CHAPTER 6

Influence of thiol stress on oxidative phosphorylation and generation of ROS in *Streptomyces coelicolor*

6.1. Introduction

6.1.1. Bacterial respiration

Bacteria generally acquire their energy by means of aerobic and anaerobic respiration. According to endosymbiont hypothesis mitochondria are evolved from the primitive aerobic bacteria (van der Beek and Stouthamer 1973). Similar to mitochondria, bacteria have their electron transport chain located on the inner cell membrane. The functional features of electron transport chain (ETC) remain similar in mitochondria and bacteria having more versatility and flexibility in the latter. Electron transport chain is basically organized into dehydrogenase and oxidase complexes connected by quinones. The quinones accept electrons from dehydrogenases and transfer them to oxidase complexes that reduce the terminal electron acceptor. There is a lot of flexibility in bacterial electron transport and it varies among different bacteria and also shows changes in response to the growth conditions. Bacteria generally possess a branched electron transport chain to oxygen with variable features as compared to mitochondria (Niebisch and Bott, 2003; Schafer and Penefsky, 2008). ETC can accept electrons from multiple substrates, since it has diverse types of single subunit dehydrogenases transferring electrons directly to quinones. Finally the electrons are transported to the molecular oxygen by quinone oxidases (Kitts and Ludwig, 1994) (Figure 6.1).

In bacteria, based on the physiology and environment of the organism, many organic and inorganic molecules such as nitrate and fumarate, can act as terminal electron acceptors instead of oxygen and thus can grow anaerobically (Kroger et al., 2002; Nishimura et al., 2007). The efficiency of oxidative phosphorylation is higher in intact mammalian mitochondria as compared to that of bacteria. Hence overall bacterial oxidative phosphorylation rate is lower than those of intact mammalian mitochondria in oxidizing the identical substrates (Harold, a and b 1972).

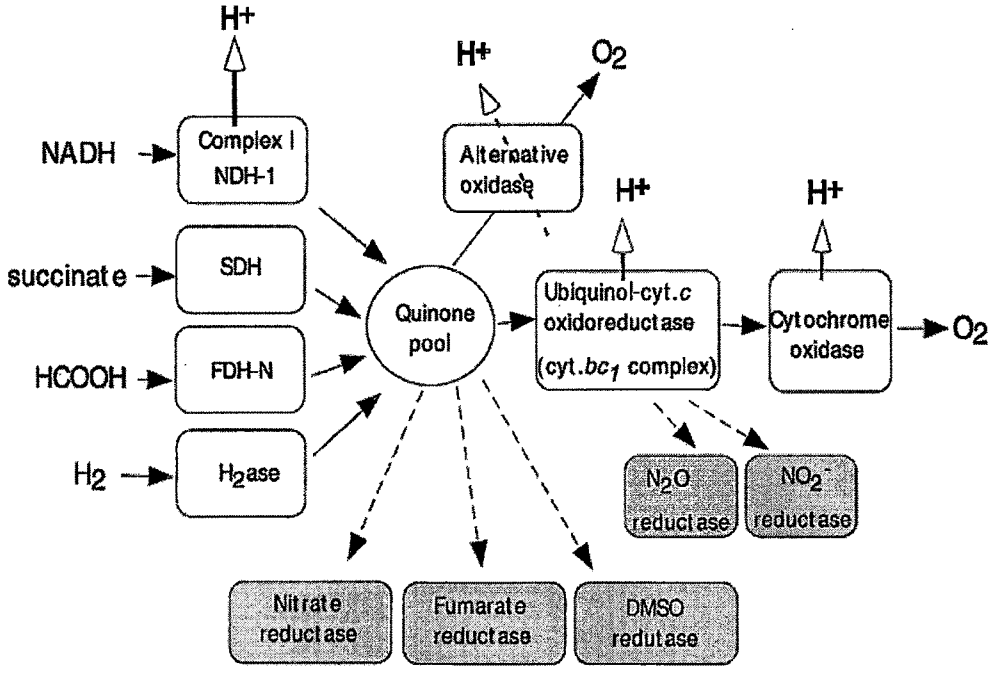

Figure 6.1: Simplified and general scheme illustrating electron pathways in respiratory chains. *White boxes* indicate electron-input units and *black arrows* the influx of reducing equivalents in the membrane. *Dashed arrows* indicate electron flux to output modules involved in anaerobic respiration (*shaded boxes*). Energy coupling sites are indicated by *open arrows* showing proton ejection.

6.1.2. Respiratory chain of *S. coelicolor*

Among bacteria, *Sreptomyces coelicolor* is a strictly aerobic organism. So the ultimate acceptor needs to be oxygen. Based on the information available from its genome sequence (Bentley, 2002) and other literature (Niederpruem and Hackett, 1961; Friedrich and Scheide, 2000; Chun et al., 2006) its ETC has many common features with the eukaryotic electron transport chain (Figure 6.2). From the figure it is clear that *S. coelicolor* possesses Complex I to complex IV with oxygen as the terminal electron acceptor. From these observations it can be presumed that under normal aerobic growth conditions it behaves like its eukaryotic counterparts with little difference.

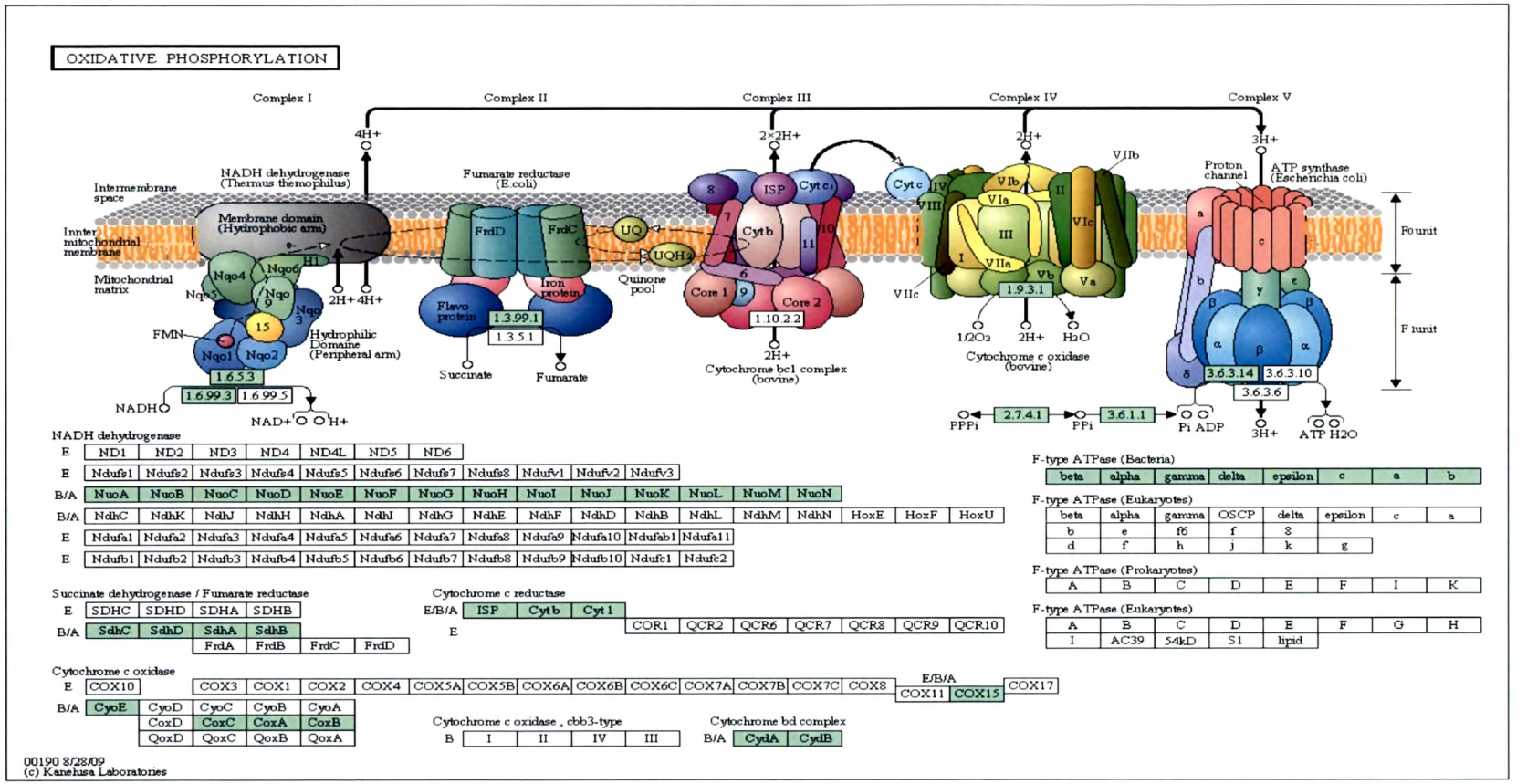

Figure 6.2: Oxidative phosphorylation pathway and comparison of Eukaryotic and bacterial components of respiratory complexes (I-V) based on homology. Highlighted boxes in green, represents the respective genes present in *S. coelicolor*. E – Eukaryotes; B/A – Bacteria/Archea. (http://www.genome.jp/kegg-bin/show_pathway?org_name=sco&mapno=00190&mapscale=1.0&show_description=show)

6.1.3. Electron transport chain and ROS generation

Molecular oxygen is a relatively stable diradical, with two spin-aligned unpaired electrons in its pi antibonding orbitals. Respiratory chain enzymes are very efficient in univalent redox reactions and thus transfer electrons to molecular oxygen leading to formation of O_2^- and H_2O_2 (Figure 6.3). Thus, respiratory chain is the major source of ROS under normal conditions. When *E. coli* respiratory vesicles are incubated in vitro with reductive substrates and oxygen, normal electron transport is accompanied by the generation of both O_2^- and H_2O_2 (Messner and Imlay, 1999). When *E. coli* grows exponentially in glucose medium, respiratory chain accounts for as much as 87% of the total H_2O_2 production and generates about 14 μM H_2O_2 per second (Seaver et al., 2001). This is mainly due to the leakage of electrons at the NADH dehydrogenase and ubiquinone sites (Gonzalez-Flecha and Boveris, 1995).

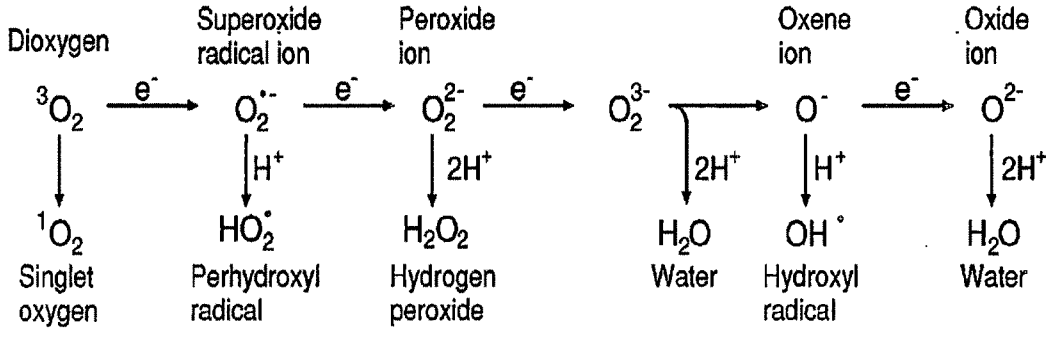

Figure 6.3: Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen.

6.2. Materials and methods

6.2.1. Oxidative phosphorylation

Clark type oxygen electrode used for Mitochondrial oxidative phosphorylation measurement was applied here with *S. coelicolor,* since it is an obligate aerobe

and its respiratory complex resembles that of the mitochondrial one. Measurements of oxidative phosphorylation were carried out at 25 °C. The respiration medium (total volume 1.6 ml) consisted of 225 mM sucrose, 20 mM KCl, 10 mM MOPS pH 7.4, 5 mM potassium phosphate buffer pH 7.4, 0.2 mM EDTA and 160 µg of BSA (i.e. 0.1 mg BSA /ml). After introducing 180µl log phase grown *S. coelicolor* culture in the electrode chamber, respiration was induced by the addition of substrates. Final concentrations of the substrates used were: glutamate (10 mM), pyruvate + malate (10 mM + 1 mM), succinate (10 mM) and ascorbate + TMPD (10 mM + 0.1 mM). Measurements with the latter two substrates were performed in the presence of 1 µM rotenone. State 3 respiration rates initiated by the addition of 80-200 nmoles of ADP and state 4 rates ensuing after the depletion of added ADP were recorded. Calculations of ADP/O ratio and ADP phosphorylation rates were as per the standard calculations as in mitochondria.

6.2.2. Measurement of hydrogen peroxide formation

H_2O_2 formation was monitored from the conversion of 2′, 7′-dichlorofluorescin diacetate to 2′,7′-dichlorofluorescein (Cathcart et al., 1983). Five ml of mid log phase *S. coelicolor* cells were centrifuged and resuspended in one ml of 100 mM potassium phosphate buffer, pH 7.2 and incubated with 10 pM dichlorofluorescin diacetate for 30 min at 30 °C. After loading with dichlorofluorescin diacetate, the cells were washed twice with potassium phosphate buffer and resuspended in the same buffer. The cells were sonicated and centrifuged at 10,000 g for 10 min to release the cytosolic dye and to remove the debris. Conversion of nonfluorescent dichlorofluorescin diacetate to highly fluorescent dichlorofluorescein was monitored with a Hitachi F-7000 fluorescence spectrophotometer, using excitation and emission wavelengths of 490 nm and 520 nm respectively.

6.3. Results

6.3.1. Respiratory rate of *S. coelicolor*

The respiration rate of *S. coelicolor* in the presence of substrates for the complexes I, II and III, in spite of oxygen uptake signal being lower for *S. coelicolor*, established that respiration in bacteria was in compliance with that of eukaryotic one (van der Beek and Stouthamer, 1973), but with slightly lower ADP/O ratio in the presence of all the four substrates (Table 1). Being an obligate aerobe, *S. coelicolor* showed induced respiration with ADP (state 3 respiration) as compared to without ADP (state 4 respiration) thus, mimicking the mitochondrial respiration. The proposed ADP/O ratio establishes the value for *S. coelicolor* and the validity of the assay with bacterial system. Hence, the protocol using these four substrates to measure the oxidative phosphorylation rate can be applied to study respiratory rates of not only *S. coelicolor* but also of other aerobic bacterial systems.

6.3.2. Effect of DTT on *S. coelicolor* respiration

State 3 and state 4 phosphorylation rates were increased in the presence of DTT (**Table 1**). These observations suggest two possibilities. First, the functionality of the respiratory complexes must have gone up under thiol stress. Second, in order to combat the reducing environment created by DTT, *S. coelicolor* must have induced the expression of respiratory complexes. However, there are no significant changes in the ADP/O ratio and the respiratory control ratio (RCR) indicating that the functionality of the respiratory complexes remains unaltered in the presence of DTT (Table 1). Increased ADP phosphorylation rate in the presence of DTT, suggests the second alternative as the most likely possibility as bacterial systems are more flexible and readily respond to external cues (Richardson, 2000).

Table 6.1: Effect of DTT on oxidative phosphorylation in *S. coelicolor* using glutamate, pyruvate + malate, succinate and ascorbate + TMPD as the substrate

Substrate	Treatment	ADP/O ratio	Respiration rate (n mole O_2/ min / mg protein)		Respiratory Control Ratio	ADP phosphorylation rate (n mole ATP formed / min / mg protein)
			+ ADP (State 3)	-ADP (state 4)		
Glutamate	No DTT (8)	2.36 ± 0.15	178.5 ± 17.5	141.8 ± 15.7	1.344 ± 0.072	1081 ± 94.98
	10mM DTT (7)	2.62 ± 0.11	249.6 ± 18.9 *	221.6 ± 19.9 **	1.213 ± 0.033	1475 ± 134.7 *
Pyruvate + Malate	No DTT (7)	2.49 ± 0.14	210.0 ± 15.4	156.5 ± 11.2	1.567 ± 0.093	1015 ± 127.9
	10mM DTT (7)	3.04 ± 0.19*	221.6 ± 25.8	155.4 ± 15.6	1.472 ± 0.061	1264 ± 197.8
Succinate	No DTT (12)	1.75 ± 0.082	196.9 ± 14.1	170.1 ± 10.1	1.226 ± 0.035	668.2 ± 38.31
	10mM DTT(10)	1.67 ± 0.127	278.2 ± 16.8 **	238.5 ± 13.4 ***	1.190 ± 0.037	904.0 ± 79.32**
Ascorbate + TMPD	No DTT (9)	0.392 ± 0.032	181.5 ± 12.0	149.6 ± 6.57	1.274 ± 0.050	140.6 ± 12.7
	10mM DTT (8)	0.387 ± 0.010	265.5 ± 8.7 ***	235.6 ± 15.4***	1.118 ± 0.040*	206.2 ± 9.8 **

Note: O_2 consuption rate was observed using Clark type oxygen electrode in control and DTT treated log phase grown cultures of *S. coelicolor* cells The cells were incubated with four substrates Glutamate, Pyruvate + Malate, Succinate and Ascorbate + TMPD and the respiratory rate was measured in the presence and absence of ADP. Based on this the ADP/O ratio, Respiratory control ratio and ADP phosphorylation rate were calculated.

6.3.3. Effect of DTT on generation of H_2O_2 in *S. coelicolor*

DTT is known to induce catalaseA in *S. coelicolor* (chapter 3 and 4). H_2O_2 generated in the presence of DTT due to Fenton reaction as well as increased respiratory rate, appear to be the chief causes for induction of catalaseA in *S. coelicolor*. Hence, H_2O_2 formation was examined for both *S. coelicolor* wild type (WT) and catalaseA mutant of *S. coelicolor* in the presence of DTT using dichlorofluorescein diacetate (Figure 6.4). It was observed that there was no significant change in the H_2O_2 production in *S. coelicolor* WT, since the induced levels of catalaseA compensate for the increased respiratory rate. In contrast catalaseA mutant showed significant increase in H_2O_2 production in response to DTT. Hence, it can be concluded that DTT induces ROS generation through increased respiratory rate.

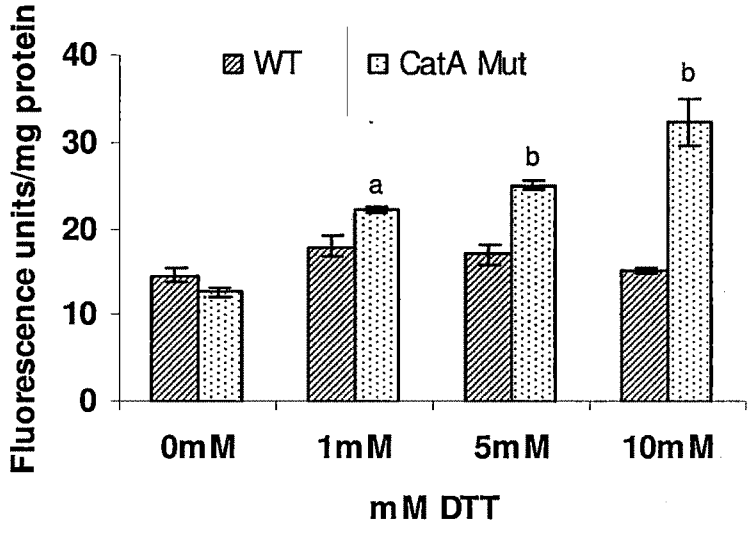

Figure 6.4: Effect of DTT on ROS generation in WT and *catA* mutant of *S. coelicolor*. Cross lined and dotted bars indicate ROS levels in WT and catalaseA mutant respectively. Values are expressed as means of six independent experiments ± SEMs. [a] $p<0.05$; [b] $p<0.01$ (as compared to their control i.e. 0 mM DTT)

6.4. Discussion

From the above studies it was found that, although the oxygen uptake signal was very low for *S. coelicolor* with respect to mitochondria, O_2 consumption was observed with higher number of the cells at the mid log phase. Also the ADP/O ratio observed with all the four substrates was in accordance with the mitochondrial values, which establishes the validity of the assay with bacterial system.

Significant increase in the state3 and state 4 respiration in the presence of DTT indicates that there is a rise in the over all respiration rate. At the same time there are no detectable changes in the ADP/O ratio and the respiratory control ratio (RCR) which states that there is no uncoupling taking place in the presence of DTT. The overall ADP phosphorylation rate is increased with all the four substrates indicating increase in the rate of ATP synthesis.

Unlike mitochondria bacteria have very versatile respiratory system. Hence, the components of bacterial respiratory system can be upregulated or downregulated in different environmental conditions and at different stages of growth (van der Beek and Stouthamer, 1973). Here, there is increase in the overall respiration rate without altering the ADP/O ratio and respiratory control ratio in the presence of thiol stress indicates that there must be upregulation of the respiratory units in order to contribute to the observed extra respiration rate.

Reducing environment leads reduction of oxygen causing partial hypoxia. In order to compensate for hypoxic stress, there seems to be a rise in the respiratory units on the inner membrane which in turn may generate more ROS. This observation is supported by increased levels of H_2O_2 in catalaseA deficient mutant in the presence of DTT. Thus, increased respiration, reduction of oxygen as a result of reductive stress and ROS generated due to Fenton reaction necessitates CatalaseA induction (Vekaria et al., 2007).