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Global proteome and phosphoproteome dynamics indicate novel mechanisms of vitamin C induced dormancy in *Mycobacterium smegmatis*

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ABSTRACT

Vitamin C has been found to affect mycobacteria in multiple ways, including increasing susceptibility to antimicrobial drugs, inducing dormancy, and having a bactericidal effect. However, the regulatory events mediating vitamin C related adaptations remain largely elusive. Ser/Thr/Tyr protein phosphorylation plays an important regulatory role in mycobacteria, contributing to environmental adaptation, including dormancy and drug resistance. This study utilised the model organism, *Mycobacterium smegmatis*, and TiO_2 phosphopeptide enrichment combined with mass spectrometry-based proteomics methods to elucidate the mycobacterial signalling and regulatory response to sub-lethal concentrations of vitamin C. After initial validation of peptide spectra, 224 non-redundant phosphosites in 154 proteins were retained with high confidence. Data analysis revealed that 30 peptides were differentially phosphorylated with Vitamin C treatment, including novel phosphosites found on both PknG and GarA. Of these significant proteins, we validated 11 by parallel reaction monitoring of high-confidence phosphopeptides. Interestingly, 17/30 phosphopeptides were annotated as part of transmembrane proteins, suggesting that it is likely vitamin C triggers typical signal transduction events in which the protein periplasmic domain perceives environmental signals and the cytoplasmic domain is then phosphorylated. Finally, the diverse nature of phosphorylated proteins involved in signalling, transport, and carbohydrate biosynthesis indicates the extent of such regulatory phosphorylation events.

Biological significance: Our findings provide new mechanistic insight into a coordinated network of signalling and regulatory responses to sub-lethal vitamin C in *Mycobacterium smegmatis* and provide evidence that vitamin C is able to act as a novel extracellular signalling molecule. Vitamin C treatment caused changes in both the proteome and phosphoproteome associated with response to oxidative stress, a shift in metabolic regulation and progression toward dormancy, as well as phospho-dependent activation of specific secretory pathways and activation of specific two component and Ser/Thr/Tyr protein kinase activities. This study confirms the potential of vitamin C as convenient means to study aspects of mycobacterial dormancy, including those regulated at post-translational level.

1. Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) disease, a major health concern worldwide. The estimated incidence of active TB in South Africa is > 1% of the population (World Health Organisation; WHO) [1], with incidence up to 8% in some communities. This is compounded by a co-incident HIV epidemic, with ~70% of all new TB cases in South Africa being HIV positive. Furthermore, the WHO estimates that ~1/3rd of the world's population is latently infected with *M. tuberculosis*, which represents a massive reservoir of potential new TB cases. During latent TB infection (LTBI), the bacilli are thought to persist in viable but more dormant-like states that can be reactivated by a variety of factors, especially if the host becomes immune compromised [2]. To date though, no *M. tuberculosis* bacilli have been observed in LTBI individuals, so the exact physiological state of *M. tuberculosis* during latent infection remains elusive. It is

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therefore essential that different experimental models are explored to derive information related to mycobacterial environmental adaptation, including dormancy.

Vitamin C is an essential dietary nutrient for humans, as it is not endogenously synthesised. It has both anti and pro-oxidant effects [3–5], but is most well-known for its anti-oxidant effects in the human body, which counter DNA damaging free radicals. Vitamin C also modulates immune function by activating macrophages and monocytes [6], and can be concentrated to millimolar levels within the macrophage [7]. Before the antibiotic era, TB susceptibility was linked to vitamin C deficiency [8,9]. In recent years, this avenue of research has been revived, as vitamin C has a direct pro-oxidant effect on *M. tuberculosis* and has been proposed to act as an intracellular signalling molecule within the macrophage [6,10–13]. Vitamin C scavenges oxygen and creates a hypoxic, oxygen radical-rich environment, thus mimicking the environment within the phagolysosome of an activated macrophage and supporting entry of mycobacteria into a more dormant state [6,11–15]. Dormancy was thought to confer phenotypic tolerance to isoniazid (INH) [12], but high concentrations of vitamin C have a sterilizing effect on mycobacteria, which may be mediated by production of reactive oxygen species (ROS) via the Fenton reaction, which actually improves susceptibility to INH [10].

Most *in vitro* mycobacterial dormancy models are based on the gradual depletion of oxygen after a long period of incubation [14]. However, the disadvantage of such models is the fact that bacterial cells experience a variety of different and even severe stresses before eventually entering dormancy, which means that the information of early molecular events leading specifically to dormancy would most probably be lost.

Post-translation modifications (PTMs) add a layer of complexity to the mechanisms of bacterial adaptation. In mycobacteria, for example, Ser/Thr/Tyr protein phosphorylation has been linked to pathogenicity, development, and the stress response, with clear differential phosphorylation patterns between fast- and slow-growing, or virulent and avirulent mycobacteria [16–18]. Modern mass spectrometry (MS) has proved to be a useful tool to assess mycobacterial PTMs, such as protein phosphorylation (reviewed in Calder et al., 2016) [19]. More recently, label free quantitative phosphoproteomics was employed to characterise the changes in *M. tuberculosis* in response to high concentrations of rifampicin [20].

Here, we have used the fast growing, non-pathogenic *Mycobacterium smegmatis* as an *in vitro* model, coupled with a label free phosphoproteomic MS-based approach, in order to characterise the phosphoproteome dynamics of mycobacteria in response to environmental challenge by sub-lethal concentrations of vitamin C. In this work, we build on previous transcriptomic [11,12] and proteomic [13] studies on vitamin C exposed mycobacteria, and use phosphoproteomics to gain a better understanding of early signalling and regulatory molecular events that mediate entry of mycobacteria into dormancy following exposure to vitamin C.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Wild type strain of *M. smegmatis* mc^2155 was grown from glycerol stocks on 7H10 Difco™ Middlebrook (Becton Dickinson; BD) agar plates, and incubated for 3 days at 37 °C. 7H9 Middlebrook media (BD) broth containing 0.2% glycerol as a carbon source and 0.05% Tween 80 was used to prevent bacterial aggregation [21]. Albumin, dextrose, and sodium chloride (ADS) supplement was made according to Vilcheze et al. [10] ADS supplement was used instead of OADC, since OADC contains catalase, which would have a protective effect against peroxides. In order to establish the effect of vitamin C on growth of *M. smegmatis*, cultures were grown to mid-log phase (OD_{600} ~0.9). Vitamin C was dissolved in ultrapure water, filtered (0.22 μm), and added to the cultures at final concentrations of 4, 8, 15, 25, 50, 100 and 200 mM. Each treatment and control condition was performed in triplicate.

2.2. Protein extraction

Cells were harvested during the exponential phase (OD_{600} ~1.2) by centrifugation at 4000 × *g* for 15 min at 4 °C, washed twice with phosphate buffered saline, pH 7.5 (PBS). Cells were snap frozen in liquid nitrogen and were later thawed and suspended in 800 μl of lysis buffer (500 mM Tris-HCl, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.15% sodium deoxycolate, 1 × protease inhibitor cocktail, 1 × phosphatase inhibitor cocktail (Roche, Mannheim Germany) and 50 mg/ml lysozyme (pH 7.0). Cells were disrupted by sonication at maximum power for six cycles of 30 s each, with one minute cooling on ice between cycles. Cellular debris was removed by centrifugation at 4000 × *g* for five minutes, and the lysate filtered through 20 μm pore size low-protein binding filters (Merck, New Jersey, USA). Proteins were precipitated using the chloroform-methanol precipitation method as previously described [22]. The pellet was re-suspended in urea buffer (6 M urea, 2 M thiourea and 10 mM Tris-HCl (pH 8)). Protein concentration was determined using a modified Bradford assay, as described by Ramagli (1999) [23].

2.3. In-solution trypsin digestion

Protein extracts were reduced with 1 mM dithiothreitol (DTT) and iodoacetic acid (IAA) was then added to a concentration of 5.5 mM, with incubation at room temperature for one hour in the dark. Proteins were pre-digested with Lysyl Endopeptidase C (LysC; Waco, Neuss, Germany) at room temperature for three hours. Samples were then diluted to a 1:5 ratio using 20 mM ammonium bicarbonate (ABC) for proteomic analysis, or water for phosphoproteomic analysis. The solution was adjusted to pH 8 before the addition of 1 μg of trypsin (New England Biolabs) per 100 μg of protein, with 20 mM $CaCl_2$. Proteolysis was carried out at room temperature for 16 h with agitation at 30 rpm. Proteolysis was quenched by addition of trifluoroacetic acid (TFA) (Sigma Aldrich, St Louis, USA) to pH 2–3.

2.4. Sample preparation (phosphopeptide enrichment with TiO_2 chromatography)

For each sample, 50 μg of protein was used for quantitative proteomic measurements (see below) and the peptides were desalted using in-house prepared C18 STAGE tips [24]. For the phosphoproteomic analysis, 5 mg of total tryptic peptides were subjected to three stages of phosphopeptide enrichment by titanium dioxide (TiO_2) chromatography. In brief, 30% acetonitrile (ACN) (Sigma Aldrich, St Louis, USA) was added to the tryptic peptides and the pH was adjusted to 2. Titasphere TiO_2 beads (10 mg; MZ Analysentechnik, Mainz, Germany) in loading buffer (30 mg/ml 2,5-dihydrobenzoic acid (Sigma Aldrich, St Louis, USA), 80% ACN), were added to the samples at a concentration of 100 mg/ml, and incubated at room temperature with rotation for one hour. The beads were pelleted and the decanted supernatant was further incubated with a fresh batch of 5 mg of beads for 30 min. This step was repeated one additional time, giving three fractions of enriched phosphoproteins bound to beads in total. Phosphopeptides bound to the beads were washed vigorously with shaking for ten minutes in 1 mL of wash buffer 1 (30% acetonitrile, 3% trifluoroacetic acid) followed by an additional ten minutes of vigorous washing with wash buffer 2 (80% acetonitrile, 0.1% trifluoroacetic acid). Phosphopeptides were loaded onto C8 STAGE tips, washed with wash buffer 2 and then eluted with 3 × 50 μl Elution buffer (40% Mass-spec grade NH_4OH [aq, 25% NH_3; Sigma Aldrich, St Louis, USA], 60% acetonitrile (pH 10.5 or higher)). Solvent was removed in a speed drying vacuum at room temperature, and peptides were resuspended in 20 μl Solvent A (2% ACN, 0.1%