Adenylylation and Catalytic Properties of Mycobacterium tuberculosis Glutamine Synthetase Expressed in Escherichia coli versus Mycobacteria*

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**Bacterial glutamine synthetases (GSs) are complex dodecameric oligomers that play a critical role in nitrogen metabolism, converting ammonia and glutamate to glutamine. Recently published reports suggest that GS from Mycobacterium tuberculosis (MTb) may be a therapeutic target (Harth, G., and Horwitz, M. A. (2003) Infect. Immun. 71, 456–464). In some bacteria, GS is regulated via adenylylation of some or all of the subunits within the aggregate; catalytic activity is inversely proportional to the extent of adenylylation. The adenylylation and deadenylation of GS are catalyzed by adenylyl transferase (ATase). Here, we demonstrate via electrospray ionization mass spectrometry that GS from pathogenic M. tuberculosis is adenylylated by the Escherichia coli ATase. The adenylyl group can be hydrolyzed by snake venom phosphodiesterase to afford the unmodified enzyme. The site of adenylylation of MTb GS by the E. coli ATase is Tyr-406, as indicated by the lack of adenylylation of the Y406F mutant, and, as expected, is based on amino acid sequence alignments. Using electrospray ionization mass spectrometry methodology, we found that GS is not adenylylated when obtained directly from MTb cultures that are not supplemented with glutamine. Under these conditions, the highly related but non-pathogenic Mycobacterium bovis BCG yields partially (~25%) adenylylated enzyme. Upon the addition of glutamine to the cultures, the MTb GS becomes significantly adenylylated (~30%), whereas the adenylylation of M. bovis BCG GS does not change. Collectively, the results demonstrate that MTb GS is a substrate for E. coli ATase, but only low adenylylation states are accessible. This parallels the low adenylylation states observed for GS from mycobacteria and suggests the intriguing possibility that adenylylation in the pathogenic versus non-pathogenic mycobacteria is differentially regulated.**
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(20–22) and include changes in the affinity of the enzyme for metal ion cofactors and substrates (24, 25). Disruption of the glnE gene in MTb yields a lethal phenotype (26, 27), suggesting that GS adenylylation is a critical process in Mycobacteria also. Thus, we hypothesized that the adenylylation cascade may provide additional pharmacological targets for tuberculosis therapy. However, the adenylylation process in MTb has not been well characterized.

Here we demonstrate the utility of mass spectrometric methods to monitor the adenylylation of MTb GS expressed in mycobacteria or E. coli. Interestingly, although the E. coli adenylylation system faithfully modifies in vitro the “correct” tyrosine on MTb GS with the expected functional effects, we observed incomplete adenylylation under conditions that yield complete adenylylation of the cognate E. coli GS. Moreover, we report differences in the extent of adenylylation of GS obtained directly from cultures of MTb versus M. bovis BCG.

MATERIALS AND METHODS

Cell Growth—The E. coli strains YMC21E and YMC21D are genetically engineered strains that yield fully adenylylated and completely unadenylylated GS, respectively, when E. coli GS is expressed in them. These strains were developed by Dr. J. Bowie of UCLA but are not extensively characterized in the literature, although they have been used extensively. Both strains lack a chromosomally encoded GS and require plasmid-encoded GS. The YMC21E also lacks ATase, whereas YMC21D lacks the uridylyl transferase that modulates the adenylylation/deadenylylation rates of ATase. Culture conditions for the expression of E. coli GS or MTb GS in these strains has been described previously (10, 25).

M. tuberculosis strain H37Rv (ATCC 27294) and M. bovis BCG-Russia (American Type Culture Collection number 35740) were grown to mid-log phase in 7H9 media with 0.05% Tween 80 and an ADC (5 g of albumin, 2 g of dextrose, and 3 mg of catalase per 100 ml) supplement and subclassed as 1-ml aliquots in 15% glycerol (final concentration) at −80 °C. For each experiment, thawed bacilli were inoculated into 30 ml of modified Sauton’s medium (28) with or without 20 μm glutamine at a starting A560 of 0.05 and harvested after twelve days of growth (29). Cell pellets were lysed as described previously (29). Filtrates were concentrated 100-fold and chromatographed over G-25 (Sigma). Purity was assessed by SDS-PAGE analysis and electrospray ionization mass spectrometry (ESI-MS) and was >95%. For MTb GS, the cell lysate obtained from sonication in Buffer 1 (10 mm imidazole, pH 7.0, 50 mA MglCl, and 1 mM MnCl2) was treated with streptomycin sulfate, and the supernatant was loaded on a DEAEP Fast Flow anion exchange column (Amersham Biosciences) equilibrated with Buffer 2 (50 mM HEPES, pH 7.4, 1 mM KCl, and 1 mM MnCl2). After washing with Buffer 2 for 10 column volumes, the GS was eluted with Buffer 2 containing 0.5 mM NaCl. Fractions containing activity as determined by the γ-glutamyl transferase assay, were concentrated and chromatographed over G-25 (Sigma). Purity was assessed by SDS-PAGE analysis and electrospray ionization mass spectrometry (ESI-MS) and was >95%. For MTb GS, the cell lysate obtained from sonication in Buffer 1 (10 mm imidazole, pH 7.0, 50 mA MglCl, and 1 mM MnCl2) was treated with streptomycin sulfate, and the supernatant was loaded on a DEAEP Fast Flow anion exchange column (Amersham Biosciences) equilibrated with Buffer 2 (50 mM HEPES, pH 7.4, 1 mM KCl, and 1 mM MnCl2). After washing with Buffer 2 for 10 column volumes, the GS was eluted with Buffer 2 containing 0.5 mM NaCl. Fractions containing activity as determined by the γ-glutamyl transferase assay, were pooled and dialyzed against Buffer 2. Purified protein was stored in 1-ml aliquots at −20 °C and found to be stable for nearly a year.

Snake Venom Phosphodiesterase Treatment—Samples containing 0.1 mg/ml GS, 1 mg/ml snake venom phosphodiesterase (Type IV, Crotalus atrox; Sigma) in Tris buffer, pH 9.0, and 5 mM MgCl2 were incubated at 37 °C for 3 h. Samples were assayed directly by the γ-glutamyl transferase assay as described below.

Site-directed Mutagenesis—The plasmid containing the MTb GS cDNA, pTrcHisB-TBGS, was denatured by 0.2 M NaOH. The presence of the cDNA was verified by double digestion with NcoI and HindIII (30). The denatured plasmid was then mutagenized with the QuikChange site-directed mutagenesis kit (Stratagene) using the forward primer 5′-CGAACAGACTCGAGTCCGCGCCG-3′ and the reverse primer 5′-GCGCGCGGCCGAGATTCGCTGGTGC-3′ (Invitrogen), with 2.5 μl of Primer Turbo polymerase reaction per well. Template amounts were varied from 500 to 15.6 ng per reaction. PCR was performed using the following program on an Eppendorf temperature cycler: (i) an initial denaturation for 60 s at 95 °C; (ii) 17 cycles of denaturation for 30 s per cycle at 95 °C; (iii) 60 s of elongation at 55 °C; (iv) 12 min of annealing at 68 °C; and (v) a final 10 min of annealing at 72 °C. Agarose gel electrophoresis (1% w/v) verified the presence of a 6 kb of PCR product, which is indicative of successful amplification of pTrcHisB-TBGS. The amount of template had no apparent effect on the amount of product obtained. The PCR-mutagenized plasmid was digested with DpnI (20 units) to degrade any template plasmids present in the PCR product mix. E. coli XL-1 Blue supercompetent cells (Stratagene) were transformed with the mutagenized plasmids and grown overnight on Luria-Bertani agar plates infused with 50 μg/ml carbenicillin. Mutagenized plasmid was purified from picked colonies using the QIAprep Spin Mini-prep protocol (Qiagen).

Forward and reverse strands of the cDNA in the mutagenized plasmids were sequenced using the BigDye 3.1 kit (Applied Biosystems) and an ABI 3730XL high-throughput capillary DNA analyzer to screen for successful mutagenesis and any spurious mutations that were introduced. The following thermal cycling program was used: (i) 60 s at 96 °C; (ii) 25 cycles of 10 s per cycle at 96 °C; (iii) 5 s at 50 °C; and (iv) 24 s at 60 °C. The DNA used for sequencing was purified by ethanol precipitation.

Catalytic Activity Assays—Enzyme activity was monitored by the γ-glutamyl transferase assay, which is based on the formation of the colored product monitored at 430 nm (31), or by the Mg2+ biosynthetic assay, which is a coupled enzyme assay that reports on the conversion of glutamate and monitors NADPH production at 340 nm (24). For the biosynthetic assay, solutions contained 100 mM HEPES (Sigma), 100 mM PIPES (USB), 100 mM KCl, 1 mM P-enolpyruvate, 190 mg of NADH, 150 μg/ml pyruvate kinase (Sigma), 150 μg/ml lactate dehydrogenase (Sigma), 50 mM MgCl2, and 1 μg of GS. Reaction was initiated with ATP, which was varied between 0 and 500 μM. For determination of the adenylylation state via the transferase assay, we used two solutions that included either Mn2+ (Solution A) or Mg2+ (Solution B). The Mg2+ form of GS is inactivated specifically by the adenylyl group so that the adenylylation state, n, is calculated as n = 12 − 12[A600], where 6 and 8 are the absorbances at 540 nm in the assay solutions B and A respectively. This assay is described in detail elsewhere.

Mass Spectrometry—Samples were chromatographed through a Poros R2 column with a linear gradient from 94.95% H2O, 5% acetonitrile, and 0.05% trifluoroacetic acid (v/v/v) to 39.95% H2O, 60% acetonitrile, and 0.05% trifluoroacetic acid (v/v/v) over 7 min and interfaced with a Fisons VG quattro II mass spectrometer fitted with a 2-M spray ESI source. Mass spectra were deconvoluted using the maximum entropy method provided in the MassLynx™ software. Calculation of the adenylylation state, n, was by n = IMW + 320/IWMW + 320 × 12, where IWMW + 320 is the intensity of the molecular ion at the molecular weight of the adenylylated species, IWMW is the intensity of the molecular ion at the molecular weight of the unadenylylated species, and 12 is the maximum adenylylation state. For some experiments with E. coli GS, a site-directed mutant, E165C, was used as a model. E165C is fully adenylylated when expressed in YMC21D and fully unadenylylated in YMC21E. The adenylylation state was calculated as with wild type, using the appropriate masses.

Molecular Modeling—Molecular models were constructed using Chimera™, and the available x-ray structure was reported in Ref. 9. The Protein Data Bank file used was 1HTQ.

RESULTS AND DISCUSSION

Mass Spectral Characterization of Adenylylation—As noted, for many enteric bacteria there is a well characterized inverse correlation between the average number of adenylyl groups/GS dodecamer and the enzymatic activity of the Mg2+-form of the enzyme. However, a detailed characterization of the functional effects of adenylylation on GS from any strain of Mycobacteria has not been performed. Moreover, the adenylylation process in Mycobacteria is not characterized. To initiate a systematic characterization of the adenylylated MTb GS in the absence of a source of MTb ATase, we recognized the potential utility of the E. coli adenylylation system for providing adenylylated MTb GS. In addition, we were interested in developing a convenient method for monitoring the adenylylation process directly in Mycobacteria. Therefore, we utilized ESI-MS to monitor the GS preparations for covariant modification. We also exploited a well established method (32, 33) for the removal of

adenylyl groups via snake venom phosphodiesterase (SVPDE) to seek a change in mass corresponding to the adenylyl moiety (Δ mass = 330 atomic mass units; Fig. 1). The validity of this strategy was established first with *E. coli* GS, wherein the previously described strains YMC21E and YMC21D produce fully unadenylylated and fully adenylylated GS, respectively (25, 34). Mass spectra for *E. coli* GS in the fully adenylylated, fully unadenylylated, and SVPDE-treated states are shown in Fig. 2. For the GS from YMC21E, the recovered mass of 51,761 Da is within the S.E. for a theoretical mass of 51,772 Da. The post-translational modification corresponding to the adenylyl group is easily observed by the mass shift of 331 atomic mass units for GS isolated from *E. coli* strain YMC21D, and this mass increment is lost upon treatment with SVPDE. The SVPDE treatment had no effect on the unadenylylated GS (not shown), thus confirming the loss of the adenylyl group as the source of the mass shift in the adenylylated preparations. These results are summarized in Table I, along with data describing functional characteristics as described below.

**Adenylylation of MTb GS Expressed in E. coli**—This approach was extended to monitor adenylylation of MTb GS expressed in *E. coli*. MTb GS was expressed in the YMC21E and YMC21D strains and purified to near homogeneity. We reasoned that if the *E. coli* adenylylation system recognized the MTb GS, then expression in both YMC21E and YMC21D strains would provide "standards" for mass spectral characterization of adenylylated and unadenylylated *M. tuberculosis* GS and for functional characterization.

Indeed, the *M. tuberculosis* GS purified from *E. coli* strain YMC21D was 25–30% adenylylated, based on the presence of two major species with appropriate masses that differed by 330 atomic mass units and their collapse to a single species with the lower mass upon treatment with SVPDE (Fig. 3). The theoretical mass of the MTb GS (53,570 Da) is slightly greater than that of the *E. coli* isoform because of 10 additional amino acids, including the N-terminal methionine, and the experimentally recovered mass is within the S.E. Notably, the adenylylated form of MTb GS was always 25–35% of the total protein present, in contrast to the *E. coli* isoform obtained from YMC21D, which was completely adenylylated.

These results were important for two reasons. First, they demonstrated that the adenylylated and unadenylylated forms of *M. tuberculosis* GS can be identified by this approach. Second, this is the first evidence for adenylylation of mycobacterial GS by *E. coli* ATase, and there is clearly some difference in the processing of MTb GS versus *E. coli* GS by *E. coli* ATase. These results are also summarized in Table I.

**Functional Effects of Adenylylation on M. tuberculosis GS**—As further validation for the mass spectrometric method, we initiated a functional characterization of the MTb GS preparation from different sources. Adenylylation of GSs that are regulated by this mechanism leads to a decrease in the activity of the Mg²⁺-bound form of GS, but not the Mn²⁺-bound form. Therefore, we also performed the Mg²⁺ biosynthetic assay. The expected decrease in activity was observed for adenylylated MTb GS obtained from YMC21D (Table I). In fact, in light of the 20–25-fold decrease in *V*ₘₐₓ for *E. coli* GS upon complete adenylylation, as observed here and by others (9, 24), it might be expected that the partially (30%) adenylylated MTb GS would have a *V*ₘₐₓ decreased by 7–8-fold as compared with the unadenylylated MTb GS. In fact, we observe a larger decrease in activity of 15-fold. Furthermore, the *K*ₘ for ATP increased...
1.4-fold upon partial adenylylation of the MTb GS, a result that is consistent with what has been established for partially adenylylated GS from *E. coli* (9, 24).

**Identification of the Adenylylation Site on MTb GS**—An issue not directly addressed by the mass spectrometry approach or catalytic studies is the location of the adenylyl group on MTb GS when the *E. coli* ATase attaches it. It is formally possible that the *E. coli* ATase could misadenylylate the MTb GS at the "incorrect" residue and still regulate the catalytic function. To address this issue, the fidelity of the reaction was validated by construction of the site-directed mutant Y406F, which eliminates the tyrosine that is homologous to the *E. coli* site of adenylylation (9, 10). Tyr-406 aligns with Tyr-397 of the *E. coli* enzyme, which is the established site of adenylylation for the latter isoform. The enzyme obtained from *E. coli* YMC21E was functionally characterized via the "transferase assay" and found to have a specific activity of 108 units/mg, compared with 120 units/mg for the wild type enzyme. Expression and purification of this mutant in *E. coli* YMC21D afforded a protein that was completely unadenylylated (Fig. 4). The theoretical mass of the unadenylylated Y406F mutant is 53,554 Da, which is within a few daltons of the recovered mass. Furthermore, treatment with SVPDE does not result in a mass shift (not shown). Apparently, the Y406F *M. tuberculosis* GS is not a substrate for *E. coli* ATase, in contrast to the case of the wild type MTb GS. Thus, the adenylylation observed in *E. coli* with the wild type MTb GS is at the expected position, Tyr-406.

**Adenylylation of GS from MTb H37Rv and *M. bovis* BCG**—The ESI-MS provides a convenient function-independent approach for monitoring the adenylylation state of GS in mycobacterial cultures. Therefore, we exploited this approach to compare cultures of pathogenic MTb and non-pathogenic *M. bovis* BCG. Two culture conditions were used to determine the

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**Table I**

| GS isoform/source | Adenylylation state via mass spectrometry* | $K_m$ of ATP | Relative $V_{max}$ |
|------------------|------------------------------------------|-------------|-------------------|
| *E. coli* GS/YMC21E | 0                                        | 190 ± 13    | 1                 |
| *E. coli* GS/YMC21D | 12 ± 0.2                                 | 709 ± 63    | 0.046             |
| MTb GS/YMC21E | 0                                        | 352 ± 22    | 1                 |
| MTb GS/YMC21D | 3.1 ± 0.2                                 | 481 ± 26    | 0.068             |

* Calculated as described under "Material and Methods." Values of "0" were reported when no peak was observed at the appropriate mass of the adenylylated form. Errors are S.E., from duplicate determination. Normalized to unadenylylated enzyme for each isoform.

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**Fig. 2.** Deconvoluted electrospray ionization mass spectra of *E. coli* GS. The enzyme expressed in strain YMC21E is completely unadenylylated (top), whereas the GS from YMC21D is completely adenylylated (center). Upon treatment of the adenylylated enzyme with snake venom phosphodiesterase, the mass shifts to the unadenylylated form (bottom). The mass difference corresponds to the adenylyl group, ~330 atomic mass units, within S.E.

**Fig. 3.** Deconvoluted electrospray ionization mass spectra of *M. tuberculosis* GS expressed in *E. coli*. The *M. tuberculosis* GS expressed in YMC21E (top) is completely unadenylylated, but ~25% adenylylated when expressed in YMC21D (53.876 Da; center). Treatment with SVPDE generates the unadenylylated enzyme (bottom).

**Fig. 4.** Deconvoluted electrospray ionization mass spectra of MTb GS Y406F obtained from YMC21D. The (GS + 330) atomic mass unit species is not observed for the Y406F mutant, and the MW is unaffected by treatment with SVPDE. The theoretical mass of unadenylylated Y406F MTb GS is 53,554 Da.
effect of nitrogen levels on the adenylylation process. Specifically, cells were grown in standard media as described under “Materials and Methods” (25), or the media were supplemented with 20 mM glutamine. For E. coli (other than YMC21E), the addition of glutamine to the culture media yields highly adenylylated GS (19, 23, 35). Interestingly, the adenylylation state of GS obtained from either M. tuberculosis or M. bovis BCG cultures was differentially affected by the addition of glutamine. Based on the ESI-MS analysis, the M. tuberculosis H37Rv GS was not adenylylated significantly in the absence of glutamine (<5%) in several preparations obtained from cultures grown under these conditions. In contrast, when this strain was grown in the presence of 20 mM glutamine, ~35% of the GS was adenylylated as indicated by the species with a mass increase of ~330 atomic mass units (Fig. 5, top left). This was observed with several cultures. Interestingly, GS from the non-pathogenic M. bovis BCG was ~20% adenylylated both in the absence and presence of glutamine (Fig. 5, right top and bottom). In other words, no glutamine-dependent change in the adenylylation state of GS was observed for the M. bovis BCG, in marked contrast to the MTb. In each case, the high molecular weight species, when present, could be converted to the lower molecular weight species by treatment with SVPDE, as was observed with M. tuberculosis GS expressed in E. coli (not shown). The results of three separate experiments are summarized in Table II. It is also striking that both mycobacterial species was differentially affected by the addition of glutamine. GS from the mycobacterial cultures is partly adenylylated (≤25%; 53,756 Da) when cultured in the absence (top right) or presence (bottom right) of glutamine. The glutamine does not significantly increase the adenylylation state of M. bovis GS.

Based on this initial characterization, the functional effects of GS adenylylation appear strikingly similar to those observed

| GS isoform/source | Adenylylation state via mass spectrometrya | Adenylylation state via transferase assay |
|-------------------|-------------------------------------------|------------------------------------------|
| E. coli GS/YMC21D | 12                                        | 11.7 ± 0.3                                |
| E. coli GS/YMC21E | 0                                         | 0                                        |
| MTb GS/M. tuberculosis | 0                              | 0.2 ± 0.2                                 |
| MTb GS/M. bovis BCG | 3.9 ± 0.0 | 4.6 ± 0.4                                |
| MTb GS/M. tuberculosis + 20 mM Gln | 4.1 ± 0.3 | 4.9 ± 0.5                                |
| MTb GS/M. bovis BCG + 20 mM Gln | 3.7 ± 0.4 | 4.3 ± 0.5                                |

a The reported values are calculated as described under “Materials and Methods,” where 12 is the maximum number of adenyyl groups per dodecamer, and the values are the mean of two or three separate protein preparations. For “0” values, no detectable species with the appropriate molecular weight was present or there was no detectable activity in the Mg²⁺ buffer.

Conclusions—In this work we demonstrate that MTb GS is chemically competent for adenylylation by the E. coli ATase. The site of MTb GS adenylylation was shown by mutagenesis to be at Tyr-406, as was expected from sequence alignments and structural models of the MTb GS (9, 10). It remains possible that the wild type MTb GS is misadenylylated at another Tyr residue, and mutation of Tyr-406 could induce a structural change that prevented this reaction. However, based on the catalytic activity of the mutant, which is nearly identical to that of the wild type MTb GS, and on the lack of other Tyr residues at the adenylylation surface, we consider this unlikely and we propose that Tyr-406 is faithfully adenylylated by the E. coli ATase. The ability of E. coli YMC21D to adenylylate MTb GS provides a convenient source of this modified enzyme without the need for growth of MTb cultures, which are significantly more challenging to handle and require additional safety precautions.

Based on this initial characterization, the functional effects of GS adenylylation appear strikingly similar to those observed
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for the E. coli GS in the physiologically relevant reaction. In this assay, with the partially adenylylated preparations of MtGBG available, we observe functional behavior that is analogous to the E. coli enzyme. Specifically, upon adenylylation the V_{max} of the Mg^{2+} form decreases by at least 10-fold with a concomitant 6–10-fold increase in the K_{m} for ATP. Our initial characterization has not revealed any differences in the regulation of MTb GS versus E. coli GS mediated by the adenylyl group.

In contrast, our results do indicate potentially important differences in the adenylylation processes for E. coli, MTb and M. bovis BCG. It is interesting that, for all of the MTb GS preparations from YMC21D, the adenylylation state was only ~25% of the subunits rather than the full adenylylation observed with E. coli GS. Possibly, the E. coli ATase, which also catalyzes the reverse (deadenylylation) reaction, reaches a different steady state with MTb GS as compared with E. coli GS. However, as elaborated below, this partial adenylylation state of MTb GS is not limited to preparations obtained from E. coli. Regardless of the reason for the incomplete adenylylation of MTb GS in E. coli, it will be interesting to study interactions between GS and ATase from the different organisms.

The difference in the adenylylation state of GS from MTb cultures versus that of the M. bovis BCG culture was unexpected. In the absence of added glutamine, the MTb GS was completely unadenylated in each of several experiments. In contrast, GS from M. bovis BCG was consistently ~25–30% adenylylated. The addition of 20 μM glutamine to cultures of MTb resulted in a substantial increase in GS adenylylation to ~35%, whereas the addition of glutamine to M. bovis BCG caused no significant increase in adenylylation. Interestingly, in the related non-pathogenic Corynebacterium glutamicum, environmental nitrogen levels do not alter the adenylylation state of GS as measured indirectly by incorporation of phosphate (37). This appears to be another example of a bacterium in which GS adenylylation is not as sensitive to nitrogen availability as in many enteric bacteria. If the regulation of GS adenylylation proves to be a general property of pathogenic mycobacteria as our data suggest, then this regulatory pathway might provide additional therapeutic targets. Obviously, our results do not prove any causal relationship between GS adenylylation and pathogenicity. The relative concentrations of several intermediate metabolites and co-factors, including ATP, UTP, glutamine, α-ketoglutarate, and metal ions, are known to affect GS adenylylation, at least in some bacteria (38). Thus, a difference in other pathways or enzyme levels could translate into differences in GS adenylylation in MTb versus M. bovis BCG. Regardless of these possible differences, it is striking that the maximal adenylylation state observed for either mycobacterial GS is ~35%, and this is mimicked by the MTb GS adenylylation in E. coli YCM21D. It is noteworthy also that E. coli ATase does adenylylate other GSs, such as the Anabaena 7120 enzyme. In in vitro studies, the E. coli ATase modified the Anabaena GS very slowly (36). However, in marked contrast to our results, the Anabaena GS was completely adenylylated. Perhaps some inherent property of mycobacterial GSs limits adenylylation to only a few subunits.

Acknowledgments—We gratefully acknowledge Drs. David Eisenberg and Marcus Horwitz for providing the plasmid encoding M. tuberculosis GS. Also, Jed Lampe and Mike Dabrowski are gratefully acknowledged for assistance with mutagenesis protein purification.

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