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Thiol specific oxidative stress response in *Mycobacteria*

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Abstract

The cellular response of mycobacteria to thiol specific oxidative stress was studied in *Mycobacterium bovis* BCG cultures. Two-dimensional gel electrophoresis revealed that upon diamide treatment at least 60 proteins were upregulated. Fourteen of these proteins were identified by MALDI-MS; four proteins, AhpC, Tpx, GroEL2, and GroEL1 are functionally related to oxidative stress response; eight proteins, LeuC, LeuD, Rv0224c, Rv3029c, AsnB, Rv2971, PheA and HisH are classified as part of the bacterial intermediary metabolism and respiration pathways; protein EchA14 belong to lipid metabolism, and NrdE, belongs to the mycobacterial information pathway category. Reverse transcription followed by quantitative real time PCR in response to diamide stress demonstrated that protein expression is directly proportional to the corresponding gene transcription.

Keywords: Mycothiol; Diamide; Mycobacteria; Disulfide

1. Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* is responsible for 2.2 million deaths annually and currently 45 million people worldwide suffer from it [1]. The ability to infect, survive and replicate within the human macrophages is one of the keys to success of *M. tuberculosis* as a pathogen. In the phagosome of infected host macrophages *M. tuberculosis* experiences endogenous stress as a result of incomplete reduction of molecular oxygen during aerobic respiration and exogenous stress as a result of the "oxidative burst" generated by NADPH oxidase in the phagosome. The reactive oxygen species that are created in these processes include peroxides, hydroxyl radicals and superoxide. In the macrophage, mycobacteria also face nitrosative stress from reactive nitrogen species that include nitric oxide, nitrite, nitrogen dioxide and nitrate. Pathogenic mycobacteria normally produce enzymes, such as peroxyredoxins, catalase, peroxidase, superoxide dismutase, and nitrosothiol reductase, to counteract the effect of reactive oxygen species and reactive nitrosative species and assist in intracellular survival and persistence in the host [2].

Thiols play an important role in the cell by maintaining a reducing environment for chemical reactions to occur and are also important in stabilizing three dimensional structure and catalytic activity of proteins. Thiol-disulfide interchange may also act as an in vivo molecular switch in signal transduction and is well documented in chloroplasts [3]. In addition, thiols may directly detoxify oxidants or act as coenzymes in reactions that detoxify oxidants. The cell maintains the redox-homeostasis of the cytosol by reversibly reducing oxidized thiols that are created in these reactions [4]. Diamide is a thiol specific probe, which has been used

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to elucidate changes in oxidation state of thiols [4]. It penetrates the cells readily and causes oxidative stress by specifically oxidizing intracellular low molecular weight thiols to their disulfide form. Thus, disulfide stress, can be considered to be a subcategory of oxidative stress that leads to the accumulation of non-native disulfide bonds in the cytoplasm.

Diamide has been used to define the thiol-disulfide stress response of multiple bacteria including *M. tuberculosis*. Disulfide stress response of *Streptomyces coelicolor* A3 was shown to involve the σ^R regulon [5]. In *M. tuberculosis*, microarray analysis and reverse transcription revealed the role of sigma factor σ^H [6]. Global analysis of *Bacillus subtilis* mRNA expression pattern and 2D differential proteomics upon exposure to diamide revealed that 350 genes were induced [7].

In this paper, we identify the mycobacterial cellular processes that are affected by thiol specific oxidative stress. By using proteomics, we describe the differential protein expression profile of *Mycobacterium bovis* BCG associated with the thiols-damaging agent diamide. We complement these studies by transcription analysis of *M. bovis* BCG genes encoding proteins identified in the proteomics section. Using these methods we have observed at least 60 proteins that are differentially expressed in response to diamide treatment and some of these proteins have been shown before to be functionally related to oxidative stress response.

2. Materials and methods

2.1. Bacterial cultures

M. bovis BCG (ATCC-35734) was grown in 200 ml Middlebrook 7H9 broth (Difco, Sparks, MO, USA) with 0.05% (v/v) Tween 80 (Sigma, St. Louis, MO, USA) by shaking at 37 °C. Middlebrook medium was supplemented with either OADC (oleic acid, albumin, catalase, dextrose) or 1% glucose.

2.2. Liquid culture for diamide induction

M. bovis BCG cultures were grown in Middlebrook 7H9 broth supplemented with OADC at 37 °C to an OD_{600} of 0.4–0.6. Bacteria were harvested by centrifugation, washed and resuspended in Middlebrook 7H9 broth containing 1% glucose (OADC was removed to eliminate the protective effect of the catalase present in this supplement). After incubating the cultures for 24 h at 37 °C, one aliquot of the cultures was treated with 5 mM diamide (Sigma, St. Louis, MO, USA) for 2 and 4 h at 37 °C. The untreated aliquot served as a control. Bacteria were pelleted by centrifugation and the cell pellets were washed three times with phosphate buffered saline (PBS, pH 7.4) containing 1% Tween 80 and then stored at −70 °C until further processing. For RNA extraction, 0.5 ml 6 M guanidinium chloride solution was added to the pellet immediately after centrifugation and the samples were stored at −70 °C.

2.3. Sample preparation for two-dimensional gel electrophoresis

Frozen cell pellets were resuspended in lysis buffer [8] containing complete protease inhibitor cocktail (Roche Applied Science). The lysis mixture was subjected to sonication four times for 30 s intermittently while keeping the tubes in ice bath. The cell lysates were centrifuged and the supernatant was removed and stored in small aliquots at −70 °C until they were required. The protein content of each sample was estimated using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA).

2.4. Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out using the sample-loading buffer (8 M urea, 4% (w/v) CHAPS, 40 mM Tris base, 65 mM dithiothreitol (DTT)) in strip holders of the IPGphor system (Amersham Pharmacia Biotech, Uppsala, Sweden). IEF was performed with Immobline Drystrips (Amersham Pharmacia Biotech Uppsala, Sweden) of either pH 4–7 or pH 3–10. Depending upon the length of strips (13, 18 or 24 cm), 50–150 μg of total protein was applied to the strips and the strips were rehydrated for 12 h. The 13, 18 and 24 cm strips were focused for 32,000, 52,000 and 62,000 Volt hours (Vh), respectively.

Second dimensional gel electrophoresis was carried out with a 12.5% SDS–polyacrylamide or Duracryl (Genomic Solutions Inc., Ann Arbor, MI, USA) gel. IPG strips were equilibrated prior to performing the second dimension gel electrophoresis. These strips were then overlaid onto vertical second dimension gels and sealed with 0.7% (w/v) agarose in 2× SDS running buffer containing trace amounts of bromophenol blue. The gels were silver stained for protein spot comparisons according to previously published protocols [9]. At least three different gels were run for each sample. For subsequent mass spectrometric analysis gels were rerun and Coomassie or silver staining was performed. The gel pictures were scanned and then analyzed using the Image Master software analysis program (Amersham Biosciences).

2.5. Matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS)

Protein spots of interest were excised from the gels, and digested in situ with trypsin (Sigma, St. Louis, MO, USA). After an overnight trypsin digestion at