Molecular Definition and Identification of New Proteins of *Mycobacterium leprae*

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This report describes N-terminal group analysis of six new proteins isolated from in vivo-grown *Mycobacterium leprae*, three of which correspond to products of the *cysA*, *ahpC*, and *rpIL* genes, which were recently defined through the *M. leprae* genome project and which encode a putative sulfate sulfurtransferase, an antioxidant enzyme, and the L7/L12 ribosomal protein, respectively.

Intracellular pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, live preferentially inside mononuclear phagocytes, and their success as intracellular pathogens resides in their ability to modulate the host cell environment to their own advantage. To understand better the molecular basis of leprosy pathogenesis, we have, in the past, identified many of the major constituents of armadillo-derived *M. leprae* (12, 18–20). This approach was undertaken in the belief that proteins expressed by the pathogen in vivo would reflect its lifestyle as well as the host cell environment, thereby allowing a better understanding of the mycobacterium-host cell relationship. The data accumulated so far point to the overexpression by *M. leprae* of proteins involved in protection against the hostile environment in macrophages. Abundant proteins are the chaperonin pair GroEL-GroES, the SodA enzyme, and a small, 18-kDa heat shock protein (12, 20, 25). More recently, a bacterioferritin probably involved in iron sequestration was characterized (19). Further substantial developments in understanding the molecular biology of *M. leprae* and its obligate intracellular parasitism will probably arise from the genome project initiated 5 years ago (1, 7, 11). Complementary to the genome project and in an attempt to define the spectrum of proteins more abundantly expressed by this obligate host-derived pathogen, the present study was initiated. The strategy involved generating N-terminal amino acid sequences of the major proteins, resolved by one- or two-dimensional gel electrophoresis, to allow correlation with DNA sequences emerging from whole-genome sequencing.

M. leprae was purified from irradiated armadillo spleens and livers by the Draper protocol (28). Buffered-water and phosphate-buffered saline (PBS)-EDTA extracts, as well as the soluble or cytosolic fraction, were prepared from thawed bacteria (300 mg [dry weight]) as described previously (18). Mycobacterial lipoglycans and glycopospholipids were removed from the soluble or cytosolic fraction by fractionation on DEAE-Sephacel equilibrated in 20 mM piperazine buffer, pH 5.7. After the column was washed with 160 mM LiClO₄, proteins were eluted in bulk with 1 M LiClO₄. These cytosolic proteins were further resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 to 20% gradient gels or by two-dimensional gel electrophoresis on ProteanII (Bio-Rad Laboratories) and subjected to Western blotting (immunoblotting) as described previously (18). Alternatively, after

electrophoresis, proteins were electrotransferred onto Immobilon-P^{SO} polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) (16), visualized with Coomassie blue, excised, and subjected to automated Edman degradation or a gas-phase sequencer as previously described (10) (work was conducted by Stanley A. Hefta, Beckman Institute of the City of Hope, Duarte, Calif.). Protein sequences were analyzed in the GenBank (release 87) and Swissprot (release 30) data banks with the Genetics Computer Group software package.

By loading small quantities of protein from these extracts (40 µg per lane) and pooling material from three Coomassie blue-stained polyvinylidene difluoride membranes, we obtained sufficient amounts of three new proteins, of moderate concentrations in these extracts, of 85 to 90 kDa (PI), 66 kDa (PII), and 34 kDa (PIII) to allow limited sequencing (Fig. 1). The cytosolic proteins of *M. leprae* were resolved by two-dimensional gel electrophoresis, stained with silver, and, in parallel, subjected to Western blotting against a collection of monoclonal antibodies with known specificities (8, 14) (Fig. 2). In this way, the amino acid sequences of a further three new proteins, PIV, PV, and PVI (23, 18, and 15 kDa, respectively), were obtained by excising Coomassie blue-stained spots from three such two-dimensional gels and pooling them (Fig. 2). As internal controls, the well-studied small, 18-kDa heat shock protein (2), the SodA homolog (24), and the bacterioferritin (19) were excised, sequenced, and shown to have sequences perfectly homologous to those described in the literature (Fig. 2).

No significant homologies were found in the GenBank and Swissprot sequence data banks when sequences of PI, PII, and PV were used as probes. On the other hand, the genes coding for the other three new proteins had already been sequenced by the genome project. The first publication of the sequence of the *M. leprae* genome reported the nucleotide sequence of the entire cosmid clone carrying the Rif-Str region, in which the *rpIL1* gene for the L7/L12 ribosomal protein is located (11). This information allowed the recognition of PVI as the L7/L12 homolog and its complete definition. Another protein, PIII, corresponded to the product of a *M. leprae* gene designated *cysA* (cosmid B2266, GenBank accession number U15182) because of its high level of similarity with the *cysA* gene of *Saccharopolyspora erythraea*, which codes for a putative thio-sulfate sulfurtransferase involved in sulfur assimilation and cysteine biosynthesis (6). As shown in Fig. 3, both *M. leprae* CysA and *Saccharopolyspora erythraea* CysA have significant homology with the human liver rhodanese (17). It is of interest that the active site Cys-247 of rhodanese (26) is conserved in both CysA proteins. Additionally, the *M. leprae* CysA polypeptide showed significant homology to another rhodanese-like protein of

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FIG. 1. Identification of new proteins of *M. leprae*. One-dimensional SDS-PAGE of the buffered-water extract of whole *M. leprae* (lane a), the PBS-EDTA extract of whole *M. leprae* (lane b), and the soluble or cytosolic fraction of disrupted *M. leprae* (lane c) is shown. Gels were stained with silver. The three new proteins (PI, PII, and PIII) present in the extracts of undisrupted bacteria (lanes a and b), but apparently not present in the cytosolic fraction (lane c), and their N-terminal sequences are indicated. X, residue not identified. Lane kDa, molecular mass markers.

a *Synechococcus* sp. located in the periplasmic space, which accumulates specifically under sulfur-limiting conditions (15).

The PIV protein is the product of an *M. leprae* gene designated *aphC* (cosmid B38, GenBank accession number L01095) because of its similarity with the C22 subunit of the alkyl hydroperoxide reductase (AhpC) from *Salmonella typhimurium*, a detoxifying enzyme that reduces organic hydroperoxides to their corresponding alcohols (23). The PIV gene sequence allowed the confirmation of the presence of a Pro instead of a Glu residue at position 10 of the N-terminal amino acid sequence.

Genes homologous to the *M. leprae* *ahpC* gene have recently been found to be widespread among mycobacterial species,

including *M. tuberculosis* (5, 21, 27). AhpC proteins from mycobacteria show even greater similarity to members of a family of thiol-specific antioxidant enzymes widely distributed in living kingdoms. It has been demonstrated that several proteins of the thiol-specific antioxidant-AhpC family are overexpressed when cells are exposed to oxidative stress (27). As in gram-negative bacteria (4, 22), AhpC proteins from most mycobacteria seem to be under the control of a positive regulatory gene, *oxyR*, located directly adjacent to the *ahpC* gene but transcribed in the opposite direction (5, 21). On the basis of these results, two of these proteins can be postulated to act as self-protection proteins to allow the leprosy bacillus to persist in macrophages. The sequence of PIII points to a rhodanese-like enzyme, and rhodanese has recently been implicated in the formation of iron-sulfur centers (3). Oxidation of iron-sulfur centers by nitrogen- and oxygen-reactive species represents a major mechanism of parasite damage by macrophages (9, 13). Therefore, the putative involvement of CysA in replacing damaged centers and perhaps in the overproduction of iron-sulfur centers can be seen as a survival mechanism for *M. leprae* in the oxidative environment provided by macrophages. On the other hand, PIV is the product of the *M. leprae* *ahpC* gene and is a member of the AhpC-thiol-specific family of enzymes with antioxidant activities. It has recently been demonstrated that *Mycobacterium avium* AhpC is readily induced when the bacterium is submitted to oxidative stress (21). In the case of *M. leprae*, data from our laboratory indicate that AhpC is present in considerable amounts among the soluble and cytosolic proteins and in the envelope fractions of the bacillus. Therefore, the *M. leprae* AhpC homolog, probably as an antioxidant enzyme, may play a key role in the survival of *M. leprae* in the midst of high concentrations of oxygen-reactive species produced by macrophages.

FIG. 2. Two-dimensional SDS-PAGE map of the soluble and cytosolic proteins of *M. leprae*. Proteins devoid of most of the soluble carbohydrates were fractionated on 12 to 20% polyacrylamide gradient gels and stained with AgNO_3 . The trivial names of known proteins are indicated in parentheses. The 71-, 65-, 35-, and 10-kDa proteins were identified with specific monoclonal antibodies as probes in a Western blot analysis. The N-terminal amino acid sequences determined for three other known proteins (the 28-kDa SodA, the 22-kDa MMP-II, and the 18-kDa heat shock protein [hsp]) and three more new proteins (PIV, PV, and PVI) are indicated. In the inset, a sample more thoroughly depleted of polyglycans permits a better visualization of the proteins recognized in that area of the gel. X, residue not identified.

human rhodanese	KASPQLCRALVSAQWVAEALRAPRAGQPLQLLDASY YLPQLGRDACQPEERHIFGAAPFDIIGQCSID	68
lep CysA	MARSDV --LVSAADWARSNLDS-----ANIVFVEVDEDTSTYDGGHIAGA--IKLDWRAD	50
<i>S. erythraea</i> CysA	MSREEV--LVSTDWABQNLT-----DGVVIAEVDDEDTAYUGGHIAGA--IKLDWKNE	50
human rhodanese	RTSPYDHMLPGARHPABYAGKLGVGGAATHVVIYDASDQGLYSAPRVWWMFRAFGHHAVSLDGGGLR	132
lep CysA	LQDFIKRDFIDTQCFKILGDRGISNDNTVILYGCNNN WFAAYAYWPKLYRHDKVKLLCGGRK	114
<i>S. erythraea</i> CysA	LQDHVRRDFVNRGFEKLLSAGKIGNDCTVILYGCNNN--WFAAYAYWPKLYGHSEVKLLDGGGRK	114
human rhodanese	HWLRQNLFLSSGKSQAPABFRAQLDPAFIKTYEIKENLESRRF-QVDSRATGRFRGTEPEP--	195
lep CysA	KWELDGRFLSTDVTRFATSYAAAPDNTIRAFRD--EVIASIKIKKLVVRSFDFPSGKLLAPAH	178
<i>S. erythraea</i> CysA	KWELDRELTKKEPXAATAYKAQEPDASIRAFRD--EVVDAIGNKLVVRSFDFPSGKLLAPAH	178
human rhodanese	---RDGIEPGHIGTWNIPFTDFLSQBLEKSPSEIRHLFQKXKVDLSKPLVATCGSGVTACHVAL	248
lep CysA	LPQEQSQPGHIGSAINIPWSKAANEDCTFKSDEQLAKLYADAGLDRLKETIVYCRIGERSSETWF	244
<i>S. erythraea</i> CysA	LPQESAQRAGHIPSAINIPWSKAANEDGTFKSEELKQVYGEAGLTDKDTIAYCRIGERSSETWF	244
human rhodanese	GA YLGGKPDVPIYDGSWVWV-YMRARFEDVIS	289
lep CysA	VLRELLGYQNVKNYDGSWTEYGSVGVPIELGS	277
<i>S. erythraea</i> CysA	VLRELLGHINVKNYDGSWTEYGSVGVPIE	274

FIG. 3. Comparison of the sequence of *M. leprae* (lep) CysA with those of CysA from *Saccharopolyspora erythraea* (6) (GenBank accession number M29612) and human rhodanese (17) (Swissprot accession number P25325). The active Cys-247 is marked with an asterisk (*). Amino acid identities (!) and conservative substitutions (:) are noted.

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