Glucose 6-Phosphate Accumulation in Mycobacteria

IMPLICATIONS FOR A NOVEL $F_{420}^-$-DEPENDENT ANTI-OXIDANT DEFENSE SYSTEM

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Glucose 6-phosphate (G6P) is a metabolic intermediate with many possible cellular fates. In mycobacteria, G6P is a substrate for an enzyme, $F_{420}^-$-dependent glucose-6-phosphate dehydrogenase (Fgd), found in few bacterial genera. Intracellular G6P levels in six Mycobacterium species were remarkably higher (~17-130-fold) than Escherichia coli and Bacillus megaterium. The high G6P level in Mycobacterium smegmatis may result from 10-25-fold higher activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, when acetate was the carbon source, suggesting a cellular program for maintaining high G6P levels. G6P was depleted in cells under oxidative stress induced by redox cycling agents plumbagin and menadione, whereas an $fgd$ mutant of $M. smegmatis$ used G6P less well under such conditions. The $fgd$ mutant was more sensitive to these agents and, in contrast to wild type, was defective in its ability to reduce extracellular plumbagin and menadione. These data suggest that intracellular G6P in mycobacteria serves as a source of reducing power and, with the mycobacteria-specific $Fgd-F_{420}$ system, plays a protective role against oxidative stress.

$M. tuberculosis$ latently infects one-third of the human population and accounts for nearly 2 million yearly deaths from active TB (1). $M. tuberculosis$ is noteworthy because of its ability to grow and persist in the inhospitable, intracellular environment of alveolar macrophage vacuoles that provide the first line of host defense against common respiratory pathogens (2). One broad but incompletely understood mechanism thought to be important for intracellular control of $M. tuberculosis$ is the complex mixture of reactive oxygen and nitrogen species such as superoxide and peroxynitrite produced by activated macrophages (3). It is also thought that mechanisms used by $M. tuberculosis$ to protect against these stress agents are similarly complex in that they are redundant and overlapping. The ability of the bacterium to adapt metabolically to this hostile and nutrient-poor intracellular vacuole environment (4) is thought to play a major role in its pathogenicity. Anti-TB drugs and the BCG vaccine provide some protection against $M. tuberculosis$, but global control of TB is far from being achieved partly because of multidrug-resistant $M. tuberculosis$ strains and the synergistic effects of TB and AIDS (5, 6). Furthermore, the molecular mechanisms of persistence are not well understood, impeding new drug development. Consequently, few anti-TB drugs have been developed in the last several decades. However, PA-824, a new nitroimidazopyran drug with strong bactericidal activity against both replication and static $M. tuberculosis$, is now in a phase II clinical trial with the Global Alliance for TB Drug Development. To become bactericidal, PA-824 requires activation involving a mycobacterial $F_{420}^-$-dependent glucose-6-phosphate dehydrogenase (Fgd) (7, 8).

Fgd, found only in $Mycobacterium$ and $Nocardia$ species, is an unusual glucose-6-phosphate dehydrogenase that transfers electrons from glucose 6-phosphate (G6P) to $F_{420}$ instead of NADP (9, 10). $F_{420}$ is produced by a limited number of genera such as $Streptomyces$, $Nocardia$, and $Mycobacterium$ species (10, 15–17). Fgd is the only known enzyme in $Mycobacterium$ and $Nocardia$ species using $F_{420}$ as an electron acceptor. Although the intracellular fate of reduced $F_{420}$ is unknown, it has been suggested that it could be required for anaerobic survival (5). $F_{420}$ is important for decolorization of malachite green, and mutants not making $F_{420}$ are more sensitive to superoxide-mediated oxidative stress (18). Although Fgd shows no significant homology with NADP-dependent glucose-6-phosphate dehydrogenase (G6PDH) (19), both catalyze similar electron transfer reactions and require glucose 6-phosphate (G6P) as the source of electrons to make reduced coenzymes $F_{420}^-$H$_2$ and NADPH, respectively. Glucose 6-phosphate, derived from glucose through the glycolytic enzymes hexokinase or glucokinase and through gluconeogenesis, generates reducing power via the pentose phosphate pathway. In mycobacteria, G6P is also an important precursor of cell wall components $l$-rhamnose and the galactan of arabinogalac-
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tan (20). Thus, because G6P is a central intermediate with many possible fates, we sought to understand the role of Fgd in G6P metabolism.

Our interest in Fgd and its role in PA-824 activation led us to examine G6P levels in mycobacteria. Previous studies have measured G6P in other species, including Escherichia coli, Streptomyces coelicolor, and Corynebacterium glutamicum (21–23). However, none examined mycobacteria. We found a remarkably high G6P level in all Mycobacterium examined, compared with E. coli and Bacillus megaterium. An investigation of related metabolic pathways revealed that mycobacteria preferentially accumulate G6P over other gluconeogenic intermediates through a significantly higher activity (compared with other bacteria examined) of the key gluconeogenic enzyme fructose-1,6-bisphosphatase (Fbp) and when cells are grown on acetate through increased GTP-dependent phosphoenolpyruvate carboxykinase (Pck) activity. We also found that G6P levels were depleted in cells under oxidative stress induced by plumbagin and menadione, which increase superoxide concentration in the cells (24). On the other hand, interruption of the fgd locus (19) to obtain pEP8300, in which the aph element was flanked with 2,741 bp DNA upstream and 4,052 bp DNA downstream. The 8,300-bp BamHI fragment from pEP8300 carrying the fgd::aph segment was cloned into the BamHI site of pJQ200KS to generate the suicide plasmid pEP8300A. M. smegmatis mc155 was electroporated with pEP8300A, grown 24 h under nonselective conditions, and then plated on LB containing kanamycin (20 μg/ml) and 10% sucrose. This sacB-mediated sucore counterselection and kanamycin-based selection step allowed the isolation of cells that acquired the aph cassette into the fgd gene on their chromosomes via a double cross-over recombination. The mutants were characterized by Southern hybridization and Fgd enzyme assay.

For complementation, Phuson high fidelity DNA polymerase (New England Biolabs) was used to PCR clone fgd from M. smegmatis mc155 chromosomal DNA into the expression vector pSMT3 (25). Primers fgd-for-EcoRV (5'-GTAGATAT-CAATGGTGCGTAAATGAGCGTCGTTACAGGC) and fgd-rev.AcE 5'-GCTAACGTTCAGTGCCAGAATCATACGCCCAG contained restriction sites for in-frame cloning. PCR product and vector were digested with EcoRV and AclI; the resulting fragments were cleaned with Qiagen gel extraction kit, ligated, and transformed into E. coli DH5α following standard cloning techniques (26). Hygromycin-resistant colonies (200 μg/ml) were isolated. The vector and two independent clones were electroporated into the fgd::kan mutant; hygromycin-resistant colonies (50 μg/ml) were purified and examined for Fgd activity and napthoquinone sensitivity.

Preparation of Cell Extracts for Enzyme and Metabolite Assays—The cells were harvested by centrifugation at 13,000 rpm for 20 min at 4 °C, washed twice with 50 mM Tris-HCl (pH 7.4), and resuspended (0.5 g of wet cells/ml of buffer). E. coli and B. megaterium were lysed by sonication for 3–5 min with successive sonication (1 min) and cooling (1 min) cycles, and
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mycobacteria were lysed by bead beating for 3 min with successive beating (30 s; 46,000 rpm) and cooling (30 s) cycles using a Mini-Beadbeater (Biospec Products) and 0.1 mm zirconia/silica beads (Biospec Products) with a cell suspension to bead ratio of 50:50 (V/V). The cell lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatants were used for enzyme assay. Protein concentration was determined by a bicinchoninic acid protein assay (Sigma). Enzyme extract aliquots were boiled for 10 min and centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatants were used for metabolite assays.

Enzyme Assays—Enzyme activities in 10 μl of cell extracts were measured spectrophotometrically at room temperature. G6PDH activity was determined by measuring the rate of increase in A340 caused by reduction of NADP. The final assay (1 ml) contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 0.7 mM NADP, and 2 mM glucose 6-phosphate. Fbp activity was determined by measuring the rate of decrease in A340 caused by reduction of coenzyme F420. Coenzyme F420 was purified as described earlier (27). The Fgd assay (1 ml) contained 50 mM Tris-HCl (pH 7.4), 0.025 mM F420, and 5 mM glucose 6-phosphate. Fbp activity was determined using an EnzCheck phosphate assay kit (Invitrogen) to follow the kinetics of Pi release monitored by the increase in A360. ATP-dependent phosphoenolpyruvate carboxykinase (ATP-Pck) activity was determined by measuring the decrease of A340 caused by reduction of coenzyme F420. Coenzyme F420 was purified as described earlier (27). The Fgd assay (1 ml) contained 50 mM Tris-HCl (pH 7.4), 0.025 mM F420, and 5 mM glucose 6-phosphate. Fbp activity was determined using an EnzCheck phosphate assay kit (Invitrogen) to follow the kinetics of Pi release from fructose-1,6-bisphosphate. Briefly, 10 μl of cell extract was mixed with the reaction buffer, the substrate 2-amino-6-mercapto-7-methylpurine riboside, and purine nucleoside phosphorylase provided with the kit to final concentrations of 1 × 0.2 mM, and 1 unit/ml, respectively. The mixture was incubated for 10 min before the reaction was started by the addition of fructose-1,6-bisphosphate to 0.1 mM. The reaction was monitored by the increase in A360. ATP-dependent phosphoenolpyruvate carboxykinase (ATP-Pck) activity was determined by measuring the decrease in A340 caused by NADPH oxidation. The final reaction (1 ml) contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 5 mM MnCl2, 75 mM NaHCO3, 1 mM dithiothreitol, 1 mM ADP, 0.3 mM NADH, and 5 units/ml malate dehydrogenase. The reaction mixture was preincubated for 15 min at 37 °C, and the reaction was started by the addition of phosphoenolpyruvate to final concentration of 5 mM. G6PDH activity was determined in the same way except that GDP was used instead of ADP. In all experiments, the reactions were initiated by substrate addition. An assay mixture with all components except substrate was used as a control for each assay. The reaction rate (ΔAbsorbance/min) calculated from the slope of linear plots (R2 = 0.95–1.0) from 0 to 3 min or 0 to 5 min was normalized to the control, and specific activities (μmol min−1 mg−1 protein) of enzymes were calculated based on extinction coefficients of NADPH and NADH (6.22 mM−1 cm−1), F420 (25 mM−1 cm−1), and 2-amino-6-mercapto-7-methylpurine riboside (11 mM−1 cm−1) (28) and the protein concentration. The experiments were independently repeated three times.

Intracellular Metabolite Assays—Intracellular metabolite concentration was determined enzymatically as described by Bergmeyer (29, 30), with modifications. Pure metabolites (>99%; Sigma) were used as standards and to confirm that boiling did not affect stability in heat treated extracts. G6P was determined by measuring the increase of A340 of a solution (1 ml) containing 0.05–0.4 μl of heat treated extracts, 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 0.7 mM NADP, and 0.5 unit/ml G6PDH. After the G6P reaction was complete, phosphoglucoisomerase or phosphoglomutase was added to 0.5 or 1 unit/ml, and the change of A340 was recorded to determine the concentration of fructose-6-phosphate and glucose-1-phosphate, respectively. 6-Phosphogluconate was determined by applying the same procedure as that for G6P except that 6-phosphogluconate dehydrogenase (0.025 unit/ml) was used instead of G6PDH. Pyruvate concentration was determined by measuring the decrease of A340 of a solution (1 ml) containing 0.2–0.5 μl of heat treated cell extracts, 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM MgSO4, 1 mM ADP, 0.3 mM NADH, and 1 unit/ml lactate dehydrogenase. After the pyruvate reaction was complete, pyruvate kinase was added to 3.8 units/ml, and the change of A340 was recorded to determine the phosphoenolpyruvate (PEP) concentration. In all of the experiments, the reactions were initiated by the addition of enzymes. Metabolite concentration, expressed as μmol g−1 protein, were calculated from ΔA340/Extinction coefficients of NADPH and NADH, and protein concentration. The experiments were repeated independently three times.

Oxidative Stress Sensitivity Assay—Sensitivity of M. smegmatis strains to plumbagin and menadione were determined by disk diffusion assay. Cell suspensions (A660 of 0.5) were prepared in phosphate-buffered saline from 48-h plate-grown cells. 100-μl suspensions were spread onto Middlebrook 7H9 plates with 0.2% glycerol to prepare a lawn of cells. Filter paper discs (7-mm diameter) containing plumbagin (0.025 μmol) or menadione (0.5 μmol) were placed on the lawns, and the cells were grown for 48 h. The diameters of clear zones were averaged from three independent experiments in triplicate.

Monitoring Reduction of Extracellular Plumbagin and Menadione—Cell-free medium obtained by centrifugation of 1 ml of bacterial suspension, collected at each time point (0, 3, 6, and 12 h after the addition of plumbagin or menadione to cells grown 48 h in Middlebrook 7H9 medium with 0.2% glycerol) from the bacterial culture were diluted in fresh culture medium and subjected to UV-visible spectral analysis. Absorbance at 419 nm (for oxidized plumbagin) and at 337 nm (for oxidized menadione) was plotted against time.

Statistics—A two-tailed t test at 95% confidence level was used to calculate p values.

RESULTS

Intracellular Glucose 6-Phosphate Concentration Is Significantly Higher in Mycobacteria than E. coli and B. megaterium—We compared the G6P level of six Mycobacterium species with E. coli and B. megaterium grown in Middlebrook 7H9 with glycerol as the carbon source and observed significantly higher G6P levels in all of the Mycobacterium species tested. G6P levels in different mycobacteria were 7–30 μmol g−1 protein, which were 17–130-fold higher than that of E. coli and B. megaterium (Table 1). With the exception of a related bacterium, C. glutamicum (where G6P was estimated at 29 μmol g−1 protein), the G6P concentration in mycobacteria is also much higher than those reported earlier for E. coli (1 μmol g−1 protein) and higher than in S. coelicolor (1–4 μmol
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g\textsuperscript{-1} protein) (21–23). Despite the fact that mycobacteria have three enzymes for entry of G6P into the pentose phosphate pathway (two G6PDH and Fgd) and use G6P for energy generation and cell wall oligosaccharide biosynthesis, G6P accumulates to surprisingly high levels. We thus sought metabolic explanations for this surplus.

Glucose 6-Phosphate Accumulation in M. smegmatis Is Preferred over Other Gluconeogenic Intermediates and Correlates with Significantly Higher Activity of the Gluconeogenic Enzyme Fructose-1,6-bisphosphatase—To obtain insight on intracellular G6P and related metabolic characteristics in mycobacteria and to investigate the mechanisms of their higher G6P accumulation, we first looked at the metabolic pathways that contribute to biosynthesis and consumption of G6P and selected related metabolic intermediates and enzymes for comparison between M. smegmatis, E. coli, and B. megaterium (Fig. 1). To follow formation and consumption of different metabolites over time, enzymes and metabolites were assayed at intervals to represent the exponential, early stationary, and stationary phases of growth.

G6P in M. smegmatis accumulated to higher levels at later phases of growth and was always present in relatively high amounts, whereas it was hardly detectable in E. coli and B. megaterium at any growth phase (Fig. 2A). 6-Phosphogluconate was hardly detectable for any species at any time. We observed consistent ratios of G6P to fructose-6-phosphate of 2.5 ± 0.46 and G6P to glucose-1-phosphate of 12.3 ± 3.3 at all phases of growth in M. smegmatis. Because of the low levels of these metabolites in E. coli and B. megaterium, reliable ratios could not be determined in these species. When intermediates at the starting point of the gluconeogenic pathway, in particular PEP, were compared for M. smegmatis, E. coli, and B. megaterium, the results were opposite to what was observed for G6P. The PEP level was remarkably lower in M. smegmatis than the other bacteria at the exponential phase (p < 0.01), although PEP levels declined in E. coli and B. megaterium at later growth phases. Pyruvate levels were similar in all three bacteria. To explain the differences in metabolite levels, we first determined enzyme activities for G6PDH and Fgd, which produce NADPH and reduced F420, respectively (Fig. 2B). G6PDH levels were generally similar in the three species at different growth phases, except that higher activity was observed in E. coli in stationary phase. The combined levels of G6PDH and Fgd in M. smegmatis represent a major route of G6P consumption, and because their sum is not significantly lower than E. coli G6PDH activity, they do not account for the high levels of M. smegmatis G6P. Because G6P accumulation was not a result of lower consumption capability, we reasoned that the biosynthetic rate of G6P could be different in M. smegmatis. Fbp and Pck are the two key enzymes of gluconeogenesis that regulate gluconeogenic carbon flow (31). Although Pck in bacteria is generally ATP-dependent, M. smegmatis has a GTP-dependent Pck but is missing the gene for an ATP-dependent Pck (32). Therefore, both ATP-dependent and GTP-dependent Pck were assayed in this study. As shown in Fig. 2B, Fbp activity was 10–25-fold higher in M. smegmatis, although Pck activity was significantly lower than that of E. coli (p < 0.05 at the exponential and stationary...

TABLE 1

Intracellular glucose 6-phosphate concentrations in different bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Glucose 6-phosphate (μmol g\textsuperscript{-1} protein)</th>
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</thead>
<tbody>
<tr>
<td>E. coli K-12</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>30.5 ± 0.9</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>17.5 ± 0.43</td>
</tr>
<tr>
<td>BCG</td>
<td>12.6 ± 0.17</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>23.0 ± 0.8</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>10.1 ± 0.6</td>
</tr>
<tr>
<td>M. phlei</td>
<td>7.1 ± 0.1</td>
</tr>
</tbody>
</table>

FIGURE 1. Pathways of carbon utilization in mycobacteria. G6P, glucose 6-phosphate; Pck, phosphoketolase; G6PDH, glucose-6-phosphatase dehydrogenase; Pyk, phosphoketolase; 6-PGDH, 6-phosphogluconate dehydrogenase; Tal, transaldolase; Pyruvate kinase; Pps, phosphoenolpyruvate synthase; Pcs, phosphoenolpyruvate carboxylase; Acy, acetyl-CoA synthetase; Acs, acetyl-CoA synthetase; 2-Ketoglutarate, 2-ketoglutarate; Sdh, succinate dehydrogenase; Fum, fumarase; Mdh, malate dehydrogenase; Ms, malate synthase; Icl, isocitrate lyase; Acy, acetyl-CoA synthetase. The pathways were drawn with the help of information in Ecocyc and KEGG databases. The enzymes and metabolites shown in bold font were analyzed in this study.
ary phases). Because the gene encoding ATP-dependent Pck is absent in *M. smegmatis*, the ATP-PCK activity observed in *M. smegmatis* is likely due to conversion of ATP to GTP, during the preincubation period, catalyzed by nucleoside diphosphate kinase (33). Because cells used for these analyses were grown in a medium containing glycerol as the carbon source, the pathways of glycerol utilization in mycobacteria (Fig. 1) suggest that G6P accumulation could occur through the higher activity of Fbp irrespective of Pck activity by using only a part of the gluconeogenic pathway. These results also explain why PEP was hardly detectable in *M. smegmatis*, whereas there was an accumulation in both *E. coli* and *B. megaterium* at least during the exponential phase.

*Mycobacteria Accumulate G6P from Various Carbon Substrates but Have Higher GTP-dependent Phosphoenolpyruvate Carboxykinase Activity for G6P Accumulation when Grown on Acetate*—Although *M. smegmatis* accumulated G6P from glycerol, likely bypassing the part of the gluconeogenic pathway that requires Pck activity, we asked whether this bacterium also makes high levels of G6P from acetate, which requires the complete gluconeogenic pathway and hence Pck activity. To address this question and to also examine whether mycobacteria accumulate G6P from different carbon substrates, we grew *M. smegmatis* with glycerol, glucose, or acetate. We found that G6P levels in *M. smegmatis* grown in glucose or acetate are less than that observed in glycerol grown cells but are still much higher than those observed in the other bacteria grown on glycerol (Fig. 3A). Less G6P in glucose- or acetate-grown cells compared with glycerol-grown cells could be related to their growth phase because the cells were grown on different substrates for the same time period, and cell growth was slower in glucose and acetate (data not shown). G6PDH, Fgd, and Fbp activities were comparable for mycobacteria grown with different substrates (Fig. 3B). However, GTP-Pck activity was significantly up-regulated (p < 0.0001) in cells grown in acetate, which could explain the mechanism of G6P accumulation in these cells, suggesting that mycobacteria are metabolically adapted to accumulate G6P.

**G6P Accumulation as Reducing Power for Mycobacteria**

To investigate whether G6P accumulation is affected by nutritional conditions, *M. smegmatis* was grown under carbon, nitrogen, phosphate, and sulfate starvation. Based on visual observations (data not shown), bacterial growth was reduced by carbon or nitrogen starvation, whereas little difference from control was observed under phosphate or sulfate starvation. Intracellular G6P concentration was significantly affected (>5-fold lower) by carbon starvation (p < 0.001), although activities of pentose phosphate pathway

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**FIGURE 2.** Higher activity of the gluconeogenic enzyme fructose-1,6-bisphosphatase correlates with an accumulation of glucose 6-phosphate in *M. smegmatis*. The bacteria were grown in Middlebrook 7H9 with 0.2% glycerol at 35 °C, and the cells were collected and harvested at different time intervals to represent cells from different growth phases. Harvesting time for *E. coli* (EC) and *B. megaterium* (BM) were: exponential, 12 h; early stationary, 15 h; stationary, 24 h. Harvesting for *M. smegmatis* (MS) was at 48, 55, and 72 h, respectively. A, concentration of glucose 6-phosphate and related intracellular metabolites at different growth phases in different bacteria. B, activities of key enzymes of pentose phosphate pathway and gluconeogenesis at different growth phases in different bacteria. Each datum represents an average of data obtained from three independent experiments. The error bars indicate S.D.
enzymes G6PDH and Fgd were very similar to those of control (Fig. 4). However, biosynthesis of G6P from glycerol could be affected by a down-regulation of Fbp activity in carbon-starved cells. Surprisingly, we observed a significantly higher accumulation of G6P in cells starved for nitrogen and sulfate (>5- and >3-fold higher, respectively; \( p < 0.01 \)). From the enzymes assayed in this study, it is not clear why intracellular G6P level increases under sulfate starvation. Under nitrogen starvation, a significantly lower (\( p < 0.05 \)) activity of G6PDH may be the cause for higher G6P accumulation. Consistent with this, a significant reduction of G6PDH activity under nitrogen starvation was also observed earlier in \textit{Saccharomyces cerevisiae} (34). On the other hand, a 50-fold reduction in the phosphate supply in the medium had no significant effect on the G6P level or the enzymes catalyzing the consumption and biosynthesis of the metabolite in \textit{M. smegmatis}.

\textbf{FIGURE 3.} \textit{M. smegmatis}, when grown under gluconeogenic conditions, still accumulates G6P, likely via up-regulation of phosphoenolpyruvate carboxykinase activity. The bacteria were grown in Middlebrook 7H9 with 0.2% glycerol, glucose, or acetate at 35 °C and harvested at 72 h. \( A \), concentration of glucose 6-phosphate and related intracellular metabolites in \textit{M. smegmatis} grown on different carbon substrates. \( B \), activities of key enzymes of pentose phosphate pathway and gluconeogenesis in \textit{M. smegmatis} grown in different carbon substrates. Each bar represents an average from three independent experiments. The error bars indicate S.D. ***, significantly different from that of cells grown in glycerol (\( p < 0.0001 \)).

\textbf{FIGURE 4.} Intracellular G6P concentration in \textit{M. smegmatis} decreases under carbon starvation but increases under nitrogen and phosphate starvation. The bacteria grown in Middlebrook 7H9 with \( 1/10 \)th carbon and nitrogen sources and \( 1/50 \)th phosphate and sulfate sources at 35 °C were harvested at 72 h. Each datum represents an average of data obtained from three independent experiments. The error bars indicate S.D. *, significantly different from that of control (\( p < 0.05 \)).

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become scarce or as a source of reducing power to generate NADPH and F$_{420}$H$_2$. We hypothesized that if G6P serves as an electron store, it will be consumed under oxidative stress to maintain redox balance. To investigate this possibility, we observed the effects of two oxidative stress-inducing agents, plumbagin and menadione (which generate superoxide radicals) (24), on G6P levels in M. smegmatis. In addition, a mutant M. smegmatis lacking Fgd activity was included to identify whether G6P is utilized in cells in an Fgd-F$_{420}$-dependent manner under oxidative stress. Although under normal growth conditions the G6P level in the fgd mutant (25.8 ± 3.3 μmol g$^{-1}$ protein) was not significantly different from that of wild type at 72 h of growth (32.5 ± 2.8 μmol g$^{-1}$ protein; Fig. 2A), the mutant was significantly more sensitive to both plumbagin and menadione (Fig. 5). Because the fgd mutant is unable to catalyze the transfer of G6P electrons to reduce coenzyme F$_{420}$, the higher sensitivity of the mutant toward oxidative stress-inducing agents suggested that the mutant could have a different G6P level under oxidative stress.

When we added plumbagin and menadione to exponentially growing M. smegmatis and the fgd mutant at 48 h of growth and monitored G6P, we observed a gradual decrease in intracellular G6P in the wild type with time (Fig. 6). On the other hand, G6P level increased from 24.7 to 30 μmol g$^{-1}$ protein in the control without plumbagin or menadione during this time period just as it was observed in M. smegmatis during transition from exponential (48 h) to early stationary phases (55 h) of growth (Fig. 2A). Consistent with data illustrating the higher sensitivity of the fgd mutant toward oxidative stress, we found that the mutant is defective in utilizing G6P even after 12 h of incubation with plumbagin or menadione. The G6P level in the plumbagin-treated fgd mutant increased over the 12-h time period (Fig. 6A), mimicking the data obtained for the untreated, control cells, where G6P concentration increased from 16 to 32 μmol g$^{-1}$ protein. Although some reduction of G6P level was observed in the fgd mutant treated with menadione, the G6P level at any time point assayed was significantly higher than the wild type, suggesting at least a partial role of the Fgd-F$_{420}$ system in defending cells from menadione-mediated oxidative stress.

To further investigate whether intracellular consumption of G6P correlates with the redox status of the oxidants, we monitored plumbagin and menadione reduction in extracellular medium. Spectral and redox characteristics of plumbagin and menadione, reported earlier (35, 36), were verified in this study by NaBH$_4$ reduction. Oxidized plumbagin and menadione give well resolved peaks at 419 and 337 nm, respectively, and the absorbance at these wavelengths decreases markedly upon reduction (data not shown). Plumbagin and menadione maintain the same spectral properties in Middlebrook 7H9 medium. We observed a time-dependent reduction of extracellular plumbagin and menadione by the wild type M. smegmatis, whereas the fgd mutant was slower in such processes (Fig. 6). To our knowledge, this is the first report of plumbagin and menadione reduction by mycobacteria. These data correlate very well with the differential utilization of G6P by the wild type and fgd mutant, suggesting that the intracellular pool of G6P is used to reduce extracellular oxidants such as plumbagin and menadione in an Fgd-F$_{420}$-dependent manner. To confirm the role of Fgd in these processes, the fgd mutant was complemented; G6P accumulation and plumbagin reduction by the complemented strain were similar to the wild type, although the ability to reduce menadione was only partially restored by complementation (Fig. 6). We suspected that F$_{420}$H$_2$ does not directly reduce menadione and plumbagin; therefore we tested whether reduced F$_{420}$ is able to reduce plumbagin and menadione by mixing the oxidants with a reaction mixture containing F$_{420}$H$_2$ (produced by using G6P and M. smegmatis cell extract as a source of Fgd) and monitoring the reaction spectrophotometrically (data not shown). However, no reduction of plumbagin and menadione was observed even after 1 h of incubation. This indicates that there is no direct chemical reduction of plumbagin or menadione by F$_{420}$H$_2$ and suggests that reduction proceeds via an enzymatic system not present in sufficient quantity.

**FIGURE 5.** fgd mutant of M. smegmatis is more sensitive than the wild type to oxidative stress induced by plumbagin and menadione. Filter paper discs (7-mm diameter) containing plumbagin (0.025 mol) and menadione (0.5 μmol) were placed on the bacterial lawns, and the cells were grown for 48 h at 35 °C. The diameter of clear zones was averaged from data obtained from three independent experiments in triplicate. ***, significantly different from that of wild type (p < 0.001).**
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DISCUSSION

G6P level in any cell is determined by a balance between catabolic and anabolic reactions in metabolic pathways such as glycolysis, gluconeogenesis, and the pentose phosphate pathway. Depending on the carbon source and cellular need, G6P in cells can be (i) derived directly from glucose, (ii) biosynthesized by gluconeogenesis, (iii) oxidized by glycolysis to generate ATP, and (iv) oxidized by the pentose phosphate pathway to generate NADPH for biosynthetic reactions in most bacteria. In mycobacteria, G6P can also generate $F_{420}H_2$ for unknown purposes. The unusually high intracellular concentration of G6P in Mycobacterium species compared with the other bacteria in this study prompted us to examine the metabolic reactions contributing to G6P accumulation and the importance of maintaining a high G6P level. The higher G6P level in all Mycobacterium species suggested that either the overall consumption of G6P is less in mycobacteria or the rate of biosynthesis is higher than the other bacteria examined.

Comparing data from exponentially growing cells, with glycerol as the carbon source, the intracellular concentration of different gluconeogenic intermediates in M. smegmatis contrasted greatly with E. coli and B. megaterium (Fig. 2). For example, concentrations of sugar phosphates G6P, fructose-6-phosphate, and glucose-1-phosphate were much higher in M. smegmatis, but PEP was barely detectable. Conversely, E. coli and B. megaterium had high PEP but extremely low or undetectable sugar phosphate levels. M. smegmatis had high FBP activity and low Pck activity, which suggests a mechanism for both the accumulation of G6P and dearth of PEP. These results suggest that mycobacteria are metabolically programmed to accumulate G6P. Conversely, E. coli and B. megaterium have low FBP activity and high Pck activity, which provides a mechanism for their low G6P levels and high PEP levels.

The level of gluconeogenic intermediates was similar when M. smegmatis was grown on other carbon substrates (Fig. 3). Notably, gluconeogenic biosynthesis of G6P from acetate, for which Pck activity could be critical, was met by an up-regulation of GTP-dependent Pck activity. Although the GTP-dependent Pck of M. smegmatis was cloned, purified, and characterized earlier, the intracellular activity of the enzyme was not previously reported (32). Here we provide the first reports of GTP-Pck activity in M. smegmatis grown on various carbon substrates and that GTP-Pck activity is up-regulated by growth on acetate. This is in agreement with an earlier report on the Pck activity of M. bovis BCG analyzed using pckA promoter-driven expression of green fluorescent protein (37).

The mechanism of G6P accumulation from acetate through up-regulation of GTP-Pck in combination with higher Fbp activity could be important for M. tuberculosis pathogenesis. First, M. tuberculosis contains a putative GTP-dependent Pck activity. Although the GTP-dependent Pck of M. smegmatis was cloned, purified, and characterized earlier, the intracellular activity of the enzyme was not previously reported (32). Here we provide the first reports of GTP-dependent Pck activity in M. smegmatis grown on various carbon substrates and that GTP-Pck activity is up-regulated by growth on acetate. This is in agreement with an earlier report on the Pck activity of M. bovis BCG analyzed using pckA promoter-driven expression of green fluorescent protein (37).

The mechanism of G6P accumulation from acetate through up-regulation of GTP-Pck in combination with higher Fbp activity could be important for M. tuberculosis pathogenesis. First, M. tuberculosis contains a putative GTP-dependent Pck encoded by pckA (32). Second, deletion of pckA in M. bovis BCG impaired virulence in macrophages and mice, and an increase in pckA mRNA was observed in mice infected with pathogenic M. tuberculosis (37, 38). Moreover, induction of pckA and genes involved in $\beta$-oxidation and the glyoxylate

in a soluble crude extract, as might be expected if the enzymes are involved in membrane-bound quinone reduction.
G6P Accumulation as Reducing Power for Mycobacteria

shunt in *M. tuberculosis* during phagosomal survival suggested the availability of fatty acids as carbon source and the importance of *pckA* in diverting carbon from β-oxidation into gluconeogenesis. Although there are alternative routes of PEP formation via pyruvate carboxylase and phosphoenolpyruvate synthase, the pyruvate carboxylase may not perform an anaplerotic conversion of oxaloacetate to PEP in *M. smegmatis* (39), and our intracellular metabolite data with much higher pyruvate compared with PEP in *M. smegmatis* suggest that phosphoenolpyruvate synthase may not play a significant role in PEP formation. Furthermore, disruption of *pck* in *M. smegmatis* inhibits growth on acetate (37), consistent with its anticipated importance in gluconeogenesis.

After determining a possible mechanism for accumulation of higher G6P levels, we then looked for nutritional conditions where they might be altered. By depriving cells of carbon, nitrogen, phosphorus, or sulfate, only carbon shortage lowered G6P accumulation, whereas a dramatic G6P accumulation was observed under nitrogen or sulfate starvation (Fig. 4). The lower G6P level in carbon-starved cells may be due to a down-regulation of Fbp. Based on a comparison of G6PDH and Fgd activity with the control, the potential for consumption of G6P through the pentose phosphate pathway appeared to be similar under control and carbon-starved conditions. Although the increase in intracellular G6P concentration under nitrogen or sulfate starvation was very interesting, its physiological advantage for mycobacteria is not clear. One possibility is that G6P can be accumulated in times of carbon excess and nitrogen or sulfur starvation with the idea that as soon as nitrogen and sulfur become available, the stored carbon can be used.

To further understand the rationale for G6P accrual in mycobacteria, we looked for conditions where cells consume the amassed G6P. In other species, G6P serves as an important source of reducing power to generate NADPH, the chosen cofactor for absorbing many oxidative stress agents. We reasoned that the Fgd-F420 system might fill that role in mycobacteria, particularly because *M. tuberculosis* has lost the oxyR stress response system, and although present in *Mycobacterium marinum*, oxyR is not critical for virulence (40, 41). Work by Guerra-Lopez et al. (18) showed that *F. tularensis* biosynthetic mutants were more sensitive to certain oxidative stress agents. Thus, we reasoned that the *M. smegmatis fgd* mutant, defective in F420 reduction, might show a similar phenotype and demonstrate that G6P serves as a source of reducing power to combat oxidative stress in mycobacteria. Under nonstress growth conditions, G6P levels of the *fgd* mutant were similar to wild type, increasing well into the stationary phase. However, when subjected to oxidative stress induced by the two naphthoquinone derivatives plumbagin and menadione (known to increase cellular superoxide concentrations), the G6P level in wild type *M. smegmatis* gradually decreased with concomitant reduction of extracellular plumbagin or menadione. On the other hand, the *fgd* mutant was defective in both consumption of intracellular G6P and reduction of extracellular oxidants. These data suggest that G6P in mycobacteria is accumulated and maintained as a reservoir of reducing power, which can be used to defend cells from oxidative stress in an Fgd-F420-dependent mechanism, and that F420H2 is the critical component for naphthoquinone reduction. We hypothesize that mycobacteria possess an F420-dependent quinone reductase, possibly a membrane-associated component of the electron transport chain that is responsible for enzymatic quinone reduction. Thus, the precise role of F420 in the protection of mycobacteria remains unknown, but it is clear that other F420-dependent proteins are involved.

Our data suggest that F420H2 takes part in yet unknown electron transfer reactions to defend cells from the toxic effects of superoxide radicals generated by plumbagin and menadione redox cycling (24). The observations from this study have implications for survival and persistence of pathogenic *Mycobacterium* species in macrophages. *M. tuberculosis* and *M. leprae* are both dysfunctional in many oxidative stress response mechanisms such as OxyR and SoxRS yet can survive and persist in macrophages rich in reactive oxygen species, probably because of a collection of redundant and overlapping protective mechanisms (3, 42, 43). Fgd and F420 are found in both of these pathogenic mycobacteria (10). Therefore, the G6P-Fgd-F420 system described in this report could play an important role in the defense of pathogenic mycobacteria against oxidative stress imposed by host macrophages.

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G6P Accumulation as Reducing Power for Mycobacteria

Glucose 6-Phosphate Accumulation in Mycobacteria: IMPLICATIONS FOR A NOVEL F420-DEPENDENT ANTI-OXIDANT DEFENSE SYSTEM
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