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Glutamine synthetase of *Mycobacterium tuberculosis*: Extracellular release and characterization of its enzymatic activity

(tuberculosis/nitrogen metabolism/pathogenesis/ammonia regulation)

GÜNTER HARTH*, DANIEL L. CLEMENS, AND MARCUS A. HORWITZ

Division of Infectious Diseases, Department of Medicine, 37-121 Center for the Health Sciences, School of Medicine, University of California, 10833 Le Conte Avenue, Los Angeles, CA 90024

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ABSTRACT **We have investigated the activity and extracellular release of glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] of *Mycobacterium tuberculosis*. The purified, homogeneous *M. tuberculosis* glutamine synthetase appears to consist of 12 most likely identical subunits of M_r 58,000, arranged in two superimposed hexagons. In the catalysis of L-glutamine, the enzyme has an apparent K_m for L-glutamate of ≈3 mM at the pH optimum of 7.5. *M. tuberculosis* releases a large proportion (≈30%) of its total measurable enzyme activity into the culture medium, a feature that is highly specific for pathogenic mycobacteria. Immunogold electron microscopy revealed that *M. tuberculosis* also releases the enzyme into its phagosome in infected human monocytes. Two potentially important roles for glutamine synthetase in the pathogenesis of *M. tuberculosis* infection are (*i*) the synthesis of L-glutamine, a major component of the cell wall of pathogenic but not nonpathogenic mycobacteria, and (*ii*) the modulation of the ammonia level in the *M. tuberculosis* phagosome, which may in turn influence phagosomal pH and phagosome–lysosome fusion.**

Mycobacterium tuberculosis is one of the world's most important pathogens. It infects 2 billion persons worldwide and causes 8 million new cases of pulmonary tuberculosis and 3 million deaths annually (1). The rising incidence of tuberculosis in the United States, in large part due to the AIDS epidemic, and the emergence of multidrug-resistant tuberculosis constitute a major public health dilemma that underscores the need for more knowledge about pathogenic mechanisms of *M. tuberculosis* (2).

M. tuberculosis is a facultative intracellular parasite that resides and multiplies within a membrane-bound phagosome in human mononuclear phagocytes, especially lung macrophages (3). Two important but poorly understood characteristics of the phagosome are that it is not highly acidified (4) and does not fuse with lysosomes (5).

We have identified glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] as one potentially important determinant of *M. tuberculosis* pathogenesis. Glutamine synthetase may influence host–pathogen interaction in two key respects. (*i*) Because of its central role in nitrogen metabolism (6), the enzyme may influence the ammonia level within infected host cells and hence contribute to the pathogen's capacity to inhibit phagosome–lysosome fusion and phagosome acidification (7). (*ii*) Our finding, described in this report, that glutamine synthetase mediates the extracellular catalysis of glutamine suggests a direct involvement of the enzyme in the synthesis of the cell wall structure poly(L-glutamic acid/glutamine) (8) found in pathogenic but not nonpathogenic mycobacteria.

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MATERIALS AND METHODS

Bacterial Cultures. *M. tuberculosis* strains Erdman (ATCC 35801), H37Rv (ATCC 25618), and H37Ra (ATCC 25177), *M. bovis* (ATCC 19210), *M. bovis* BCG (bacille Calmette–Guérin, ATCC 19274), *M. phlei* (ATCC 11758), and *M. smegmatis* (ATCC 14468) were grown in 7H9 medium (Difco) at pH 6.7 and 37°C in a 5% CO_2 atmosphere. *Escherichia coli* DH5α (9), *Legionella pneumophila* Philadelphia 1 (10), *Bacillus cereus* (ATCC 14579), and *Bacillus subtilis* (ATCC 6051) were grown as described. For comparative glutamine synthetase assays, all bacteria were grown in 7H9 medium at pH 6.7 and 7.5 or in Sauton's medium (11).

Purification of Glutamine Synthetase. Supernatant from 18 liters of *M. tuberculosis* Erdman strain cultures was filtered through Tuffryn 0.45- and 0.22-μm filters (Gelman) and concentrated by tangential flow through a polyethersulfone membrane (Filtron Technology, Northborough, MA). Proteins in this concentrate were precipitated with ammonium sulfate at 100% saturation, pelleted by centrifugation, and dialyzed against sorbitol buffer (10% sorbitol/10 mM potassium phosphate, pH 7.0/5 mM 2-mercaptoethanol/0.2 mM EDTA). The proteins were applied to DEAE-Sepharose CL-6B (Pharmacia) and glutamine synthetase was eluted at 0.5–1 M NaCl. The enzyme was further chromatographed on thiopropyl-Sepharose 6B (Pharmacia), eluted at 150–250 mM 2-mercaptoethanol, concentrated to 2.5 ml in a Diaflo unit (Amicon), and finally size fractionated on Sepharose 6B (Pharmacia). Enzymatically active fractions were pooled and stored at 4°C.

Protein concentrations were determined by the bicinchoninic acid reagent (Pierce). Proteins in the active fractions were analyzed by SDS/10% PAGE and stained with Coomassie brilliant blue R or silver nitrate. The N-terminal sequence of glutamine synthetase was determined on poly(vinylidene difluoride) membranes at the University of California, Los Angeles, protein microsequencing facility with a Porton 2090 E amino acid sequencer.

Assays of Glutamine Synthetase Activity. The enzyme was assayed both in the biosynthetic (forward) assay (glutamate + ATP + ammonia → glutamine + ADP + P_i) and in the transfer assay (glutamine + hydroxylamine → γ-glutamylhydroxamate + ammonia) as described (12). One unit of glutamine synthetase was defined as the amount of enzyme producing 1 μmol of P_i per min in the biosynthetic reaction or 1 μmol of γ-glutamylhydroxamate per min in the transfer reaction.

The pH optima of glutamine synthetase were determined for both assay systems for the range pH 6.0–9.0. The enzyme's cation requirements were also examined for both reactions. Cobalt(II) chloride, magnesium chloride, manganese chloride, or zinc(II) chloride was added at 50 mM for the biosynthetic reaction and at 3 mM for the transfer reaction.

*To whom reprint requests should be addressed.

The enzyme's substrate specificity was analyzed by testing substrate analogs at the same concentration as the standard substrate. The apparent K_m of the enzyme for L-glutamate in the forward reaction and L-glutamine in the transfer reaction was determined by varying either the L-glutamate concentration from 0.5 mM to 100 mM or the L-glutamine concentration from 0.3 mM to 90 mM. Inhibition of enzymatic activity by EDTA (13), L-methionine *S*-sulfoximine (14), and limited proteolysis (15) was assessed for both assay systems as described.

Extracellular Release of Glutamine Synthetase. The activity of *M. tuberculosis* Erdman glutamine synthetase associated with the cell pellet or released into the culture medium was determined by the standard transfer reaction for bacteria cultured for 7, 14, 21, or 28 days. Bacterial culture supernatants were obtained by filtering cultures through 0.2-μm Acrodisk membranes (Gelman) and concentrating the filtrates 5-fold. Bacterial cell extracts were obtained by first lysing the bacteria by treating them with lysozyme/Triton X-100 and vortexing for 40 sec with 60-mesh crystalline alumina beads (Fisher) and then centrifuging the bacteria and retaining the supernatant.

Measurement of Enzyme Substrates and Products in Infected Culture Medium. Ammonia production by *M. tuberculosis* was determined as described (16). The presence of ATP in culture supernatants was determined by apyrase treatment and firefly luciferase assays. The presence of L-glutamate and L-glutamine was demonstrated by thin-layer chromatography (GHL-Uniplate, Analtech) in 1-butanol/acetic acid/water, 15:6:4 (vol/vol).

Immunologic Reactivity of Glutamine Synthetase. Polyvalent, glutamine synthetase-specific antibody was raised in rabbits by immunization with 50 μg of purified enzyme in Syntex adjuvant (17) containing 1.5 μg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine, followed by three booster immunizations at 12-day intervals with 25 μg of enzyme. IgG preparations from each of two rabbits had a reciprocal titer of <100 before immunization and $\approx 10^7$ after immunization, as determined by immunoblotting. Antibody to glutamine synthetase in sera obtained from *M. tuberculosis*-infected guinea pigs (18) and from uninfected control animals was assessed by immunoblotting.

Electron Microscopy. The presence of *M. tuberculosis* glutamine synthetase in phagosomes of infected human monocytes was explored by incubating cryosections of infected monocytes first with pre- or postimmune anti-glutamine synthetase IgG at 10 μg/ml and then with goat anti-rabbit IgG conjugated to 10-nm gold particles. The stained sections were evaluated with a 100 CX II JEOL transmission electron microscope (19). The enzyme's ultrastructure was analyzed by the single-carbon-layer technique (20). Glutamine synthetase was adjusted to 80 μg/ml in 10 mM imidazole chloride, pH 7.2/1 mM $MnCl_2$, adsorbed to a carbon film, and negatively stained with 2% uranyl acetate.

Chemicals and Enzymes. All chemicals and enzymes were purchased from Sigma unless indicated otherwise and were of the highest grade available.

RESULTS

Purification of Glutamine Synthetase and Measurement of Its Enzymatic Activity. In view of the central role of glutamine synthetase in nitrogen metabolism and the potential importance of ammonia production to intraphagosomal survival, we purified glutamine synthetase from the highly virulent Erdman strain of *M. tuberculosis*. The yield after several purification steps that resulted in a 30-fold enrichment of the enzyme was about 40% (Fig. 1 and Table 1). The enzyme was homogeneous as determined by SDS/PAGE and staining with Coomassie blue (Fig. 1) or silver nitrate (data not shown). One band of $M_r \approx 58{,}000$ was observed, a mass very similar to that of other characterized glutamine synthetase subunits (13, 21). In both assay systems (12), the purified glutamine synthetase exhibited high enzymatic activity.

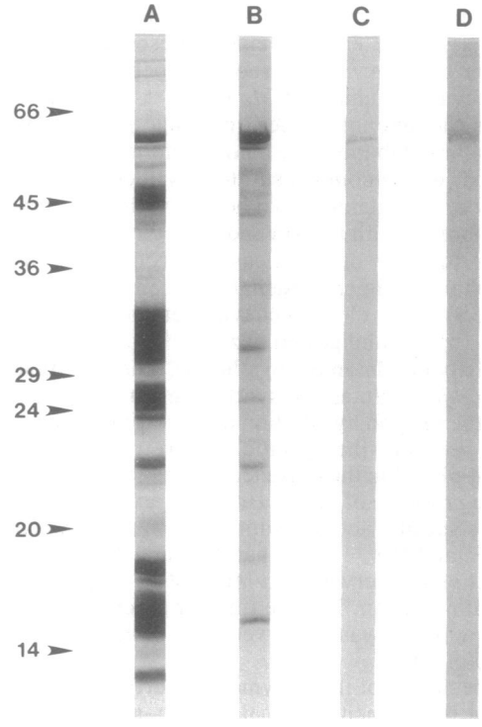

FIG. 1. Purification of *M. tuberculosis* glutamine synthetase from culture filtrate. After each purification step, aliquots from pooled glutamine synthetase-containing fractions were electrophoresed in SDS/10% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue R. Lane A, ammonium sulfate precipitation; lane B, DEAE-Sepharose CL-6B; lane C, thiopropyl-Sepharose 6B; lane D, Sepharose 6B. Numbers at left refer to molecular weight standards presented as $M_r \times 10^{-3}$.

Loss of enzymatic activity was observed under three different conditions. (*i*) Incubation of the purified enzyme in 10 mM imidazole, pH 7.0/10 mM EDTA for 15 min at 20°C inactivated the enzyme as measured by the loss of ≥99% of its activity in the transfer reaction, which is evidently dependent on the presence of metal ions. (*ii*) Incubation of the enzyme with the potent specific inhibitor L-methionine *S*-sulfoximine readily inactivated it. Measured in both assays, the activity of the enzyme at inhibitor concentrations of 0.5 and 2.5 mM declined to 16% and 9%, respectively, of its uninhibited value during a 5-min incubation and to 8% and 1.5%, respectively, during a 15-min standard incubation. Even at an inhibitor concentration of only 0.1 mM, the enzyme's activity

Table 1. Purification of *M. tuberculosis* glutamine synthetase

Step	Protein, mg	Specific activity, units/mg: Forward reaction	Specific activity, units/mg: Transfer reaction	Yield, %
Ammonium sulfate precipitation	360	3.8	4.1	100
DEAE-Sepharose CL-6B	56	20.4	21.9	83
Thiopropyl-Sepharose 6B	20	43.0	45.5	62
Sepharose 6B	5	110.0	125.0	41

Starting material for the purification of glutamine synthetase was 18 liters of culture filtrate, equivalent to $\approx 6 \times 10^{12}$ cells.

declined to 18% during a 15-min incubation. (*iii*) Limited proteolysis of the enzyme in the presence of 1% (wt/wt) trypsin or chymotrypsin at room temperature revealed two main cleavage products of M_r 37,000 ± 3000 and 20,000 ± 2000. As the amount of these cleavage products increased over a 24-hr incubation, the amount of the M_r 58,000 subunit decreased, with a commensurate loss in enzymatic activity. Incubation of glutamine synthetase with both proteinases together or first with trypsin and then with chymotrypsin did not alter the size of the cleavage products (± M_r 2000). Electrophoresis under nondenaturing conditions showed that the proteolytically cleaved enzyme retained the same mobility as the native undigested enzyme.

Specificity of Glutamine Synthetase. The specificity of the *M. tuberculosis* glutamine synthetase was assessed first by varying the pH and by addition of activating cations. For the forward reaction, the specific activity of the enzyme was highest at pH 7.5 in the presence of Mg^{2+} and Co^{2+} and at pH 7.0 in the presence of Mn^{2+}. However, at the pH optima, the enzyme's specific activity with Mg^{2+} was much higher than with Co^{2+} and Mn^{2+}, which had 14% and 23%, respectively, of the activity observed with Mg^{2+}. Zn^{2+} was ineffective as an activating cation. For the transfer reaction, high specific enzyme activity was observed only with Mn^{2+} at a pH optimum of 7.0.

The specificity of the enzyme was further investigated by analyzing the capacity of analogs of L-glutamate, ATP, or ADP to replace standard substrates. In the forward reaction, L-glutamate was specifically required; D-glutamate was a poor substrate, and DL-glutamate showed about 50% of the standard specific activity. GTP, but not other nucleotides (CTP, UTP, and dTTP) could replace ATP to some extent (45% of the standard specific activity). In the transfer reaction, GDP could substitute for ADP reasonably well (90% of the standard specific activity); however, CDP, UDP, and dTDP were not readily utilized by the enzyme.

The apparent K_m values of the enzyme for L-glutamate in the forward reaction and L-glutamine in the transfer reaction were calculated to be 2.7 ± 0.2 mM for L-glutamate and 2.9 ± 0.3 mM for L-glutamine. The enzyme's specific activity of 110 μmol of P_i per min per mg of enzyme in the forward reaction and 125 μmol of γ-glutamylhydroxamate per min per mg of enzyme in the transfer reaction yielded turnover numbers of ≈70,000 and ≈87,000 mol of product per min per mol of enzyme, respectively.

Structure of Glutamine Synthetase. Size fractionation on Sepharose 6B yielded a M_r of ≈680,000 for the native glutamine synthetase. This suggests that the molecule is composed of ≈11.7 subunits of M_r 58,000, the size of the band observed on denaturing gels. This number of subunits is in good agreement with the value of 12 subunits described for other glutamine synthetases (12, 22). The identity of the M_r 58,000 molecule as a member of the glutamine synthetase family (21) was further established by determination of the N-terminal amino acid sequence of the molecule (Table 2).

Electron microscopy of negatively stained homogeneous enzyme revealed several structural features characteristic of glutamine synthetases (20, 23). In one orientation (top view), the enzyme subunits appeared arranged in a hexagonal or ring form (Fig. 2*A*), while in a second and third orientation (side views), the enzyme appeared as two protein layers projecting either as four spots in a rectangular array (Fig. 2*B*), each spot presumably representing three superimposed subunits, or as two parallel strips (Fig. 2*C*), each strip presumably representing three pairs of superimposed subunits in a row. The long diameter of the hexagonal form in the first orientation was 14.0 ± 0.6 nm and the height of the molecule in the second and third orientations was 9.2 ± 0.3 nm.

Extracellular Presence of Glutamine Synthetase and Its Substrates. Surprisingly, we observed that enzyme activity amounting to about one-third of the total activity present in the cell pellet and supernatant was released into the culture medium. The level of detectable enzyme activity correlated well with the amount of glutamine synthetase protein present in the cultures. This finding prompted us to compare the release of glutamine synthetase by *M. tuberculosis* with its release by other mycobacterial species and both Gram-negative and Gram-positive bacteria (Table 3). Of the bacteria studied, all mycobacterial species exhibited more total glutamine synthetase activity than the Gram-negative and Gram-positive microorganisms. Most strikingly, only the pathogenic mycobacteria *M. tuberculosis* and *M. bovis* released substantial amounts of the enzyme into the culture medium. A ratio of 1:2–4 for the released/cell-associated enzyme activity was consistently found for these pathogenic species. The nonpathogenic mycobacteria *M. phlei* and *M. smegmatis* released <1% of the enzyme units released by the pathogenic mycobacteria, and the ratio of released to cell-associated enzyme activity was <1:100. *E. coli*, *L. pneumophila*, *B. cereus*, and *B. subtilis* did not release any detectable enzyme activity into the culture medium under the conditions studied. Culturing the bacteria in 7H9 medium at pH 7.5 instead of 6.7, or in Sauton's medium instead of 7H9, did not alter these results.

Analysis of Substrates and Products of the Biosynthetic Reaction. The extracellular release of glutamine synthetase by pathogenic mycobacteria prompted an analysis of the enzyme's substrates (L-glutamate, ammonia, and ATP) and products (L-glutamine) in the culture medium. Ammonia was determined over a 4-week growth period. Initially present as ammonium sulfate at 3.8 mM in 7H9 medium, the ammonia concentration increased to almost 10 mM during the first 2 weeks of culture and leveled off. The detectable glutamine synthetase activity correlated inversely with the ammonia

Table 2. N-terminal sequences of various glutamine synthetases

Species	Sequence
M. tuberculosis	TEKTPDDVFKLAKDEKVLYL
M. smegmatis	AEKTSDDIFKLIKDENVEYV
M. phlei	AEKTADDILKLIRDEDVEYG
B. subtilis	AKYTREDIVKLVKEENVKYI
E. coli	SAEHVLTMLNEHEVKFVDLR

The *M. tuberculosis* N-terminal glutamine synthetase sequence was determined after transfer to poly(vinylidene difluoride) membrane. All other glutamine synthetase sequences were reported earlier (21).

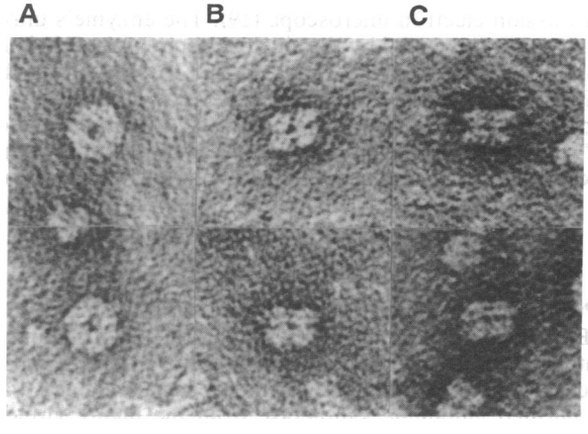

FIG. 2. Ultrastructure of glutamine synthetase. Glutamine synthetase was adsorbed to a carbon film and negatively stained with 2% uranyl acetate. The three characteristic orientations are shown: hexagonal or ring form (*A*), side view with four spots in a rectangular array (*B*), and side view with two parallel strips (*C*). (×536,000.)

Table 3. Extracellular release of glutamine synthetase by various microorganisms

Bacteria	Activity*		Ratio B/A
	Cell extract (A)	Culture supernatant (B)	
M. tuberculosis			
Erdman	63.7	28.7	1:2.2
H37Rv	42.3	18.1	1:2.3
H37Ra	36.5	16.4	1:2.2
M. bovis BCG	49.6	11.4	1:4.3
M. smegmatis	6.3	0.06	1:105
M. phlei	6.9	0.06	1:115
E. coli DH5α	2.1	≤0.01	≤1:210
L. pneumophila	2.1	≤0.01	≤1:210
B. subtilis	2.8	≤0.01	≤1:280
B. cereus	1.6	≤0.01	≤1:160

Enzyme activity (milliunits per 10^8 cells) by transfer assay in bacterial cell extract (A) and cell-free culture supernatant (B).

concentration in the medium. Increasing the ammonium sulfate concentration to 38 mM led to a 10-fold reduction in detectable enzyme activity and glutamine synthetase protein.

The presence of ATP in the culture medium was verified by the addition of apyrase to the supernatant fluid of a 3-week *M. tuberculosis* Erdman culture. A significant increase in the medium's phosphate concentration, from 1.07 ± 0.04 mM to 1.24 ± 0.03 mM, was observed, indicating an ATP concentration of 170 μM. A comparable concentration of 150 μM ATP was also demonstrated by several luciferase assays.

The presence of L-glutamine was investigated in culture filtrates of all the bacterial species examined in Table 3. The presence of glutamine synthetase in culture filtrates correlated well with the presence of a readily identifiable L-glutamine spot on TLC plates (Fig. 3), clearly distinguishing pathogenic mycobacteria from nonpathogenic mycobacteria and all other microorganisms examined. L-Glutamine was present at ≈500 ng/ml (3.4 μM) in culture supernatant of *M. tuberculosis*. L-Glutamate, initially present at 500 μg/ml (3.4 mM) in 7H9 medium, declined to ≈10 μg/ml (68 μM) in *M. tuberculosis* culture supernatant.

Immunogenicity of Glutamine Synthetase in Infection. The antigenicity of glutamine synthetase in mycobacterial infection was assessed by measuring antibody to the enzyme in sera from guinea pigs infected by aerosol with *M. tuberculosis*. Infected animals but not uninfected animals demonstrated a strong antibody response to purified glutamine synthetase on immunoblots. The reciprocal titer of these antibodies was ≈1000 for infected animals versus ≤50 for control animals.

Immunolocalization. Glutamine synthetase was localized in infected human monocytes by the cryosection immunogold technique. Abundant specific staining for mycobacterial glutamine synthetase was observed both within the mycobacteria and extracellularly in the phagosomal space (Fig. 4). Negligible immunogold staining for glutamine synthetase was observed in the host cell cytoplasm or in sections stained with control rabbit antibody, confirming the specificity of the anti-glutamine synthetase antibody. The ratio of released to cell-associated enzyme in *M. tuberculosis* phagosomes was ≈1:3 [2.2 ± 0.2 (mean ± SD) gold particles in phagosomal space versus 6.3 ± 1.2 gold particles inside bacteria], similar to that in broth cultures.

DISCUSSION

Glutamine synthetase purified from *M. tuberculosis* to apparent homogeneity was characterized as a member of the family of bacterial glutamine synthetases. The enzyme's N-terminal 20 amino acids align with the N termini of other well-characterized glutamine synthetases (21). The highest degree of homology, >80% for the N-terminal 20 amino acids, is to the *M. smegmatis* enzyme. The homology with the *E. coli* enzyme drops to ≈40% (21).

Glutamine synthetases characterized to date possess an oligomeric structure. Our data indicate that the *M. tuberculosis* enzyme is composed of 12 subunits of $M_r \approx 58{,}000$, yielding a native protein of $M_r \approx 680{,}000$. The homogeneity of the M_r 58,000 molecule by gel electrophoresis and amino acid sequence analysis suggests that all 12 subunits are identical. Electron microscopy and X-ray crystallography of the *E. coli* and *Salmonella typhimurium* glutamine synthetase enzymes support a structural model of two overlying planes, each combining 6 subunits in the form of a hexagon (20, 23). Our electron microscopy data on the *M. tuberculosis* enzyme are consistent with this concept.

The activity of characterized glutamine synthetases requires the presence of metal ions (24). Removal of divalent cations by EDTA inactivates the enzyme and, in conjunction with denaturants such as urea, leads to dissociation of the native molecule to subunits (12, 13). Bacterial enzymes have been reported to be further regulated by ammonia, modification of subunits by AMP, and a variety of metabolites acting as feedback inhibitors (12, 23), although the *B. subtilis* enzyme, for example, lacks the regulation by adenylylation (22). Our study shows that the *M. tuberculosis* enzyme is also easily inactivated by EDTA. In addition, the *M. tuberculosis*

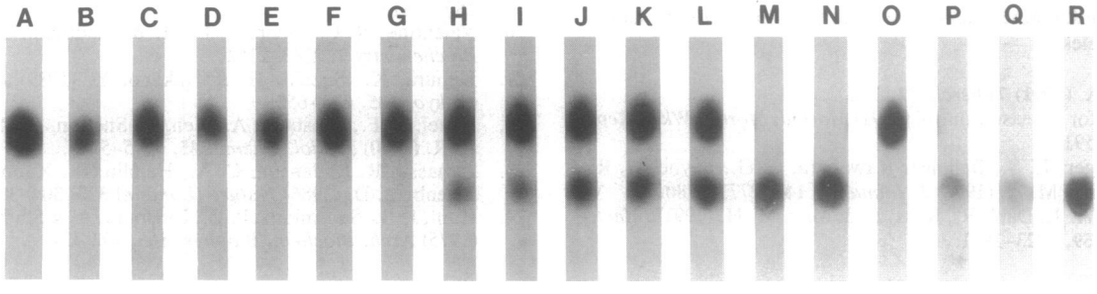

FIG. 3. Detection of extracellular L-glutamine in cultures of *M. tuberculosis* and other bacteria by TLC. Lanes: A, 5 μg of L-glutamic acid (7H9 medium); B, *E. coli* DH5α; C, *L. pneumophila*; D, *B. cereus*; E, *B. subtilis*; F, *M. phlei*; G, *M. smegmatis*; H, *M. bovis*; I, *M. bovis* BCG; J, *M. tuberculosis* H37Ra; K, *M. tuberculosis* H37Rv; L, *M. tuberculosis* Erdman; M, *M. tuberculosis* Erdman (Sauton's medium with L-asparagine); N, *M. tuberculosis* Erdman (Sauton's medium with L-glutamate); O, *M. tuberculosis* Erdman (7H9 medium, 38 mM ammonium sulfate); P, *M. tuberculosis* Erdman (Sauton's medium with 38 mM ammonium sulfate); Q, 5 μg of L-asparagine; R, 5 μg of L-glutamine. L-Glutamic acid (upper spot) and L-glutamine (lower spot) stain red and appear dark. L-Asparagine migrates the same distance as L-glutamine; it stained yellow on the original chromatograph but appears light on this reproduction (lanes P and Q). Both L-glutamine and L-asparagine are present in lane M, giving a spot with a red (dark) center and a yellow (light) halo on the original chromatograph; the halo is not visible on the reproduction.

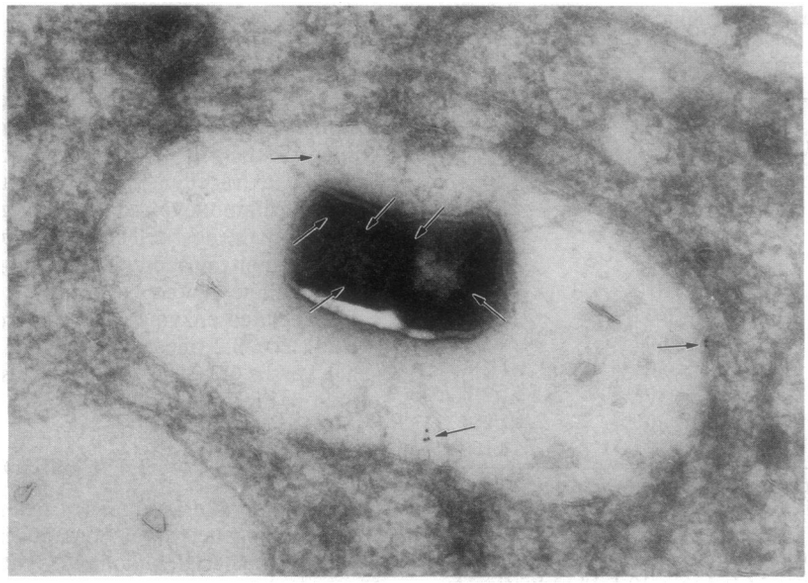

FIG. 4. Immunolocalization of *M. tuberculosis* glutamine synthetase in infected human monocytes using ultracryomicroscopy. Staining for *M. tuberculosis* glutamine synthetase with 10 nm immunogold particles (arrows) is present within the bacteria and outside the bacteria in the phagosomal space. (×30,000.)

glutamine synthetase shows an inverse relationship between the ammonia concentration in the culture medium and detectable enzyme activity.

Our study suggests two potentially important roles for glutamine synthetase in the pathogenesis of *M. tuberculosis* infection. First, the enzyme's release into the growth medium and the presence there of all its substrates and reaction products indicate that the extracellular enzyme catalyzes the synthesis of glutamine, a major cell wall component of only pathogenic mycobacteria (8). How the poly(L-glutamate/glutamine) heteropolymer is synthesized and attached to the cell wall is not clear. Second, the enzyme's involvement in nitrogen metabolism and ammonia production may contribute to the capacity of *M. tuberculosis* to inhibit phagosome acidification in infected monocytes and phagosome–lysosome fusion (4, 5, 7).

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1. Kochi, A. (1991) *Tubercle* **72,** 1–6.
2. Centers for Disease Control (1991) *Morbid. Mortal. Wkly. Rep.* **40,** 585–591.
3. Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R. & Horwitz, M. A. (1990) *J. Immunol.* **144,** 2771–2780.
4. Crowle, A. J., Dahl, R., Ross, E. & May, M. H. (1991) *Infect. Immun.* **59,** 1823–1831.
5. Armstrong, J. A. & D'Arcy Hart, P. (1971) *J. Exp. Med.* **134,** 713–740.
6. Magasanik, B. (1977) *Trends Biochem. Sci.* **2,** 9–12.
7. Gordon, A. H., D'Arcy Hart, P. & Young, M. R. (1980) *Nature (London)* **286,** 79–80.
8. Hirschfield, G. R., McNeil, M. & Brennan, P. J. (1990) *J. Bacteriol.* **172,** 1005–1013.
9. Hanahan, D. (1983) *J. Mol. Biol.* **166,** 557–580.
10. Mengaud, J. M. & Horwitz, M. A. (1993) *J. Bacteriol.* **175,** 5666–5676.
11. Eidus, L. & Hamilton, E. J. (1963) *Am. Rev. Resp. Dis.* **90,** 258–260.
12. Woolfolk, C. A., Shapiro, B. & Stadtman, E. R. (1966) *Arch. Biochem. Biophys.* **116,** 177–192.
13. Shapiro, B. M. & Ginsburg, A. (1968) *Biochemistry* **7,** 2153–2167.
14. Manning, J. M., Moore, S., Rowe, W. B. & Meister, A. (1969) *Biochemistry* **8,** 2681–2685.
15. Lei, M., Aebi, U., Heidner, E. G. & Eisenberg, D. (1979) *J. Biol. Chem.* **254,** 3129–3134.
16. Sugawara, K. & Oyama, F. (1981) *J. Biochem. (Tokyo)* **89,** 771–774.
17. Allison, A. C. & Byars, N. E. (1986) *J. Immunol. Methods* **95,** 157–168.
18. Pal, P. G. & Horwitz, M. A. (1992) *Infect. Immun.* **60,** 4781–4792.
19. Clemens, D. L. & Horwitz, M. A. (1993) *Infect. Immun.* **61,** 2803–2812.
20. Valentine, R. C., Shapiro, B. M. & Stadtman, E. R. (1968) *Biochemistry* **7,** 2143–2152.
21. Kimura, K., Suzuki, H. & Nakano, Y. (1989) *J. Biochem. (Tokyo)* **105,** 648–652.
22. Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E. & Stadtman, E. R. (1970) *J. Biol. Chem.* **245,** 5195–5205.
23. Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N.-H. & Eisenberg, D. (1986) *Nature (London)* **323,** 304–309.
24. Hunt, J. B., Smyrniotis, P. Z., Ginsburg, A. & Stadtman, E. R. (1975) *Arch. Biochem. Biophys.* **166,** 102–124.