mM HO.

DISCUSSION

We have previously reported that Haloferax IRU1, an extreme halophilic archaeon, is highly resistant to the lethal effects of (Asgarani et. al. 2007).

Defense mechanisms against oxidative stress in extremely halophilic Archaea have previously been determined, for instance in Halobacterium salinarum which exhibits high resistance to the lethal effects of DNA-damaging agents. In fact intracellular KCl has been found to have a protective role in the resistance of H. salinarum (Shamohammadi et. al. 1997). Bacterioruberin, one of the major pigments of the extremely radioresistant bacterium, Rubrobacter radiotolerans (Saito et. al. 1994), has been documented as a very efficient scavenger of OH radicals (Saito et. al. 1997). This pigment has also been found in Haloferax species (D’Souza et. al. 1997, Asgarani et. al. unpublished data). Research has shown that a colourless mutant of H. salinarum is more radiation sensitive than the wild type, and that bacterioruberin has a protective role against DNA-damaging agents including ionizing radiation (Shamohammadi et. al. 1998). Therefore, the resistance of Haloferax could also be partly due to the high concentration of intracellular KCl, the presence of the bacterioruberin and also extra enzymatic activity (antioxidant enzymes such as catalase and DNA repair enzymes).

In the present study, we have demonstrated the presence of adaptive response in Haloferax IRU1 that may function in the tolerance to oxidative stress produced by H$_2$O$_2$. Although hydrogen peroxide is not itself a free radical, it generates hydroxyl radicals, the most toxic species of the reactive oxygen species. Catalase is an antioxidant enzyme, which catalyzes the dismutation of H$_2$O$_2$, forming O$_2$ and H$_2$O.
In conclusion, the present results suggest that catalase of Haloferax IRU1 protect this organism against oxidative stress, although it may not be the sole mechanism for the resistance of this Archaeon to DNA-damaging agents. The additional protective role of DNA repair presumably present in this organism remains to be elucidated.

ACKNOWLEDGMENTS

The research was supported by National Institute of Genetic Engineering & Biotechnology, and Alzahra University. The authors are grateful to H. Terato and P. Shariati for their contribution to the improvement of this paper through useful suggestions and detailed comments.

References


Previous findings have suggested that catalase may function to protect different groups of organisms against H2O2 (Engelmann et al. 1996, Gaetani et al. 1994, Gaetani et al. 1989, Gill and Sigler, 1995, Jamieson 1992, Mutoh et al. 1999). In this study catalase was found to have an important role in the tolerance to the H2O2 stress by Haloferax IRU1 which exhibited high levels of catalase expression and activity. Adaptive response is observed in all organisms in response to a number of different cytotoxic agents. One of these agents, oxidative stress, is known to induce an adaptive response in several organisms by the induction of many proteins. The main physiological benefit of the adaptive response is protecting of cells and organisms from high doses of a toxic agent. Therefore understanding oxidant adaptive response in more detail and identifying the protective proteins involved may prove to be of clinical benefit (Crawford and Davics 1994). Valuable information on the adaptive response process and the mediating enzymes involved, such as catalase, in several organisms such as Saccharomyces cervisiae (Jamieson et al. 1994, Jamieson 1992), Schizosaccharomyces pombe (Mutoh et al. 1995, Mutoh et al. 1999), Haloferax et mediteranei (D’Souza et al. 1997) and Escherichia coli (Assis et al. 2002, Jeggo et al. 1977) have been obtained. An important early study by Samson and Cairns nicely demonstrated a strong adaptive response in Escherichia coli following exposure to alkylating agents (Samson and Cairns 1977).

In the present study, the adaptive response of Haloferax IRU1 to oxidative stress has been examined in exponential phase cells. Cells that pretreated with 0.03, 3 and 3 mM H2O2 for 0.5, 1, 4 and 16 h, significantly increased survival in the presence of such lethal doses (fig. 2). Catalase activity also was increased in pretreated cells with 0.01 MIC, 0.1 MIC, and MIC after 16 h (fig. 3)

SDS-PAGE and western blot analysis shows moderately increasing expression levels of catalase in pretreated cells in comparison with untreated ones (fig. 6 a, b), thus indicating the existence of an adaptive response to oxidative stress in this strain with catalase being identified as the inducible protein in this response. In addition, catalase expression, activity and specific activity rose with increasing concentrations of hydrogen peroxide. Therefore catalase can play a key role in the resistance of Haloferax IRU1 against oxidative stress.

In conclusion, the present results suggest that catalase of Haloferax IRU1 play a key role in the resistance of Haloferax IRU1 against concentrations of hydrogen peroxide. Therefore catalase can activity and specific activity rose with increasing protein in this response. In addition, catalase expression, this strain with catalase being identified as the inducible role in the existence of an adaptive response to oxidative stress in comparison with untreated ones (fig. 6 a, b), thus indicating increasing expression levels of catalase in pretreated cells in SDS-PAGE and western blot analysis shows moderately increasing expression levels of catalase in pretreated cells in comparison with untreated ones (fig. 6 a, b), thus indicating the existence of an adaptive response to oxidative stress in this strain with catalase being identified as the inducible protein in this response. In addition, catalase expression, activity and specific activity rose with increasing concentrations of hydrogen peroxide. Therefore catalase can play a key role in the resistance of Haloferax IRU1 against oxidative stress.
Importance of catalase in adaptive response and resistance to hydrogen peroxide in Haloferax IRU1

Importance of catalase in adaptive response and resistance to hydrogen peroxide in Haloferax IRU1

Author Information

Homeira Amirkhani, M.Sc of Microbiology
Department of Biology, Faculty of Science, Alzahra University, Tehran, Iran

Ezat Asgarani
Assistant professor of Genetics, Department of Biology, Faculty of Science, Alzahra University, Tehran, Iran

Mahvash Khodabanode
Assistant professor of Biochemistry, National Institute of Genetic Engineering & Biotechnology, Tehran, Iran