Synopsis of the thesis on

Effect of thiol stress in *Streptomyces coelicolor* at biochemical and genetic levels

To be submitted to

The Maharaja Sayajirao University of Baroda

For the degree of

Doctor of Philosophy in Biochemistry

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For the organism to survive it is very important to maintain its homeostasis at cellular and organismal level. There are some external factors which may cause disturbance to homeostasis which may lead to alterations in the normal metabolism leading to the condition widely known as stress. In order to overcome this stress, the organism tries to adopt itself by altering its functions at various levels like gene expression, protein synthesis, transcription, and protein folding. There are many kinds of stress, most common among them are oxidative, osmotic and heat shock.

Evolution started in the reducing environment in the absence of oxygen, after the evolution of photosynthetic organisms gradually there was rise in the oxygen level. Oxygen, due to its high reactivity and formation of reactive oxygen species (ROS), was proved to be fatal for the existing organisms. So the protection mechanism against this oxidative stress was also evolved during the course of evolution of aerobic organisms. There are lots of studies about the oxidative stress responses, but there is still very little understanding about the reductive stress which is not as common as oxidative stress and its consequences in the aerobic conditions.

The redox environment with in the cell is more reducing than extracellular space. This is very important to be maintained, in order to prevent the disintegration of the cellular matrix. For this cell has to regulate the redox balance towards the reducing side. At the same time cells take up many activities such as respiration, where it requires oxidizing conditions as well. As oxidative stress is harmful to the cell, so is reductive stress which is the other extreme in destabilizing redox balance and altering the normal homeostasis. Hence, there is a need to balance the redox conditions accurately. Thiols play a very significant role in the regulation of the redox status. In eukaryotes this is partially fulfilled by means of compartmentalization. Most of the oxidative pathways are located in the endoplasmic reticulum (ER), where oxidizing conditions are maintained for protein folding. Under anaerobic conditions ER is known to exert reductive stress leading to unfolded protein response (Trotter and Grant 2002). Reports in literature indicated under hypoxia rat hepatocyte mitochondria are known to generate ROS in response to the reducing conditions and thus participate in lethal cell injury during chemical hypoxia. It was observed that desferrioxamine which is an Inhibitor of iron catalyzed hydroxyl radical formation delayed cell killing due to hypoxia (T. L. Dawson *et. al* 1993).

E. coli, under oxidative stress activate an array of enzymes by a transcription factor OxyR, which is the major regulator of redox balance. The transcription factor oxyR and other proteins act as switches to control the redox state of the cell. Since bacteria are

exposed to very different conditions they might have developed the special response against the reductive stress which may be different from oxidative type. There is very less information about the reductive stress in prokaryotes. Thioglycerol, dithiothreitol (DTT) and β-mercaptoethanol are common reducing agents, which are known to suppress the aerobic growth in *E. coli.* There is an array of proteins whose expression is induced in the presence of these reducing compounds. These compounds may cause the following effects: lowering of S-adenosylmethionine levels, inhibition of respiration, interfering with the formation of disulfide bonds in the periplasmic space and outer membrane proteins, finally leading to reductive stress (G.T. Javor 1989). Thus, it would be important to explore this response in detail.

In this study *S. coelicolor* was chosen for the study to understand the reductive stress response. *Sreptomyces coelicolor A3(2)* is a Gram positive actinomycete, highly aerobic and versatile organism having the largest genome size (8.66Mbp) found among the prokaryotes. It differs from other bacteria in many aspects like it shows mycelial growth, high G+C content of 72.1% and also lacks glutathione. It is very well known for its secondary metabolic characteristics and produces a wide array of antibiotics and other important substances. It is also studied for its other regulations like stress and metabolic responses. Under oxidative stress induced by hydrogen peroxide (H_2O_2). *S. coelicolor* causes the induction of catalaseA in the log phase. There are three types of catalases in *S. coelicolor* CatA, CatB and CatC. CatA is the major catalase expressed in the log phase and involved in the oxidative stress response (YH Cho and JH Roe 1997). CatB is expressed in the stationary phase and the function of CatC is not much clear but known to express in the late log phase to early stationary phase.

CatalaseA is under the regulation of a repressor CatR which is a redox sensitive protein. CatR binds to the CatA promoter in the reduced state. Under oxidative stress the cysteine thiols of CatR get oxidized and is released from the CatA promoter leading to its overexpression (Ji-Sook Hahn *et. al.* 2000). Here in the present study we have checked the effects of thiol stress on the redox balance in *S. coelicolor.* The proteomic studies were carried out to identify the proteins involved in the regulation of thiol stress. One of the major proteins is CatalaseA which is generally induced in the oxidative stress. Further studies were carried out in order to understand the role of catalaseA in reductive stress and probable mechanism of its induction.

The objectives of the present study are –

1. To standardize the conditions for thiol stress and to establish its reductive nature.
2. To compare the differential protein expression profile of *S. coelicolor* and identification of proteins involved in thiol stress mediated by DTT (Dithiothreitol)
3. To assess the role of catalseA (one of the induced proteins) in the DTT mediated thiol stress and to study its mechanism of induction.
4. To determine the role of CatR in DTT mediated catalaseA induction.
5. To study the redox status of *S. coelicolor* under thiol stress and its effect on the membrane bioenergetics.

1. ***To standardize the conditions for thiol stress and to establish its reductive nature.***

In order to study the effect of sulphhydryl stress, DTT (Dithiothreitol) was chosen as a reducing agent. *S. coelicolor* was grown in YEME (Yeast Extract Malt Extract) medium and DTT stress was given at the mid log phase at different concentrations of DTT and at different time intervals. Since the organism has mycelial growth and grows as tangled balls in liquid culture it is not possible to determine its growth using optical density measurements or colony count methods. Hence, the growth was measured in terms of protein concentration. There was a decrease in the growth with increasing concentration of DTT. In order to check the redox status of the cell, the cells were sonicated and checked for the total thiol content which was found to be increasing under DTT stress. Hence the cells are experiencing the strong reducing conditions as expected. Also we checked the total ROS status of the cell by a redox sensitive fluorescent dye DCFH-DA, which was found to be unchanged under DTT stress. Overall these results show that the nature of the stress is of reductive type.

2. *To compare the differential protein expression profile of S. coelicolor under thiol stress mediated by DTT (Dithiothreitol) and identification of proteins involved in thiol stress.*

In order to investigate the effect of thiol stress, proteome analysis was done using SDS-PAGE and 2D-PAGE followed by MALDI-TOF analysis. From the differential protein expression profile around eighteen spots were found to show induction and six spots were down regulated under DTT stress. These spots were trypsinized and analyzed by MALDI-TOF. Out of them mass spectra could be generated for 19 spots. On the basis of MASCOT search the major identified proteins were CatalaseA, putative tetR-family transcriptional regulator, N-acetyl-γ-glutamyl-phosphate reductase, metalloproteinase, actinorhodin polyketide ketoreductase and β chain of putative DNA-polymerase III. All these proteins might have some role in the DTT mediated thiol stress response which can be examined further.

CatalaseA, which showed the most prominent expression even under one dimensional SDS-PAGE, was surprising since catalases are widely known to be induced under oxidative stress. Hence our studies lead to understand the role of catalaseA in DTT mediated thiol stress.

3. *To assess the role of catalseA in the DTT mediated sulphhydryl stress and to put forward its probable mechanism of induction.*

On the basis of biochemical analysis it was observed that catalaseA was induced under DTT in a dose dependent manner. Besides this there was no change in the superoxide dismutase (SOD) levels, which suggest that there is not much effect on the generation of superoxide radicals.

It was assumed that the induction of catalaseA is due to augmentation of $^{\bullet}OH$ radical generation under thiol stress by means of Fenton reaction. Under aerobic conditions there is a continuous generation of free radicals like O_2^{-}, H_2O_2, $OH^{\cdot}$. In normal conditions H_2O_2 is not much detrimental to the cell until it is reduced to $OH^{\cdot}$ radicals or some other forms of free radicals. According to Fenton reaction Fe^{2+} acts as

a reducing agent to drive this reaction. There is a very limited pool of Fe inside the cells which is not sufficient to drive Fenton reaction continuously. But a strong reducing agent like DTT can recycle Fe^{3+} to Fe^{2+} ions. This may lead to enhance the production of $OH^{\cdot}$ radicals causing all types of cellular damage. Hence the cell tries to adapt itself from the damage by inducing catalaseA activity thus breaking down the H_2O_2 into H_2O and O_2.

In order to prove this, the Fenton reaction was blocked by a Fe chelator O-phenanthroline. O-phenanthroline treatment prior to DTT stress failed to induce catalaseA, which suggest the role of the fenton reaction in the induction of catalaseA. So, it can be concluded that *S. coelicolor* shows the induction of catalaseA as a protective mechanism against the accelerated Fenton reaction.

4. To ***determine the role of CatR in DTT mediated catalaseA induction.***

In the presence of H2O2, *S. coelicolor* is known to induce catalaseA in the log phase. This induction is mediated by a repressor CatR. In order to find the role of CatR in DTT mediated thiol stress, CatR promoter was PCR amplified and cloned prior to a reporter gene enhanced green fluorescent protein (EGFP) in a shuttle vector pIJ8660 and was named pIJ6022. pIJ6022 was transferred to *S. coelicolor* and was found that there is not much change in the expression of EGFP under DTT stress. This suggests that CatR is not playing an important role in DTT mediated catalaseA induction as in case of H_2O_2 stress.

In order to confirm it, catalaseA mutant was generated in our laboratory by random chemical (EMS) mutagenesis. The cloned pIJ6022 was then transferred to catalaseA mutant. If catR is involved in DTT stress, the EGFP expression should be exaggerated in catalaseA mutant as compared to wild type since the mutant lacks catalaseA. EGFP expression studies in the mutant are in progress.

5. ***To study the redox status of S. coelicolor under sulphhydryl stress and its effect on the membrane bioenergetics.***

In order to study the role of DTT on the redox balance of the cell, as mentioned earlier, the total thiol content was determined and was found that the total thiol content was found to be higher under thiol stress as compared to control. The total ROS was measured by a redox fluorescent dye Dichlorofluorescein Diacetate (DCFH-DA). There was a significant rise in the ROS level in the presence of DTT and this difference was further increased in the CatalaseA mutant.

The membrane bioenergetics was done by means of oxidative phosphorylation studies using clark type oxygen electrode. Different substrates like glutamate, pyruvate + malate, succinate and ascorbate + TMPD were used for determining the phosphorylation rate for electron transport chain. From these studies it was found that there was significant increase in the overall respiration rate (with succinate and ascorbate + TMPD as substrates) in the presence of DTT. Also there was increase in the ADP phosphorylation rate in the presence of DTT. From the above results it shows that there is concomitant effect of both oxidative as well as reductive stress in the presence of DTT. Reductive with respect to thiol group and oxidative stress by means of fenton mediated reactions. Also there is increase in the overall respiration rate and ADP phosphorylation rate, probably to overcome the anaerobic stress, which may lead to more slippage of electrons, and thus the generation of ROS.

Conclusion

From the above studies it is clear that the redox status of the cell under DTT stress is reducing. Still there is induction of catalaseA. Normally under aerobic conditions there is continuous generation of ROS mainly through electron transport chain, which is independent of redox status of the cell. The generation of H_2O_2 in this case is not destructive, as it gets degraded faster by physiological level of catalase than its rate of conversion in to ·OH radicals. Under thiol stress there is a continuous supply of electrons by a reducing agent to reduction of Fe2+ ions to Fe3+. Hence fenton reaction is driven continuously leading to burst of ·OH radicals and thus to overall cellular damage. In order to combat this stress catalaseA is induced which clears H_2O_2 at a faster rate than its reduction by fenton reaction. Besides this under thiol stress the cell

experiences hypoxic conditions by altering the physiology of ETC, which may lead to induction of oxidative phosphorylation rate. This in turn again leads to more slippage of electrons, thus more generation of ROS. So under thiol reductive stress there is no direct involvement of oxidative stress. But the basal level of ROS generated is channalized in to more harmful free radicals at a faster rate than under normal physiological state. All these studies explain the reason for a very high induction of catalaseA under thiol stress.

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