A Magnetosome-specific GTPase from the Magnetic Bacterium *Magnetospirillum magneticum* AMB-1*

Yoshiko Okamura, Haruko Takeyama, and Tadashi Matsunaga‡

From the Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

Magnetic bacteria produce intracellular vesicles that envelope single domain magnetite crystals. Although many proteins are present in this intracellular vesicle membrane, five are specific to this membrane. A 16-kDa protein, designated Mms16, is the most abundant of the magnetosome-specific proteins, and to establish its function we cloned and sequenced its gene from *Magnetospirillum magneticum* AMB-1. This was achieved by determination of the N-terminal amino acid sequence of the protein following two dimensional polyacrylamide gel electrophoresis, and sequencing of the gene was performed by gene walking using anchored polymerase chain reaction. Mms16 contains a putative ATP/GTP binding motif (P-loop). Recombinant Mms16 with a hemagglutinin tag, was expressed in *Escherichia coli* and purified. Recombinant Mms16 protein could bind GTP and showed GTPase activity. GTP was the preferred substrate for Mms16-catalyzed nucleotide triphosphate hydrolysis. These results suggest that a novel protein specifically localized on the magnetic particle membrane, Mms16, is a GTPase. Mms16 protein showed similar characteristics to small GTPases involved in the formation of intracellular vesicles. Furthermore, addition of the GTPase inhibitor AlF₄⁻ also inhibited magnetic particle synthesis, suggesting that GTPase is required for magnetic particles synthesis.

*Magnetospirillum magneticum* AMB-1 contains a 16-kDa protein, designated Mms16, which is the most abundant of the five BMP-specific proteins. In this work, we therefore cloned, sequenced, and partially characterized this 16-kDa protein. AlF₄⁻ inhibited magnetic particle synthesis, suggesting that GTPase is required for magnetic particles synthesis.

Received for publication, July 9, 2001, and in revised form, September 6, 2001
Published, JBC Papers in Press, September 13, 2001, DOI 10.1074/jbc.M106408200

*This work was funded in part by Grant in Aid for Scientific Research on Priority Area (A) 10145102 and Specially Promoted Research 13002005 from the Ministry of Education, Culture, Science, Sports and Technology of Japan. It was also supported by the New Energy and Industrial Technology Development Organization’s Proposal-based Advanced Industrial Technology Research and Development Program 1413. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB051019.

† To whom correspondence should be addressed: Dept. of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-Chō, Koganei, Tokyo 184-8588, Japan. Tel.: 81-423-88-7020; Fax: 81-423-85-7713; E-mail: tmsunaa@cc.tuat.ac.jp.

‡ The abbreviations used are: BMP, bacterial magnetic particle; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; bp, base pair(s); HA, hemagglutinin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ARF, ribosylation factor.

Abstract

Magnetic bacteria synthesize intracellular magnetite particles that are aligned in chains of around 10 particles per cell. They are covered with a thin lipid membrane and have an average diameter of 50–100 nm. The morphology of these bacterial magnetic particles (BMPs) is species-dependent and it is reasonable to hypothesize that species-specific biological factors located in the BMP membrane mediate magnetite crystallization (1). However, the mechanism of BMP synthesis, including vesicle formation, still remains unclear. A complete understanding of biomineralization and of membrane vesicle formation at the molecular level will also have important implications for studying biomineralization in general and vesicle formation in prokaryotes in particular.

Several processes are involved in BMP synthesis, and one of the most important is vesicle formation. Numerous studies have been devoted to the investigation of eukaryotic intracellular vesicle formation, and hence the molecular machinery is well understood (for reviews, see Refs. 2 and 3). In contrast, there are few molecular studies of vesicle formation or of the events leading to invagination of the cytoplasmic membrane in prokaryotes. We have hypothesized that the BMP membrane is derived from the cytoplasmic membrane and formed through the invagination of the cytoplasmic membrane by a process similar to that which occurs in eukaryotes (4).

A second process in BMP synthesis is magnetite crystallization, and this has been studied in more detail. It appears that ferric iron is reduced on the cell surface, taken into the cytoplasm, transferred into vesicles (magnetosomes), and finally oxidized to produce magnetite (5). Several proteins appear to be required for magnetite crystallization, and the first of these reported, *magA*, was cloned from a non-magnetic mutant obtained by transposon mutagenesis of *Magnetospirillum magnetum* AMB-1 (6). Internal localization analysis of the MagA protein using a *MagA*-luciferase fusion protein indicated that MagA is localized on the BMP membrane where it transports iron into the BMP vesicles (6, 7). Thus magnetosomal membrane proteins play an important role in magnetite crystal formation.

Other proteins on the BMP membrane have been partially characterized. Gorby *et al.* observed two unique proteins in the BMP membrane fraction from *M. magnetotacticum* MS-1 (8). Okuda *et al.* identified three additional specific proteins in the BMP membrane of MS-1, and they determined the DNA/amino acid sequence of a 22-kDa protein (9). However, since gene transfer and its use in studying gene expression has not been successful in strain MS-1, the function of these proteins is still unknown. In strain AMB-1, three BMP membrane-specific proteins were identified and partially characterized. One of these, MpsA, which shows homology with acetyl-CoA carboxylase (transferase), containing acyl-CoA binding motif (4). Recently, we have found two additional BMP-specific proteins, a 12-kDa and a 16-kDa protein (10) bringing the total number in strain AMB-1 to five (Table I). The 16-kDa protein was found to be the most abundantly expressed of the five BMP-specific proteins. In this work, we therefore cloned, sequenced, and partially characterized this 16-kDa protein. Although the MagA protein is important for BMP formation, it is not specific to the BMP membrane but is also present on the
cytoplasmic membrane (7). However, the 16-kDa protein is confirmed as a specific protein expressed on the BMP membrane.\(^2\) Thus, this is the first report of the experimental function of BMP-specific protein. We conclude that the 16-kDa protein is a GTPase with properties similar to eukaryotic small GTPases that control vesicle trafficking. Furthermore, inhibition of GTPase by aluminum fluoride prevents BMP synthesis suggesting that GTPase activity is required for BMP synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—[\(\alpha\text{-}^{32}\text{P}]\)GTP, [\(\gamma\text{-}^{32}\text{P}]\)GTP, and [\(\text{S}\text{S}\)]GTP\(\gamma\)S were purchased from PerkinElmer Life Sciences prodios, Inc. ATP, CTP, GTP, UTP, ADP, and GDP were obtained from Sigma. G-proteins, bovine brain as standard, were purchased from Calbiochem.

**Strains and Growth Conditions**—Escherichia coli DH5a was used for gene cloning and protein expression. *E. coli* was cultured in Luria broth at 37 °C after adding the appropriate antibiotics. *M. magneticum* AMB-1 (ATCC 700264) (12) was grown anaerobically at 26 °C after adding the appropriate antibiotics. The gene for the 16-kDa protein with a hemagglutinin (HA) tag was amplified against AMB-1 genomic DNA using forward primers (20), the gene for the 16-kDa protein with a hemagglutinin (HA) tag with reverse primers with HA tag with EMBL DNA data bases.

**Cloning and Sequencing of a Gene Encoding a 16-kDa Protein**—The BMP membrane protein sample was prepared from 10° cells according to the previously described (10). The 16-kDa protein from 200 μg of protein sample was isolated by two dimensional polyacrylamide gel electrophoresis (PAGE) using automatic electrophoresis, TEP-2 unit (Shimadzu, Kyoto, Japan) according to the procedure described by Nokihara et al. (14). Cloning was further analyzed by performing homology searches using the EMBL DNA data bases.

**RESULTS**

**Isolation of the Gene Encoding Mms16 and Its Sequence Analysis**—58 amino acid residues from the N-terminal sequence of the 16-kDa protein, designated Mms16 (magnetic particle membrane-specific protein), were determined (Fig. IA). Primers (S1 primer: 5’-CATAAGCAGACGCCAGCGACGTTCTC

---

\(^2\) M. Takahashi, Y. Okamura, H. Takeyama, and T. Mastunaga, manuscript in preparation.
Magnetosome-specific GTPase 48185

Overexpression of Mms16-HA Tag Fusion Protein—Overexpression of Mms16-HA tag fusion protein in E. coli was performed. Although remarkable induction of Mms16-HA tag fusion protein in transformant was not observed at the existence of IPTG, a specific band was observed as shown in Fig. 2A, lane 4 (indicated by arrow) after beads purification, and it was confirmed to be the Mms16-HA tag fusion protein by Western blots (Fig. 2B). The bands of H and L chain were derived from Sepharose beads on which anti-HA antibody was immobilized. The purified Mms16-HA tag fusion protein was used for GTPase assay.

Activity of GTP Binding and GTP Hydrolysis of Mms16—Fig. 3A shows GTP cross-linking to the Mms16-HA tag fusion protein. The Mms16-HA tag fusion protein from transformant, which was confirmed by Western blot (Fig. 3A, a), cross-linked with [$^{32}$P]GTP/S (Fig. 3A, b). The ability of the purified protein to convert [α-32P]GTP to [α-32P]GDP is shown in Fig. 3B where Mms16-catalyzed GTP hydrolysis occurred. Changes in amount of GTP hydrolysis increased depending on time (Fig. 3C). For biochemical characterization of the isolated Mms16, the activity of GTP hydrolysis was measured under varied GTP concentrations from 1 μM to 20 μM. Results showed that activity increased with increasing GTP concentration dependently (Fig. 4). These results indicate that Mms16 is a GTPase.

The specificity of Mms16 GTPase activity was tested by adding nucleotide competitors (Fig. 5). Excess unlabeled GTP could reduce Mms16 GTPase activity, whereas remarkable reduction of activity was not observed in the presence of other NTPs (CTP, ATP, UTP, and ITP). On the other hand, GDP could inhibit transport on an in vitro system that reconstitutes vesicular transport (29, 30). To investigate the relationship between GTPase and BMP synthesis, increasing concentrations of AlCl$_3$ with 5 mM NaF were added to AMB-1 culture. Although the AlF$_4$ complex did not inhibit growth, cells lost magnetism as the concentration of AlF$_4$ increased (Table II). Observation by electron microscopy reveals that cells grown with AlF$_4$ contained interrupted chains of BMPs (Fig. 6B). The number of BMP decreased com-
pared with that of normal condition cell. Thus, magnetism of cells becomes weaker following the increased concentration of AlF4/H11002. These results suggest, at least, that GTPase activity is required for BMP synthesis.

DISCUSSION

This work reports the functional analysis of a magnetosome-specific protein from a magnetic bacterium for the first time. Mms16 protein, identified as the most abundant protein in magnetosomes, contains a putative P-loop sequence; however, this putative P-loop sequence has only three residues of any combination of four between Gly and Gly-Lys, and it contains only one motif of the four conserved sequence motifs in the GTPase superfamily (31). Despite these characteristics, Mms16 protein shows GTPase activity. The Ras, Rho, Rab, and Ran of small GTPase family were also observed to have spontaneous GTPase activity (32). These results suggest that the isolated Mms16 is similar to these GTPases. For example, the intrinsic GTP dissociation rates of Ras are about 10^5 times lower than in Ras in the presence of an exchange factor (33). Thus, interactions of other proteins would be required for binding to the membrane, GTP/GDP exchange, or stimulation of the activities in situ similar to that in eukaryotic cells. Probably, this Mms16 is also controlled by some factors in vivo, hence GTPase is expected to act in much lower levels of GTP concentration.
The Mms16 was localized in the cytoplasm of *E. coli* but was strongly associated with the BMP membrane since it was not removed when subjected to over 20 times stringent washing with sonication. Therefore, it appears to be directly associated with the membrane. Motif analysis of Mms16 indicated that it contained a myristoylation site; however, the site is not at the N terminus. ARF, one of the family of mammalian small GTPases, is anchored in the membrane by myristoylation of Gly at the N terminus (34). From observation of such a membrane-bound form and GTP hydrolysis activity, the Mms16 has similar properties to eukaryotic small GTPases. Therefore, it is anticipated that its function may also be similar.

It does not seem to be similar to other prokaryotic GTP-binding proteins that have also been widely studied and fall into five groups; (i) initiation factor (IF-2), elongation factor (EF-Tu) (35), (ii) cytokinesis protein PtsZ, which forms a ring at the leading edge of the cell division site (36) and is a GTPase that has significant structural similarity to tubulin (37), (iii) signal recognition particle (SRP) and SRP receptor α subunit (38), (iv) Era protein, which encodes an essential protein (39–41) that may play a role in DNA replication or chromosome partitioning in *Bacillus subtilis* (44, 45). Although its precise cellular function is unknown, Era and Obg have been implicated in a wide array of cellular functions.

Eukaryotic cells have an elaborate network of organelles, many of which are in constant and bi-directional communication through a flow of small transport vesicles. The small transport vesicles that mediate membrane trafficking between intracellular organelles are encased in a protein coat. The small GTPase family is involved in the priming and budding of trafficking vesicles. Well studied examples of vesicle formation include the Golgi-derived COP (coat protein)-coated vesicles (46, 47). In events leading to the formation of COP-coated vesicles, N-myristoylated ARF (48) anchored in the membrane is recruited concomitantly with GTP/GDP exchange at the Golgi surface (49) where it triggers assembly of the coatamer (50, 51). Coat assembly drives vesicle budding (52). GTP hydrolysis is required to release the coatamer complex and ARF from the vesicles (46, 47).

In several bacteria, intracellular membranal structures clearly originate from the cytoplasmic membrane. Although the origin of the thylakoid membrane of cyanobacteria are not known, in most phototrophic bacteria, intracytoplasmic membranes that are invaginations of the cytoplasmic membrane, are the site of the photosynthetic apparatus (53). Also chemolithotrophic, nitrifying bacteria possess complex arrangements of internal membranes (54). Spheroplasts of *Nitrosomonas* do not contain internal vesicles (55). This suggests that internal membranes of those organisms are invaginations. Methanotrpha generally contain intracytoplasmic membranes (56), which appear to be involved in the methane oxidation pathway. *Azotobacter* sp. BH72 is a chemoheterotroph, and their intracytoplasmic membranes, related to nitrogen fixation, often appear as stacks of flattened vesicles similar to those of many phototrophic bacteria (57). Surprisingly, the process of intracytoplasmic membrane formation is poorly understood in prokaryotes and no genes involved in this process have yet been isolated.

In magnetic bacteria, the origin of membrane vesicles that envelope the magnetite particles is unknown. We can hypothesize that magnetosomes arise through invagination of the cytoplasmic membrane. The BMP specific GTPase may also be involved in vesicle formation through a process similar to that which occurs in eukaryotic vesicle formation. Inhibition of GTPase activity by AlF₄⁻ causes inhibition of BMP synthesis, suggesting that GTPase activity is required for BMP formation.

In previous studies, we have reported two proteins, MagA and MpsA, presented on the BMP membrane. MagA is expressed on both BMP and cytoplasmic membrane and showed iron transport activity. Interestingly, MagA topology is inversely oriented between the cytoplasmic membrane and the BMP membrane (6, 7), something that would occur if magnetosomes were formed by membrane invagination. MpsA, acyl-CoA transferase homologue, is present specifically on the BMP membrane in higher amounts than MagA, but its function is unknown. In eukaryotes, ARF requires acylation for membrane invagination (58). Taken together, our previous studies and this study, we have proposed a mechanism for the primary formation of BMP. The Mms16 binds with the membrane. And this serves to prime the invagination from the cytoplasmic membrane. Acyl-CoA and GTP hydrolysis might be required during vesicle budding. MagA takes up ferric iron into the vesicle released from cytoplasmic membrane.
