Metabolic and Bactericidal Effects of Targeted Suppression of NadD and NadE Enzymes in Mycobacteria

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ABSTRACT ***Mycobacterium tuberculosis*** **remains a major cause of death due to the lack of treatment accessibility, HIV coinfection, and drug resistance. Development of new drugs targeting previously unexplored pathways is essential to shorten treatment time and eliminate persistent *M. tuberculosis*. A promising biochemical pathway which may be targeted to kill both replicating and nonreplicating *M. tuberculosis* is the biosynthesis of NAD(H), an essential cofactor in multiple reactions crucial for respiration, redox balance, and biosynthesis of major building blocks. NaMN adenylyltransferase (NadD) and NAD synthetase (NadE), the key enzymes of NAD biosynthesis, were selected as promising candidate drug targets for *M. tuberculosis*. Here we report for the first time kinetic characterization of the recombinant purified NadD enzyme, setting the stage for its structural analysis and inhibitor development. A protein knockdown approach was applied to validate bothNadD and NadE as target enzymes. Induced degradation of either target enzyme showed a strong bactericidal effect which coincided with anticipated changes in relative levels of NaMN and NaAD intermediates (substrates of NadD and NadE, respectively) and ultimate depletion of the NAD(H) pool. A metabolic catastrophe predicted as a likely result of NAD(H) deprivation of cellular metabolism was confirmed by ^{13}C biosynthetic labeling followed by gas chromatography-mass spectrometry (GC-MS) analysis. A sharp suppression of metabolic flux was observed in multiple NAD(P)(H)-dependent pathways, including synthesis of many amino acids (serine, proline, aromatic amino acids) and fatty acids. Overall, these results provide strong validation of the essential NAD biosynthetic enzymes, NadD and NadE, as antimycobacterial drug targets.**

IMPORTANCE **To address the problems of *M. tuberculosis* drug resistance and persistence of tuberculosis, new classes of drug targets need to be explored. The biogenesis of NAD cofactors was selected for target validation because of their indispensable role in driving hundreds of biochemical transformations. We hypothesized that the disruption of NAD production in the cell via genetic suppression of the essential enzymes (NadD and NadE) involved in the last two steps of NAD biogenesis would lead to cell death, even under dormancy conditions. In this study, we confirmed the hypothesis using a protein knockdown approach in the model system of *Mycobacterium smegmatis*. We showed that induced proteolytic degradation of either target enzyme leads to depletion of the NAD cofactor pool, which suppresses metabolic flux through numerous NAD(P)-dependent pathways of central metabolism of carbon and energy production. Remarkably, bactericidal effect was observed even for nondividing bacteria cultivated under carbon starvation conditions.**

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With the continuing problem of tuberculosis (TB) compounded by the HIV epidemic and the emergence of increasing rates of drug resistance, there remains a need for new and more effective antibiotics aimed at previously unexplored targets. Among potential targets identified by a variety of genomic techniques, there are many indispensable metabolic enzymes (1, 2). While central metabolic processes were found to be recalcitrant to antibiotic development, certain enzymes responsible for the synthesis of the essential cofactor folate (FolA and FolP) were proven to be reliable targets for antibacterial drugs (trimethoprim and sulfonamides). Biosynthesis of the nicotinamide adenine dinucleotide (NAD) cofactor appears to be another prominent candidate target pathway in many bacterial pathogens (3–8), including *Mycobacterium tuberculosis* (9, 10). Indeed, NAD(H) and its phosphorylated derivative NADP(H) are irreplaceable cofactors in ~17% of 849 enzymatic reactions included in an *M. tuberculosis* metabolic model (11). Among these are the key pathways required to produce ATP, pathways already validated by the newly ap-

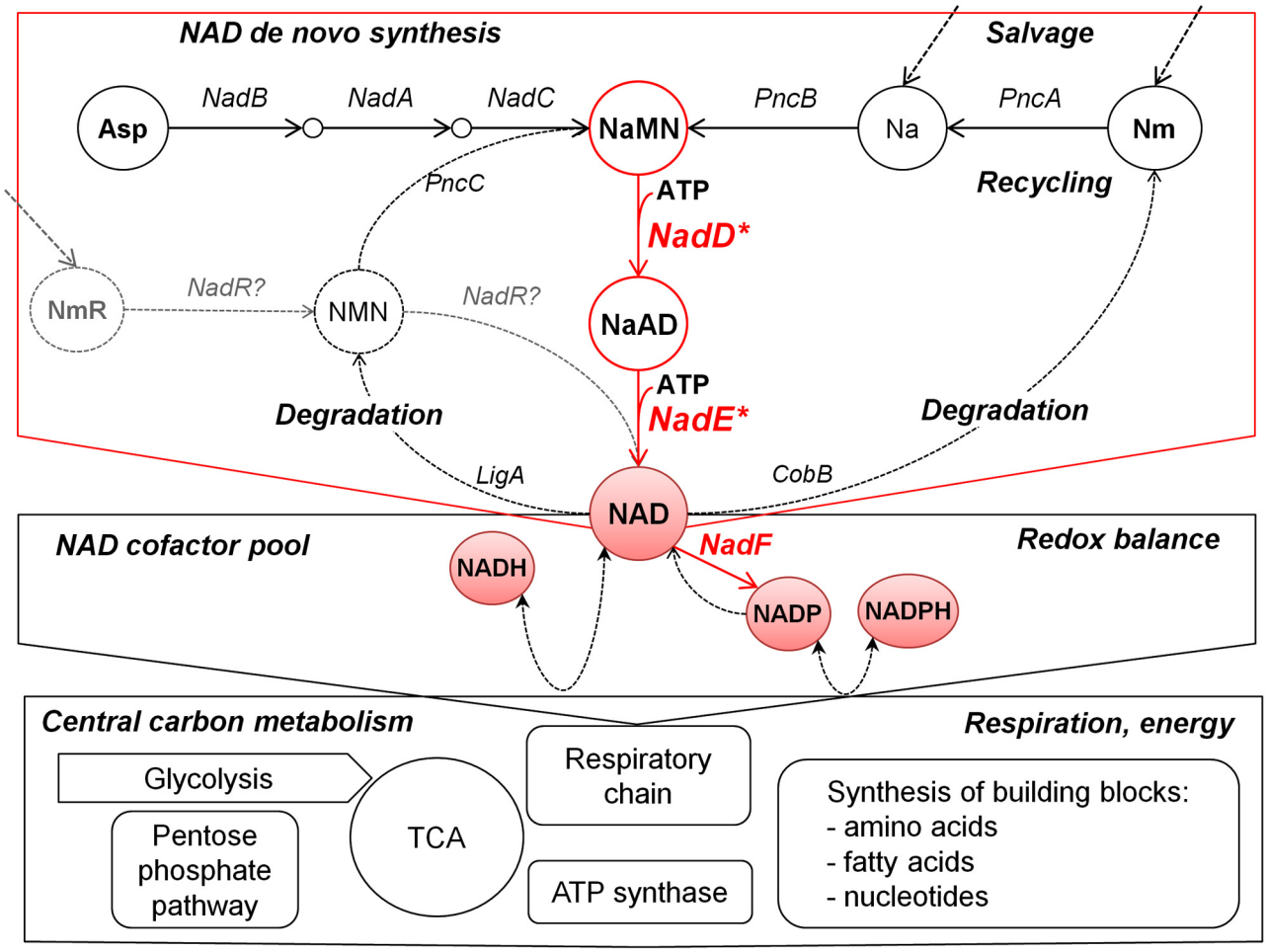

FIG 1 Targeting indispensable enzymes of NAD biosynthesis is expected to cause global metabolic catastrophe. In the simplified diagram of genomics-based reconstruction of NAD biogenesis and homeostasis (upper panel; for details, see Table S1 in the supplemental material), the key intermediary metabolites are shown by abbreviations in circles as follows: Asp, aspartate; Na, nicotinic acid; Nm, nicotinamide; NmR, nicotinamide riboside; NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; NaAD, nicotinic acid adenine dinucleotide. Enzymes are indicated as products of the respective genes above corresponding biochemical transformations (arrows). Essential enzymatic steps are outlined in red, and two enzymes selected as targets in this study, nicotinate mononucleotide adenylyltransferase (NadD) and NAD synthetase (NadE), are marked by an asterisk (*). Our study results suggest that the NadR-dependent pathway of NmR salvage is nonfunctional in *M. tuberculosis* (shown by grey and indicated by question marks), which has no typical PnuC-like NmR transporter. The NadR homolog, which is present in *M. tuberculosis* but not in *M. smegmatis*, appears to lack both of the enzymatic activities, corresponding to NmR kinase and NMN adenylyltransferase, reported for the bifunctional NadR enzyme from some gammaproteobacteria. As NAD constantly undergoes nonredox degradation (only some of NAD-degrading enzymes are shown), blocking its *de novo* synthesis, salvage, and recycling pathways via inactivation of a downstream enzyme(s), NadD or NadE, would impair the homeostasis of all four functional forms of NAD-related cofactors (middle panel). A resulting cofactor shortfall would suppress metabolic flux in hundreds of redox reactions comprising all essential pathways of central carbon and energy metabolism (lower panel).

proved drug bedaquiline (12, 13). Moreover, NAD homeostasis likely plays a key role in survival in limited oxygen environments in *M. tuberculosis*, which utilizes the anaerobic electron transport chain to maintain a necessary redox balance within a pool of NAD-related cofactors to persist in a nonreplicating state (14). In addition to its role as an essential redox cofactor in over a hundred central metabolic reactions, NAD is subject to rapid degradation in the cell (with a turnover half-life [$t_{1/2}$] of ~90 min in *Escherichia coli* [15]) by a variety of nonmetabolic enzymes utilizing NAD as a substrate in DNA repair, protein deacetylation, ADP ribosylation, etc. Therefore, biosynthetic NAD replenishment is required to maintain the pool of NAD cofactors, as they cannot be taken up by microbial cells (except for some obligate intracellular pathogens such as *Chlamydia* [16]).

Genomics-based reconstruction (7, 10) (Fig. 1) and experimental data (9) suggest that the two upstream routes of NAD biogenesis, *de novo* synthesis (enzymes NadA, NadB, and NadC) and vitamin B_3 salvage (PncA and PncB), are functionally redundant and are, therefore, poor candidates for the development of inhibitors. On the other hand, the last two consecutive steps of NAD biosynthesis from a nicotinate mononucleotide (NaMN) precursor via a nicotinate adenine dinucleotide (NaAD) intermediate (see Fig. 1) appear to be irreplaceable. The respective enzymes, NaMN adenylyltransferase (NadD) and NAD synthetase (NadE), are conserved in most bacterial species, being quite distinct from their human counterparts (7). These bacterial enzymes have been extensively characterized (17, 18) and targeted for drug development in both Gram-positive and Gram-negative bacteria (6, 19–22). Both *nadD* and *nadE* genes are conserved in all sequenced mycobacterial genomes (see Table S1 in the supplemen-

tal material) and were implicated as essential by genome-scale studies in *M. tuberculosis* (23–25).

Therefore, one of the main goals of this study was to assess the potential utility of both enzymes, NadD and NadE, as antimycobacterial drug targets. NadE from *M. tuberculosis* has been previously characterized in detail, including structural analysis (26–28); some NadE inhibitors inhibited *M. tuberculosis* growth *in vitro* (9). Here, for the first time, we report cloning, expression and enzymatic characterization of NadD from *M. tuberculosis*. We used a recently described targeted protein degradation approach (29) to test the essentiality of NadD and NadE and determine the biochemical and biological consequences of depletion of these target enzymes. We found that both are required for not only the growth but also the survival of the model organism *Mycobacterium smegmatis*. We also found that a homolog of NadR protein from *M. tuberculosis* is not able to complement NadD or NadE depletion in *M. smegmatis*, supporting the idea of the universal essentiality of these target enzymes. Using mass spectrometry and stable isotope tracers, we confirmed expected perturbations in NAD biosynthetic pathways resulting from inactivation of NadD and NadE enzymes and global changes in central carbon metabolism triggered by the induced depletion of the NAD(H) cofactor pool. The observed network-scale impact, which could not be assessed by traditional genetic techniques, provided an additional strong rationale for pursuing these enzymes as antimycobacterial drug targets.

RESULTS

NadD and NadE are indispensable enzymes of NAD biosynthesis conserved in all mycobacterial genomes. Comparative genomics-based reconstruction of NAD metabolism (captured by us in the relevant SEED subsystem [30]) showed that most features characteristic of *M. tuberculosis* (10) are conserved in all other sequenced genomes of mycobacterial species. Briefly, they include the following (Fig. 1): (i) *de novo* synthesis of NaMN from aspartate (*nadABC* genes); (ii) a redundant salvage/recycling (*pncAB*) route of NaMN synthesis from nicotinamide (Nm) or nicotinic acid (Na); and (iii) downstream conversion of NaMN to NAD via an NaAD intermediate by two consecutive reactions (genes *nadD* and *nadE*). Based on this network topology, the latter two enzymes appear to be indispensable in all mycobacteria, in agreement with some experimental findings in *M. tuberculosis* (9, 23). In contrast, a NadR homolog (NadRh) is present in only some species of mycobacteria, e.g., in *M. tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium microti* but not in *M. smegmatis* and many others (see Table S1 in the supplemental material). Although the prototype NadR protein is known to drive two-step NAD synthesis in *Haemophilus influenzae* following the uptake of exogenous nicotinamide riboside (NmR) via a committed transporter, PnuC (8, 31), the actual function of NadRh in *M. tuberculosis* (28% identity with *hi*_NadR) is unclear. No homologs of PnuC are present in NadRh-containing species of mycobacteria, suggesting that NadRh may be involved in NAD recycling rather than biosynthesis or may play another, yet unknown, functional role. Indeed, a recombinant purified NadRh from *M. tuberculosis* did not show any detectable NmR kinase or NMN adenylyltransferase activity, whereas both activities of recombinant NadR proteins from *Salmonella enterica* or *H. influenzae* could be readily measured by the same assays (see Fig. S1 in the supplemental material).

NadD from *M. tuberculosis* is an NaMN-preferring adenylyltransferase. While previous attempts to express NadD from *M. tuberculosis* failed to yield functionally active enzyme, here we resolved this problem by correcting the position of the translational start of the *nadD* gene (see Fig. S2 in the supplemental material). Indeed, expression in *E. coli* of the full-size protein with the N terminus extended by 8 amino acids yielded functionally active NaMN adenylyltransferase. In contrast, no activity was observed when a shorter form of *M. tuberculosis* NadD (within the original boundaries of GenBank GI:15609558) was expressed and purified using the same expression vector. The purified recombinant full-size enzyme displayed the same specific activity before and after proteolytic removal of the N-terminal SUMO-$(His)_6$ tag. The steady-state kinetics analysis confirmed a strong substrate preference for NaMN (with apparent values of $K_m = 0.34 \pm 0.03$ mM and $k_{cat} = 4.5 \pm 0.1\ s^{-1}$) over NMN ($K_m = 4.0 \pm 1.1$ mM and $k_{cat} = 0.043 \pm 0.005\ s^{-1}$) at saturating ATP, which translates to a nearly 1,000-fold preference at the level of k_{cat}/K_m (see Fig. S2 in the supplemental material). Such a stringent substrate preference is characteristic of the members of bacterial NadD family, distinguishing them from the human counterpart enzyme (NMNAT), which shows nearly equal levels of activity with both substrates (32).

NadD or NadE knockdown has strong bactericidal effects in an *M. smegmatis* model. A protein depletion method recently developed and validated in mycobacteria (29) allowed us to assess bactericidal effects and explore the metabolic consequences of depleting NadD and NadE enzymes in an *M. smegmatis* model system. The induction of targeted proteolysis in each of the two engineered strains (Fig. 2A) led to ≥90% degradation of NadD or NadE enzymes within the first 2 h by both Western blot and enzymatic activity analyses (Fig. 2B and C). These effects were target specific; depletion of one target enzyme had no effect on the activity of another enzyme. Inactivation of either enzyme led to a complete growth arrest by 6 h after induction (Fig. 2D). Moreover, the knockdown (KD) of either NadD or NadE had an apparent bactericidal effect, as evidenced by a >1,000-fold decrease in the number of viable cells (in CFU/ml) compared to the uninduced control whereas the drop in the overall cell number was only <10-fold (Fig. 2E). A comparable bactericidal effect was observed upon induction of NadD or NadE degradation after incubation for 3 days under carbon starvation conditions (dormancy) (see Fig. S4 in the supplemental material). Taken together, these results provide the first direct validation of both the NadD and NadE enzymes as potential targets for the development of bactericidal agents against mycobacteria. The relevance of this conclusion for *M. tuberculosis* is supported by the observation that the expression of the *nadRh* gene from *M. tuberculosis* in *M. smegmatis* did not suppress the bactericidal effect of the induced degradation of NadD or NadE enzymes even when NmR was added to the growth media (see Text S1 in the supplemental material).

NadD or NadE elimination blocks NAD synthesis, leading to depletion of the cofactor pool. Liquid chromatography-mass spectrometry (LC-MS)-based determination of NAD and its key metabolic intermediates in cell extracts allowed us to directly confirm and assess the dynamics of blocking NAD biosynthesis upon induced degradation of either target enzyme (Fig. 3; see also Table S2 in the supplemental material). NadD knockdown led to nearly complete depletion of the NaAD product within the first 2 h and a somewhat slower (at 4 to 6 h after induction) accumulation

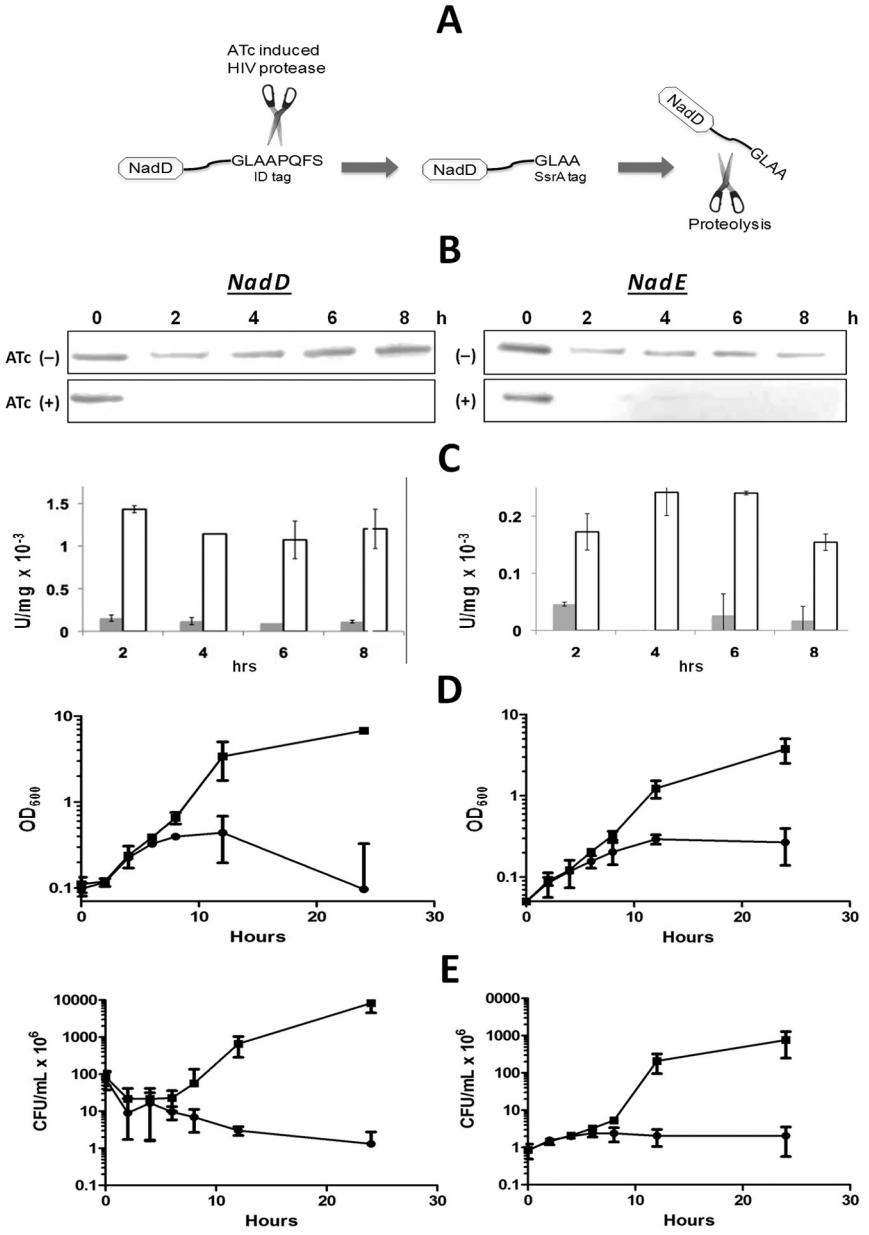

FIG 2 Induced degradation of NadD and NadE has a strong bactericidal effect in the *M. smegmatis* model system. (A) Identification number (ID)-tagged NadD and NadE are stable in the absence of HIV-2 protease, which is expressed only upon induction with anhydrotetracyclin. (B and C) When induction occurs, proteolytic removal of the HIV-2 protease recognition site exposes the SsrA recognition site and Clp-mediated proteolysis of NadD or NadE ensues. Induced and uninduced cells are indicated as ATc (+) and ATc (−), respectively. Induction by ATc leads to rapid (within 2 h) depletion of target enzymes as shown by Western blot analysis with anti-Myc-tag antibodies (B) and measurement of respective enzymatic activities in crude extracts (C). Activity was measured by HPLC-based assays; data are presented in units/mg of total protein. (D) Depletion of either enzyme leads to growth arrest, monitored by OD_{600}, within 10 h. (E) In both cases, the bactericidal effect is observed as confirmed by CFU analysis. Growth curves for NadD or NadE ATc (+) are shown with the symbol "●"; growth curves for ATc (−) are shown with the symbol "■."

of the substrate (NaMN). As a result, the overall pool of NAD cofactor gradually decreased up to ~9-fold compared to uninduced control samples (normalized per total protein) within 8 h. The depletion of the NAD^+ pool was accompanied by a comparable depletion of the reduced (NADH) cofactor pool, as independently assessed by a colorimetric assay (Fig. 3). Importantly, NADH could not be replenished from NAD^+ even though the latter remained in excess. After depletion of NadE, the accumulation of both precursors, NaMN and NaAD, was observed (Fig. 3), with the most remarkable (>100-fold) increase of the latter pool, up to the level of NAD^+ in uninduced control. The overall dynamics and extent of depletion of NAD(H) pools were similar to those observed in the case of NadD knockdown.

Notably, in both cases, a substantial accumulation of ^{13}C-labeled nicotinic acid (Na) in the medium was observed using a gas chromatography-MS (GC-MS) approach at 6 h after depletion

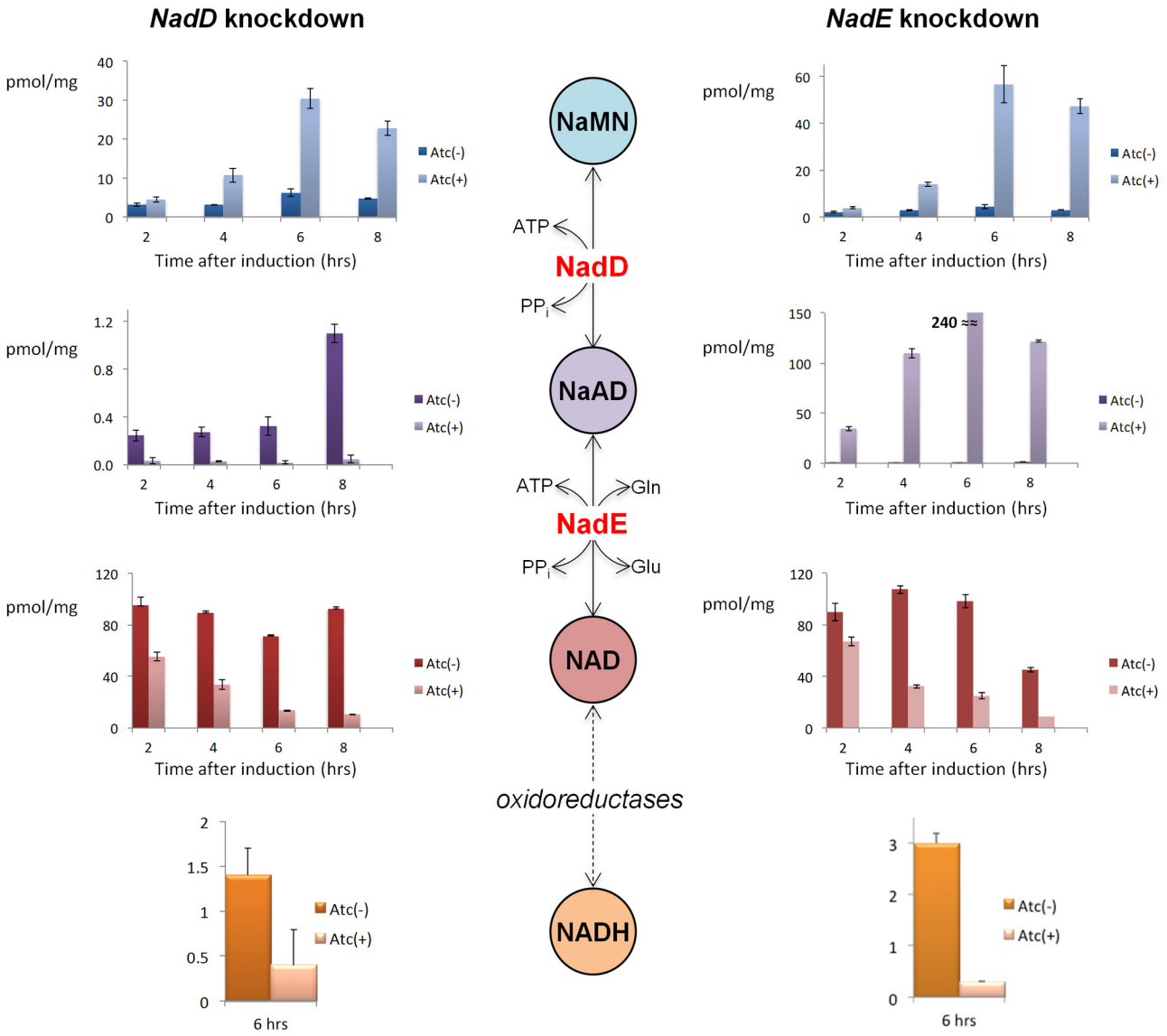

FIG 3 NadD and NadE inactivation yields accumulation of NAD metabolic precursors and depletion of the NAD(H) pool. LC-MS analysis of crude extracts showed rapid accumulation of NaMN and depletion of NaAD (the NadD substrate and product, respectively) upon induction of NadD degradation followed by the depletion of NAD pool. The same approach showed accumulation of both precursors, NaMN and NaAD (the NadE substrate), and depletion of NAD (the NadE substrate) upon NadE knockdown. A colorimetric coupled assay confirmed proportional depletions of the NADH pool in both NadD and NadE knockdown experiments (bottom panels).

of either enzyme. The latter is a likely result of NAD degradation not compensated for by recycling (which requires both NadD and NadE activities), thus leading to product inhibition of PncB by a buildup of NaMN.

NAD cofactor pool depletion leads to suppression of numerous central metabolic pathways. To explore mechanisms underlying bactericidal effects of NAD depletion, we assessed induced changes in major central metabolic pathways using biosynthetic ^{13}C labeling followed by GC-MS profiling of extracted metabolites. The percentage of synthesis from glucose of metabolites was calculated as the ratio of metabolite ^{13}C labeling to ^{13}C-glucose labeling (33). We hypothesized that the strongest effects on synthesis would be observed for the endpoint metabolites whose *de novo* syntheses include indispensable NAD(P)-dependent steps. We focused primarily on the time points at ~6 h postinduction corresponding to a substantial depletion of the NAD pool but before there was an appreciable difference in the CFU titers of induced and uninduced cultures. No difference in the uptake rates of the main carbon sources, glutamate and [U-$^{13}C_6$]glucose, from the medium were observed within the time frame of the experiment, supporting the idea of the relevance of this comparison. We obtained similar results with NadD (Fig. 4) and NadE (see Table S3 in the supplemental material) knockdown.

NAD cofactor pool depletion strongly affected the synthesis of various amino acids (Fig. 4). Synthesis of phenylalanine and tyrosine (specifically, fragments of these amino acids containing α and β carbons) decreased with NAD depletion, while synthesis of the common precursor, phosphoenolpyruvate (PEP), was unaf-

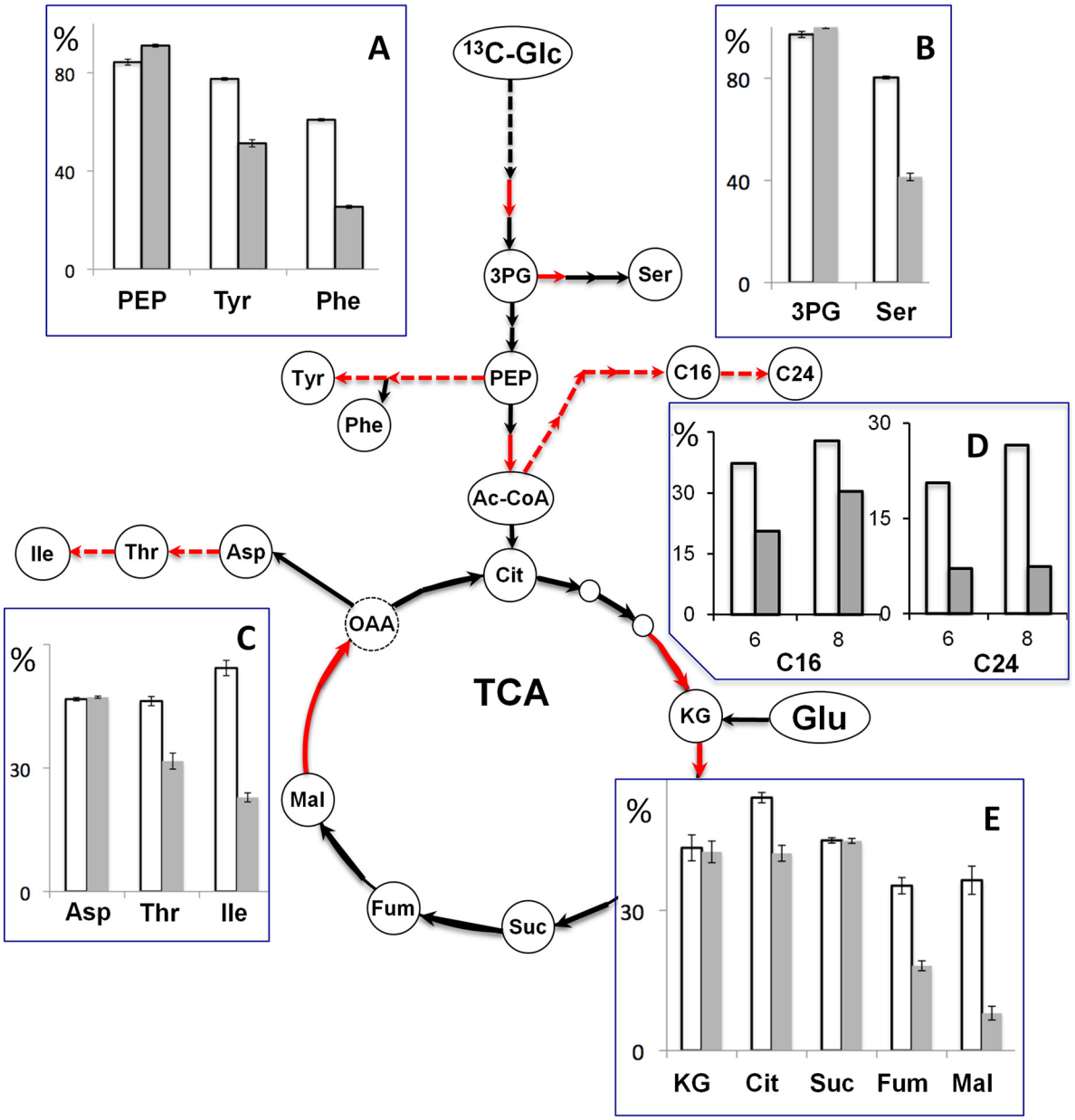

FIG 4 Depletion of the NAD cofactor leads to suppression of metabolic flux over numerous essential NAD-dependent pathways. A stronger early response was observed in pathways where multiple consecutive steps required NAD(P) cofactors as illustrated by several examples from [^{13}C]glucose (^{13}C-Glc) labeling and GC-MS metabolite profiling studies (for additional data, see Table S3 in the supplemental material). In a simplified diagram of central metabolism, metabolites are shown by abbreviations in circles as follows: 3PG, 3-phosphoglycerate; Asp, aspartate; Thr, threonine; Ile, isoleucine; PEP, phosphoenolpyruvate; Tyr, tyrosine; Phe, phenylalanine; Ser, serine; Gly, glycine; Ac-CoA, acetyl-coenzyme A (acetyl-CoA); KG, α-ketoglutarate; Cit, citrate; Suc, succinate; Fum, fumarate; Mal, malate; OAA, oxaloacetate; Glu, glutamate; Glc, glucose; C16, C16:0 fatty acid; C24, C24:0 fatty acid. Redox reactions utilizing NAD(H) or NADP(H) cofactors are marked by a red arrow. Dashed lines reflect multiple reactions. In graphs A to E, the percentages of *de novo* synthesis from glucose versus other biosynthetic, salvage, or recycling fluxes are compared for samples without (white bars) and with (gray bars) knockdown of NadD. Analyzed samples were typically obtained at 6 h postinduction by ATc (except for C16 and C24, where measurements at 6 and 8 h are indicated) when the growth rates of induced and noninduced cells remained comparable (as described for the lower panel in Fig. 1). Induction of NadE typically showed similar effects. (A) Chorismate pathway. (B) Serine and glycine synthesis. (C) Amino acids derived from aspartate. (D) Fatty acid synthesis. (E) tricarboxylic acid (TCA) cycle.

fected (Fig. 4A). The synthesis of both amino acids includes a common NAD-dependent enzyme, shikimate dehydrogenase; therefore, suppression of its activity by the depletion of the NAD cofactor pool could be responsible for the observed changes. Importantly, the shikimate pathway plays a central role in the biosynthesis of other essential aromatic compounds along with amino acids (e.g., quinones). It includes a number of experimentally demonstrated essential genes that are considered potential drug targets (34, 35). Another strong inhibitory effect could be deduced from serine synthesis, which was decreased ~2-fold upon NAD depletion, whereas there was no change in synthesis of its upstream glycolytic intermediate, 3-phosphoglycerate (3-PG;

Fig. 4B). This may be explained by the cofactor-limiting suppression of 3-phosphoglycerate dehydrogenase, the first committed step of the pathway. In addition to producing the amino acids serine and glycine, this pathway contributes to essential folate-mediated C1 metabolism. Biosynthesis of several aspartate-derived amino acids was also responsive to NAD depletion. Thus, synthesis of threonine and, to a greater extent, isoleucine decreased upon NadD knockdown (Fig. 4C). On the other hand, synthesis of their precursor, aspartate, was unaffected (Fig. 4C). This likely reflects the impact on the NAD-dependent enzyme homoserine dehydrogenase in the synthesis of threonine, which is further converted to isoleucine via a pathway containing an additional NADP-dependent enzyme, ketoacid reductoisomerase. The involvement of downstream NAD-dependent enzymes did not always translate to notably decreased synthesis of pathway endpoints. Thus, lysine synthesis, which also includes one committed downstream NAD-dependent enzyme, dihydropicolinate reductase, in addition to aspartate semialdehyde dehydrogenase (shared with the Thr/Ile pathway), did not reveal any difference between depleted and control cells (see Table S3 in the supplemental material). The absence of detectable differences may be due to a variety of factors such as a relatively low dilution of ^{13}C label from unlabeled exogenous or endogenous metabolites.

Lack of NAD biosynthesis has an even more striking effect on biosynthesis of fatty acids, as NAD is required for every elongation cycle in fatty acid propagation. *De novo* synthesis of C16:0 and C24:0 fatty acids was substantially (30% and 70%) diminished upon depletion of NadD (Fig. 4D). The effect was stronger for the longer-chain fatty acid, in keeping with a higher demand for NAD-derived cofactors in its synthesis.

Finally, ^{13}C labeling data also pointed to inhibitory effects of NAD depletion on the functioning of the tricarboxylic acid cycle. Indeed, while α-ketoglutarate and succinate were derived equally from glucose in NadD knockdown and control cells (Fig. 4E), the synthesis from glucose of fumarate and malate was decreased in knockdown cells, suggesting reduced activity of upstream NAD-dependent enzymes (e.g., α-ketoglutarate dehydrogenase) allowing dilution of a depleted fumarate/malate pool by non-^{13}C sources, e.g., aspartate.

DISCUSSION

One of the earliest antibiotics used to treat TB, isoniazid, was derived from the antituberculosis compound nicotinamide, which is also a precursor in a salvage route of NAD biogenesis and an inhibitor of NAD *de novo* synthesis in eukaryotic cells. While isoniazid clearly has a different mode of action, NAD homeostasis is important for mycobacterial infection, as demonstrated in mice for *M. bovis* (36). NAD biosynthesis represents an attractive target pathway for antibiotic development because of the central role of NAD(P) cofactors that are driving at least 10% to 15% of the chemical reactions in bacterial metabolic networks (2, 7, 10). Genes encoding NadD and NadE enzymes, conserved among bacterial species and proven to be essential in *M. tuberculosis*, are attractive and actively pursued drug targets (6, 9, 19, 21). Here we found that the inability to synthesize NAD resulting from targeted degradation of NadD or NadE enzymes led to rapid cell death in *M. smegmatis* (Fig. 2). Given the pleiotropic effects on metabolism, this is likely to be true in other mycobacteria, including *M. tuberculosis*. Moreover, this pathway has other characteristics that make it an attractive target for antibiotic development. There is only limited similarity between mycobacterial NadD and the functionally equivalent human NMNAT (7). Therefore, it should be possible to produce specific inhibitors, as has been done for other bacterial NadD enzymes (6). In addition, the protein knockdown system we employed does not completely eliminate targeted proteins (29). Thus, pharmacological agents that produce only partial inhibition could be sufficient to kill mycobacteria.

A metabolic profiling approach used in this study provided us with the experimental evidence of NAD and NADH depletion (Fig. 3) triggering global suppression of many central metabolic pathways (Fig. 4). The observed effects were prominent in pathways heavily relying on redox reactions driven by NAD(P) cofactors. Among them are known essential pathways of fatty acid synthesis (37), cholesterol catabolism (38), and synthesis of aromatic compounds (35). An observed disruption of the Krebs cycle would affect production of energy from respiration, which is important for targeting the dormant form of *M. tuberculosis*. During a monitored early phase of interference with NAD synthesis (prior to appreciable growth arrest), the ATP level slightly increased rather than decreased. This transient accumulation likely reflects sharply reduced consumption of ATP upon NAD(P) synthesis. Indeed, the synthesis of 1 molecule of NADP from NaMN consumes 3 molecules of ATP. A similar accumulation was observed for aspartate, the starting-point metabolite of NAD *de novo* synthesis.

NadD and NadE are essential in *M. tuberculosis* despite the presence of a distant homolog of NadR protein (termed NadRh), which is absent from *M. smegmatis* and many other species of mycobacteria. In our hands, NadRh did not appear to contribute to NAD biogenesis in *M. tuberculosis*, pointing to the overall resemblance of NAD metabolism in *M. smegmatis* to that in the slowly growing mycobacterial pathogens. Altogether, our results suggest that targeting NAD biosynthesis could lead to production of highly effective bactericidal antituberculosis compounds. The mechanism of action of inhibitors that could be developed against presently characterized NadD or NadE enzymes is expected to include the depletion of the NAD cofactor pool, resulting in the collapse of the entire central metabolic network. Our results suggest that many central metabolites could be used as reporters of NAD biosynthesis, providing a starting point for screens for compounds directed toward this pathway.

MATERIALS AND METHODS

Reagents and bacterial strains. Recombinant purified enzymes for coupled assays, NAD synthetase (NadE) from *Bacillus anthracis* and NMN adenylyltransferase (NadM) from *Acinetobacter baylyi*, were from previous studies (3, 5). Other reagents can be found in Text S1 in the supplemental material. Bacterial strains derived from *M. smegmatis* and *M. bovis*, plasmids, and primers used in this study are listed in Table S4 in the supplemental material.

Cloning of *M. tuberculosis nad* genes, heterologous expression, and purification. NadD and NadRh proteins were purified using standard protocols and refolding procedures (NadRh) as described in Text S1 in the supplemental material.

Enzyme activity assays and steady-state kinetic analysis. Enzymatic characterization of purified proteins was performed using a series of specific assays, including continuous and discontinuous coupled enzyme assays and direct high-performance LC (HPLC) analysis slightly modified from references 5 and 8 and described in Text S1 in the supplemental material.

Induced degradation of NadD and NadE enzymes in the *M. smegmatis* model. To deplete levels of a targeted protein, we used the Clp

protease system, which is widely conserved among bacteria (29). We utilized the natural proteolytic process of the *M. smegmatis* Clp system, specifically, ClpP and several accessory proteins, which identify protein targets and help degrade them. We employed this system to generate knockdown strains of NadD and NadE in *M. smegmatis* (see Text S1 in the supplemental material).

Effects of NadD and NadE knockdown on viability. The detailed protocol for NadD and NadE knockdown growth experiments is described in Text S1 in the supplemental material. Total protein lysates were prepared and standardized by values corresponding to the optical density at 600 nm (OD_{600}) and then lysed by bead beating. Western blot analysis was performed as previously described (39). Detection was performed using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) according the manufacturer's protocol.

Effects of NadD and NadE knockdown on levels of NAD-related metabolites by LC-MS analysis. Levels of NAD, NaAD, and NaMN in *M. smegmatis* were quantified using a reverse-phase liquid chromatography—multiple-reaction monitoring–mass spectrometry (LC-MRM-MS) assay as described in Text S1 in the supplemental material.

^{13}C biosynthetic labeling and GC-MS-based metabolic profiling. The labeling experiments were performed with the engineered *M. smegmatis* strains NadD KD and NadE KD. The analyses of polar metabolites and fatty acids by GC-MS were done as described previously (33). Protocols used for sample collection, derivatization, and GS-MS analysis are provided in Text S1 in the supplemental material.

Assessing the functional role of NadRh. (i) To study the effect of *M. bovis* NadRh expression on the ability to rescue NadD and NadE knockdown in *M. smegmatis* model, the corresponding gene was introduced on pUC57:Amp into the respective engineered *M. smegmatis* strains and was followed by knockdown viability experiments as described in Text S1 in the supplemental material. (ii) To assess the functional importance of NadRh, the corresponding gene was knocked out in *M. bovis* (see Fig. S3 in the supplemental material).

Relative ATP activity levels. Fresh cells from knockdown growth curves were mixed 1:1 with BacTiter-Glo (Promega) reagent per the specifications of the manufacturer. Luminescence was determined in a 96-well-format Fluoroskan Ascent FL plate reader (Thermo).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00747-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 1.8 MB.
Figure S2, TIF file, 1.2 MB.
Figure S3, TIF file, 0.8 MB.
Figure S4, TIF file, 0.5 MB.
Table S1, DOCX file, 0 MB.
Table S2, DOCX file, 0 MB.
Table S3, DOCX file, 0 MB.
Table S4, DOCX file, 0 MB.

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