Self-assembly of biomineralization protein Mms6 and its function as a ferric iron reductase that associates with lipid membranes

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Self-assembly of biomineralization protein Mms6 and its function as a ferric iron reductase that associates with lipid membranes

by

Shuren Feng

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular, and Developmental Biology

Program of Study Committee:
Marit Nilsen-Hamilton, Major Professor
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Iowa State University
Ames, Iowa
2015

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DEDICATION

To my family who have been supporting me unconditionally through the years, to my beloved wife Fan, and my daughter Eileen who have been my sources of impetus and inspiration in life…
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ACKNOWLEDGMENTS

I would like to thank my major professor Dr. Marit Nilsen-Hamilton for her constant support and guidance through the years. I am grateful for her constant support and patient help all through the process of completing this thesis. I am appreciative of her efforts to teach me to evaluate data objectively, to think critically, and to effectively manage my time and resources. These training that I got will be invaluable for the future of my career, no matter what career path I might be going for. Without her devoted help and guidance, I could not have made it to the completion of this thesis. I am especially grateful for the caring, encouragement, and kind help from Dr. Marit Nilsen-Hamilton and her husband, Dr. Richard Hamilton, when I had to go through some hard times of my life. “Hardship is good for you, young man…” I was told these words by Dr. Richard Hamilton. These words always popped up on my mind and helped me to move on when I was in times of trouble and at the brim of giving up.

Also, I would like to thank my committee members, Dr. Edward Yu, Dr. Eric Henderson, Dr. Gregory Phillips, Dr. Mark Hargrove, and my previous committee member Dr. Alan Dispirito for their guidance, constructive critiques, and kind help through the years. Special thanks are given to our lab manager Lee Bendickson who has been always there to help, with him being our lab manager, my mentor, and friend all at the same time.

I would like to thank our collaborators from Ames Laboratory, Department of Chemical Engineering, and Department of Material Science and Engineering. Dr. Surya Mallapragada as our project leader has been always supporting, and working hard to make sure that our research moves towards the right directions. I would like to thank all our collaborators
including Dr. David Vaknin, Dr. Wenjie Wang, Dr. Mufit Akinc, Dr. Monica Lamm, Dr. Klaus Schmidt-Rohr, Dr. Tanya Prozorov, Dr. Xunpei Liu, Keith Fritzsching, and Honghu Zhang for their collaborations, hardwork, and thoughtful discussions during the years. I really enjoyed and learned a lot from this multidisciplinary group, and some of the friendships developed will be cherished for the rest of my life.

Also, my current and past colleagues in MNH lab; Dr. Alison Pappas, Dr. Lijun Wang, Dr. Wei Zhao, Dr. Xiaoling Song, Dr. Muslum Ilgu, Dr. Ilchung Shin, Supipi Liyamali Auwardt, Dr. Judhajeet Ray, Samir Mehanovic, Dr. Pierre Palo, Dr. Tianjiao Wang, Dr. Lijie Zhai, Shambhavi Shubang, Michael Zeller, Mathew Luriano and any other rotational, SULI and visiting students. This inspiring group has been a constant source of friendship, good advice and acompany.

I would like to thank my family for their unconditional love and support during good and difficult times throughout this work. I would like to thank my wife for her constant love and support during my PhD life. I am appreciative of my parents, my sister Shiren Feng, and my brother-in-law Hong Shi for their unconditional love and support. Lastly, I want to thank god to send an angle to me and my family-Eileen. She teaches so many things as a four-year-old, far more than what I could have imagined. And, Eileen, dad loves you forever.
ABSTRACT

Biomineralization by living organisms provides excellent examples of controlled mineral synthesis for us to learn how to produce materials with desired morphologies and properties under ambient conditions. Magnetotactic bacteria (MTBs) are an example of biomineralizing organisms that produce magnetic nanoparticles. MTBs are a diverse family of organisms that are capable of producing nano-sized magnetic particles inside the cell-body with finely controlled particle size and magnetic properties. A single protein, Mms6, from these bacteria was shown to direct the biomineralization of magnetite nanoparticles from iron solutions in vitro. Previous work showed that Mms6 forms micelles in solution, with the hydrophobic N-terminal domain incorporated in the micelle and C-terminal domain exposed on the surface of the micelle. Evidence was obtained for a structural change of Mms6 when it binds with Fe$^{3+}$ as shown by CD spectroscopy and by measuring intrinsic tryptophan fluorescence. But how the protein forms micelles and undergoes structural change upon contact with iron regulates the crystallization process was still to be determined.

By using TEM and AFM microscopy, I visualized the spherical micelles formed by Mms6. The results reported in chapter 2 of this thesis are consistent with the view that the N and C-terminal domains interact with each other within one polypeptide chain and across protein units in the assembly. From protein mutational studies to determine the amino acid residues important for self-assembly, I identified the unique GL repeat in the N-terminal domain with additional contributions from amino acids in other positions, throughout the molecule. Analysis by CD spectroscopy identified a structural change in the iron-binding C-terminal domain in the presence of Fe$^{3+}$. A change in the intrinsic fluorescence of tryptophan in the N-terminal domain showed that this structural change is transmitted through the
protein. Thus, self-assembly of Mms6 involves an interlaced structure of intra- and inter-molecular interactions that results in a coordinated structural change in the protein assembly with iron binding.

Mms6 displays two distinct types of tryptophan fluorescence spectra when tested in quartz cuvettes and plastic 96 well plates. Further investigation showed that Mms6 adsorbs onto hydrophobic plastic surfaces. In chapter 3, we report that Mms6 undergoes structural rearrangements on binding iron that can be measured by intrinsic trp fluorescence. Both phases of iron binding (high affinity stoichiometric and low affinity, high capacity) were linked to the fluorescence changes. The high affinity and stoichiometric binding measured at pH 7 demonstrated the same high affinity as was determined by direct iron binding with 55Fe filter capture assays. This fluorescence change is proposed to be an intramolecular structural change as it is not temperature-dependent. The high capacity and low affinity binding of iron is cold sensitive as is the fluorescence change that could be measured at low pH with high molar ratios of iron to protein. Trp119 was identified as the residue for which the signal was measured. Thus intrinsic fluorescence spectroscopy reveals a complex combination of structural changes in Mms6 that probably involve both intra and inter-molecular interactions.

The observation, made by our colleagues, that Mms6 binds ferric and not ferrous iron brought up the question of how Mms6 regulates the crystallization of magnetite, which contains both ferric and ferrous iron. The results, reported in Chapter 4 of this thesis, showed that Mms6 is a ferric reductase, which can catalyze the reduction of ferric iron to ferrous iron using NADH and FAD as electron donors and cofactors, respectively. Higher reductase activity was observed when Mms6 was integrated into either liposomes or bicelles. These
results are consistent with a hypothesis that Mms6 is a membrane protein which promotes the formation of magnetite by a mechanism that involves reducing iron.
CHAPTER 1: GENERAL INTRODUCTION

1.1 Thesis Organization

This dissertation contains five chapters. Chapter one is a general introduction of the information related to the structural and functional studies of Mms6 protein presented in this dissertation. A review of biomineralization theories and examples is given first that is followed by an introduction of the structural properties of biomineralization proteins. The molecular mechanism of magnetosome biogenesis is then reviewed, followed by the discussion of membrane proteins involved in magnetosome biogenesis. The chapter concludes with a general discussion of magnetic nanoparticles and their synthesis and applications.

Chapter 2 is revised from a paper that was published in the *International Journal of Molecular Sciences*, ISSN 1422-0067, 2013. In this paper we reported the mechanistic study of Mms6 self-assembly and characterization of Mms6 self-assembly when it binds iron. As the first author, my contributions to this work include: TEM analysis of Mms6 micelles, mutagenesis and purification of Mms6 mutants in the N-terminal domain (Trp to Ala or Phe mutants), mutagenesis and purification of Mms6 mutants in the C-terminal domain (Leu/Ile to Gly mutants); analysis of Mms6 and its mutants for micelle formation by FPLC, analysis of Mms6 peptides for multimer formation by FPLC in the presence and absence of Fe\(^{3+}\), Trp tryptophan fluorescence spectroscopic analysis of Mms6 with iron binding, and I-TASSER modeling of Mms6. The co-authors are Dr. Lijun Wang (conducted FPLC analysis of Mms6, m2Mms6, and m3Mms6 and C21 peptide without iron, CD spectroscopy of peptides with iron binding), Dr. Pierre Palo (Expressed and purified Mms6(A155C), Mms6(A157C), and Mms6(GL-GA) mutants), Dr. Xunpei Liu (conducted some of the TEM analysis, AFM
imaging, and the SANXS spectroscopy), Professor Surya Mallapragada (participated in the design and discussion of this work, revised the manuscript), and Professor Marit Nilsen-Hamilton (corresponding author, mentored Shuren Feng, Lijun Wang, initiated the project, oversaw the project including data analysis and drafted and revised the manuscript).

Chapter three is a manuscript prepared for submission to The Journal of Biological Chemistry. In this manuscript we reported the characterization of Mms6 by tryptophan fluorescence spectroscopy and mutational studies to understand how Mms6 self-assembles, and how the protein structure changes when Mms6 interacts with iron. We found that Mms6 can adsorb onto hydrophobic plastic surface that gives it a different structure. My contributions as the first author of this work include: generate and prepare the mutants of trp and C-terminal Leu and Ile residues, conduct FPLC analysis of these mutants, conduct and analyze the fluorescence experiments at pH3 with iron binding. The coauthors are Dr. Pierre Palo (generated and purified the Mms6(GL-to-GA) mutant), Dr. Lijun Wang (conducted the iron binding test at 4°C, and trp fluorescence with iron binding at 25°C in quartz cuvette), and Professor Marit Nilsen-Hamilton (corresponding author, mentored Shuren Feng, initiated the project, oversaw the project including data analysis and manuscript review).

Chapter four is a manuscript prepared for submission to The Journal of Biological Chemistry. In this manuscript we reported the characterization of Mms6 as a ferric reductase that associates with lipid membranes, which results in increased ferric reductase activities. We also report the discovery of ferric reductase activity in the C-terminal domain of Mms6. My contributions as the first author of this work include: optimizing the reductase conditions, generated and prepared five of the C-terminal OH/COOH-to-Ala mutants, tested ferric
reductase of Mms6 with lipid membranes, determined the pH optimum, conducted the DLS test for Mms6 interaction with lipids). The coauthors are Dr. Lijun Wang (studies of the C-terminal domain of Mms6 as a ferric reductase, identification of NADH and FAD as electron donors and cofactors), Dr. Pierre Palo (generated and purified five of the C-terminal domain OH/COOH-to-Ala mutants, conducted iron binding assay), Samir Mehanovic (preliminary test of ferric reductase), and Professor Marit Nilsen-Hamilton (corresponding author, mentored Shuren Feng, initiated the project, oversaw the project including data analysis and manuscript review).

Chapter five is the general conclusion which summarizes the major findings in this thesis.

1.2. Literature Review

1.2.1 Biomineralization theories and examples

1.2.1.1 Biomineralization: concepts and basic theories

Biomineralization refers to the process by which organisms produce purely inorganic or organic-inorganic composite materials with distinct features for various functions such as protection, structural support, motion, storage, orientation, detoxification (1,2). Biomineralization actively controlled by living organisms is different from “bio-induced mineralization” that refers to the passive sedimentation of minerals around biomolecules or dead bodies of organisms (2). The distinctive crystallographic, mechanical, or physical properties of biomineralized materials that are precisely controlled by genetic information of various living species have attracted increasing interest of researchers. Since the first discussion of “Biomineralization” in book by W. J. Schmidt in 1924 (3), the study of
biomineralization has developed into a multidisciplinary subject encompassing, but not limited to, biology, chemistry, biochemistry, and material sciences (1).

Biominerals, containing small portions of organic macromolecules, are often structurally more complex and mechanically more suitable for their biological functions than their inorganic counterparts. The unique properties of biominerals are believed to originate from their organic constituents, most of which are proteins and polysaccharides. For example, 95% of the mass weight of nacre from molluscs is CaCO$_3$, but the fracture resistance of nacre is 3,000 fold more than pure CaCO$_3$ minerals. Furthermore, with the same thermodynamic driving forces as inorganic crystallization, most biomineralization happens at ambient temperatures and pressures and under physiological conditions. A comparable level of morphological control cannot be achieved in the laboratory using conventional chemical synthesis methods. Understanding the mechanism(s) of how organic biomolecules interact with inorganic ions to control the synthesis of biominerals at molecular, cellular, and physiological levels would enable the fabrication of better artificial materials for application (4).

Research since the 1970s has focused on the structural and functional roles of biomacromolecules in the biomineralization process to elucidate the mechanism(s) by which crystallization is directed (5). With the advance of various techniques in chemistry, material science, and biological sciences, we have gained deeper insights into the mechanism of biomineralization in recent years (6). Despite the fact that the detailed mechanisms of biomineralization are still under debate, there are several well recognized strategies that living organisms commonly use to achieve controlled mineralization.
1.2.1.1 Strategy I: Localized enrichment of crystallizing ions—spatial boundaries that allow for supersaturation

Supersaturating of ions is a prerequisite for crystallization and it can be achieved in various ways, e.g., increase of ionic concentrations, chemical reactions, temperature/pressure changes, compositional changes (7). However, most environments that organisms inhabit normally don’t have the high concentrations of ions needed for crystallization. Organisms from different stages of evolution employ similar ways to enrich the ion(s) they need to produce localized microenvironments that make supersaturation possible, which is essential for the nucleation and controlled crystal growth (8). The most commonly used boundaries are lipid vesicles that are associated with biomineralization of different organisms. In bacteria that produce intracellular magnetic nanoparticles, supersaturating concentrations of iron are achieved by accumulating iron in the magnetosome vesicles that originate from the inner cell membrane (9). Recent research showed that the iron transport is likely mediated by the cation diffusion facilitator (CDF) family of proteins, MamB and MamM that are located on the magnetosome membrane and involved in both iron transport and control of magnetite size and shape (10,11). Higher plants producing calcium oxalate crystals in the vacuoles of specialized cells have compartmentalized intra-vacuole chambers to concentrate calcium oxalate for nucleation and crystal growth (12). Sea urchin skeletal spicules originate from intracellularly sequestered deposits in mesenchymal cells and filopodial templates (13). Interestingly, even extracellular calcification of vertebrate bone, teeth, and cartilage starts within the matrix chamber with calcium channels and other macromolecules originating from plasma membrane of specialized cells. The release of matrix chambers from the cells helps to establish the calcium/phosphate rich environment in pre-formed extracellular matrixes of
collagen fibers and cell bodies that delineate the spaces in which the crystals of hydroxyapatite (HA) will form (14,15).

In addition to the compartments formed by lipid membrane vesicles, proteins and polysaccharides are also commonly used to form supramolecular structures that function as biomineralization boundaries and regulate the further steps of crystal formation (8). Indeed, enrichment of needed ions at high concentrations often involves formation of compartments either inside the cells in the form of lipid vesicles or outside the cells in the form of supramolecular assemblies formed by macromolecular frameworks or cells (2,7). The compartmentalization both provides passive boundaries for accumulation of target ions to high concentrations and allows for fine tuning of the chemical compositions of crystallizing ions inside the compartment by controlling the influx and efflux of ions across the compartment (2). Selective concentration of ions in a compartment also minimizes possible toxicity from external concentrated metal ions that are not found under normal physiological conditions (16).

1.2.1.1.2 Strategy II: Nucleation control mechanisms—structural control (acidic proteins, sulfated glycans, and supermolecular assemblages)

Ionic enrichment in biological compartments mostly doesn’t reach the same degree of supersaturation required for the conventional formation of nucleation cores in pure inorganic systems (8). Supersaturated ions may nucleate by spontaneous clustering into tiny grains only when the free energy of forming a solid phase in the aqueous solution is balanced by the energy released during the formation of bonds in the crystal and the free energy associated with the solid-liquid phase change (2,17,18). The energy barrier of nucleation is a similar
concept to the activation energy of a chemical reactions, which depends on the degree of supersaturation and interfacial energy of ionic clusters in the supersaturated ion solutions (2,7). Higher degrees of supersaturation will decrease the activation energy of nucleation, and will lead to uncontrolled nucleation once over a certain threshold (2,19). Biomineralizing organisms often control the degree of supersaturation in a narrow range by fine tuning the concentration of ions in the mineralizing compartments through ion transporters, chemical reactions, or metabolic activities (1,5,7).

The effect of interfacial energy on nucleation is exemplified in a beaker of water placed in a freezer: ice crystals firstly form on the surface, wall, and bottom of the beaker since these areas are water-air or water-glass interfaces where interfacial energies are lower than the bulk of water. Biomineralizing organisms control interfacial energies by introducing organic surfaces (proteins, lipid membranes, and polysaccharides) and ion-clustering macromolecules in solution to regulate nucleation (2,20,21). The interface between the organic surface of a biomineralizing compartment and its inner ionic solutions also decreases the interfacial energy that a supersaturated solution has to overcome for nucleation to happen (2). More often than not, these organic surfaces are made of proteins that self-assemble into a matrix/template that can actively interact with the crystallizing ions through charge-charge interactions, hydrogen-bonding, enzymatic catalysis, and templating effects (1,4,22).

Traditional views of protein-mediated crystallization events involve the formation of metastable prenucleation clusters in supersaturated solutions, and then coalesce of these prenucleation clusters into a stable nucleation product (23). However, recent progress in analyzing early stage of crystallization mediated by these proteins supports a different model
The new model of nucleation involves the formation of more “solute”-like prenucleation clusters that are in dynamic equilibrium with the ionic solution environment that is not necessarily supersaturated. These prenucleation clusters then aggregate into larger amorphous nucleation clusters that can rearrange into a crystalline lattice on which crystals can grow into their mature form (25,26). In this new nucleation model, one of the roles of biomineralization proteins is to bind and stabilize the “solute”-like prenucleation clusters. The fact that biomineralization proteins like Mms6 and amelogenin bind crystallizing ions with high stoichiometries suggests that they either bind or mediate the formation of prenucleation aggregates (27,28).

Proteins rich in acidic residues are widely found to play a role at biomineralization interfaces, which function to promote the formation of prenucleation clusters by clustering ions with these acidic residues (29-31). Early research on calcification in mollusca showed that both aspartic acid-rich proteins and proteins with serine-linked polysaccharides are intimately involved in the interactions with biomineralized crystals (22). The organic matrixes of demineralized spicules of sea urchn embryos were also shown to contain many glycoproteins rich in aspartic acid, glutamic acid, and serine residues (20). Acidic matrix biomacromolecules from animal skeletons often contain aspartic acid-rich domains and covalently linked sulfated polysaccharides, which are believed to be involved in the binding of calcium, creating the supersaturation necessary for nucleation (32). The same strategy also seems to be used by magnetotactic bacteria for the synthesis of superparamagnetic nanoparticles. The protein, Mms6 (together with Mms5, Mms7, and Mms13), tightly associated with magnetosome magnetites isolated from the bacteria, also contains a C-terminal domain rich in hydroxyl/carboxyl amino acid residues that is responsible for iron
binding (27,33). Artificial peptides and macromolecules mimicking the properties of these biological molecules were also shown to promote the nucleation of minerals in vitro (34,35).

1.2.1.1.3 Strategy III: Crystal growth and morphological control mechanisms

One of the major challenges faced by material scientists is to control the sizes, shapes, and properties of materials. Organisms control biomineralization to almost perfection by genetically encoded proteins. One well-recognized function of these proteins is that they self-assemble into specific supramolecular structures that can template and actively regulate the crystal growth and maturation. One such example comes from the biomineralization of HA in animal bones, which are composed of well-organized HA crystals formed along collagen fibrils (36). It has been shown that organization of collagen fibrils provides spatial localization of precipitation and constraints on mineral development in addition to an initial nucleation function (37). With the advance of high-resolution microscopy, current research has shown that collagen is an active scaffold, playing multiple roles in crystallization from pre-nucleation clustering, nucleation, and structural guidance (21).

The strategies for biomineralization don’t stand alone. Compartmentalization also plays a role in the control of crystal size and shape because the membrane vesicles and their macromolecular frame work of proteins and (or) polysaccharides) function as physical restraints on the growth of crystals (6,21). Some of the macromolecules promoting nucleation also participate in crystal growth, exerting further control on the morphology and properties of biominerals (1). The aforementioned mechanisms are simplified models of the biomineralization process. The control of crystallization in living organisms is more complex, being orchestrated by metabolic activities and changing local environments.
1.2.1.2 Biomineralization of calcium minerals

As one of the essential elements for living organisms, calcium minerals are probably the most common examples of biomineralization. They include the exoskeletons of single cell organisms such as coccolithophores and green algae, the shells of marine mollusks, the exoskeletons of crustaceans, egg shells, and animal bones and teeth. Based on the basic chemical compositions of these minerals, they can be categorized into two major classes: calcium carbonate and calcium phosphate. Calcium carbonates, with the same chemical composition but varying structures, are members of a heterogeneous group that includes calcite, aragonite, vaterite, amorphous calcium carbonate, and calcium with several different hydration states (38). Calcites, aragonites, and vaterites are the most commonly seen biomineralized calcium carbonate minerals. In different organisms, the morphologies and functions of calcium minerals may vary, but the mechanisms by which these organisms form these minerals have a lot in common (2,7).

One of the well-studied examples of biomineralized calcium carbonate minerals are the shells of marine mollusks. These shells normally contain both calcites and aragonites (39). Nacre, made of multilayered “brick wall”-like aragonite minerals, is the inside layer of most shells, with the outside layer containing mainly prismatic calcite minerals (39). Biological control of this differentiated crystallization on the growing shell is executed by a layer of outer epithelium cells and occurs in the extrapallial space and fluids that lie at the growing frontier of the two crystallizing layers (2,40-43). During the growth of shells, the outer layer, consisting of calcite minerals, first forms the base. Crystallization of the nacre aragonite minerals on this base is mediated by a protein-polysaccharide matrix of about 30nm in
thickness (44,45). The protein-polysaccharide matrix is formed by macromolecules secreted by the outer epithelium cells and assembled in the extrapallial space into a matrix structure (46,47). Structural characterization showed that these matrix structures may limit the thickness of aragonite crystals, thus reducing the formation of voids during the crystal growth (47,48). The organic matrix intercalated between the inorganic layers of aragonite bricks is also believed to contribute to the high fracture resistance properties of nacre because the comparatively elastic organic matrix is more resistant to cracks and better at dissipating energies associated with an expanding defect in the crystals (2,40,49). Biochemical characterization of demineralized nacre revealed that this organic matrix consists of a macromolecular acidic layer, a silk-fibroin-like protein layer and a beta-chitin layer (40). The silk-fibroin-like protein layer can assemble into an hierarchical sheet structure, upon which the macromolecular acidic layer are extensively inter-associated (50). Such an assembled structure is a typical biomineralization interface in which the acidic residues patterned on the matrix can actively interact with crystallizing ions and regulate the nucleation and crystal formation in the extrapallial space (50-52).

Biomineralization of calcium phosphate (HA) minerals is mainly observed in bone and teeth. The chemical compositions of bone and teeth are more complex than the calcium carbonate classes because they contain larger fractions of organic materials in the biominerals and the proportions of these organic materials are normally constantly changing due to physiological and cellular regulations. By comparison, inorganic HA crystals contain more of other ions, e.g., CO$_3^{2-}$, Mg$^{2+}$, Na$^+$, F, Cl, OH$^-$ (2). Similar to the improved mechanical properties of calcium carbonate minerals by the inclusion of organic materials, the mechanical properties of bones are distinctively different from their pure inorganic
counterparts due to the presence of organic components, which are mainly collagen fibrils and other proteins (2,7,51). On the cellular level, a network of cells interconnected by pores and channels in the bones are known to regulate the dynamic growth, dissolution, and remodeling of bones in response to the changes in hormonal levels and the outer environments (2). The network of osteocytes, osteoblasts, and osteoclasts segregates bone of bigger sizes into individual compartmentalized regions, in which they secret various biomacromolecules to modulate the mineralization and demineralization process in response to the physiological requirements of the body (2,6,21).

At the molecular level, biomineralization of bone are regulated by a supramolecular network formed through self-assembly of collagen and other secreted macromolecules (2,6,36). Collagen assembles into a triple-stranded helix structure before being secreted into the extracellular space, where the triple-stranded helix filaments assemble into collagen fibrils that can be further cross-linked into the mature collagen fibril networks by enzymatic reactions (1,36,50). In the mature collagen fibril networks, each collagen molecule is about 300 nm in length along the axis of fibril and axially displaced from each other by about 67 nm. This arrangement maximizes the number of cross-links between adjacent collagen molecules (2,36). The large number of inter-molecular crosslinks created during the assembly of collagen networks is believed to increase the strength and stability of the collagen matrix and improve properties of HA crystal lattices (2). The displaced arrangement of collagen molecules also results in regularly-spaced gaps along the collagen fibril known as hole zones, which are believed to be sites of nucleation and formation of plate-like HA crystals (36). Recent studies on HA biomineralization have revealed evidence of collagen matrix actively participating in bone biomineralization from the early stage of calcium
prenucleation cluster formation, to the formation of calcium phosphate, the transformation of calcium phosphate into oriented HA nucleation core, and to the stage of HA crystal growth into the mature HA minerals (24,53). This has challenged the traditional view of collagen functioning only as a passive scaffold for HA biomineralization (24,36,54,55).

Despite the well-known structural properties of collagen and the matrix network it forms, biomineralization of bones at the molecular level also involves other non-collagen proteins like acidic glycoproteins (glycoproteins rich in aspartate and glutamate), phosphoproteins (proteins rich in highly phosphorylated Ser residues), gamma-carboxyglutamic acid-rich proteins, and proteoglycans. Most of these non-collagen proteins are rich in anionic groups that can potentially interact with calcium ions, and they are found to interact specifically with the collagen matrix at the hole zones. The means by which these proteins interact with the collagen matrix and calcium ions are still elusive due to the difficulty of structurally characterizing these complex multimolecular complexes.

Other major calcium phosphate biominerals are the enamels formed on the outside layers of teeth, inside which the dentine is made of bone-like minerals rich in a collagen matrix (56,57). Different from bones in which a significant mass of collagenous matrix is intercalated in HA minerals (about 35 percent on average), mature enamels contain less than five percent of organic materials. The increased brittleness of the enamel HA with less matrix protein is counterbalanced by a special arrangement of HA crystals that are interwoven into ribbon-like bundles of crystals with unique length to width ratios as revealed by the microscopic structures of these minerals (2). The formation of enamel minerals is also mediated by extracellular matrix acidic glycoproteins, dominated by the protein named
amelogenin. These proteins (mainly amelogenin) are abundant during the early stages of enamel formation. As enamel minerals mature, the extracellular matrix proteins are gradually cleaved and removed from the mineralization sites by proteases (56-60).

As the major protein that self-assembles into the extracellular matrix for enamel biomineralization, the N-terminal and C-terminal domains of amelogenins are highly conserved among different species due to their important functions in enamel formation (61). Amelogenin consists of about 180 aa residues, in which the N-terminal domain (1-42 aa), named the TRAP domain (Tyrosine-Rich Amelogenin Polypeptide), is rich in tyrosine and C-terminal domain (157-173aa) is highly hydrophilic with mostly negatively charged residues. The central region of amelogenin is highly hydrophobic and rich in proline and leucine residues. The hydrophobic central region of amelogenin is believed to be responsible for its formation of multimers in solution and its self-assembly into nanospheres of about 20nm in diameter (62,63) in which the hydrophilic C-terminal domain and N-terminal domain are believed to be exposed to the outside thus susceptible to protease cleavage (64-66). The nanospheres of amelogenin can further self-assemble to form linear nanochains consisting of individual nanospheres (67,68). The nanospheres can bind to specific crystalline planes of the growing HA crystals with its C-terminal charged residues and are believed to block the growth of crystals at these crystalline planes thus constraining the growth of crystals into specific directions (2,66). In addition to its function of regulating HA crystal growth, the C-terminal region was also shown to be important for the self-assembly of HA into a stable nanochain structures (69,70). Further characterization of amelogenin interacting with HA crystals by single molecule atomic force microscopy demonstrated that the C-terminal residues specifically bind to the (100) plane of growing HA crystals probably
through charge-charge interactions between calcium ions and the negatively charged amino acid residues, which results in the inhibition of crystal growth on the 100 plane direction whereas allows crystal elongation at the (001) plane direction (71). Characterization of amelogenin under varying experimental conditions (e.g., in bulk solution, on surfaces of different charges, or on membranes) reveals different assembly patterns of amelogenin, which might indicate the different functioning states of amelogenin in enamel formation under varying physiological conditions of the host. (36,72-76).

In addition to the essential roles of amelogenin in enamel formation, other proteins like enamelin are important in this biomineralization process. Similar to amelogenin, enamelin is expressed and secreted by ameloblasts into the enamel matrix of developing teeth at early stages of enamel formation (56,60). The primary sequence of enamelin is rich in glutamate, aspartate, and glycine residues, which are believed to be involved in its interactions with the positively charged calcium ions during enamel formation (77). Two phosphorylated serine residues in the enamelin sequence are believed to be important for binding calcium and regulating enamel formation(78,79). The in vitro observations of enamalin and amelogenin functioning together to promote nucleation of HA crystals in a cooperative manner and their in vivo expression pattern all point to the probability that the two proteins interact extensively during the biomineralization of enamel and both play important roles in regulation of enamel formation (80-86). However, how amelogenin, enamelin, and other enamel proteins coordinate to regulate the dynamics of enamel biomineralization is still under investigation.

Although the functions, shapes, mechanical properties, and chemical compositions of these nacre, bones, and enamel biominerals vary, the basic strategies for their controlled
biomineralization are similar. As discussed later in this section, the same strategies used in calcium biomineralization regulation are also used in biomineralization of different minerals and in different organisms.

1.2.1.3 Biomineralization of silica minerals

Silica minerals are different from other biominerals in that they are not ionic salts. Instead, they are amorphous and tightly packed colloidal silica materials (2,87). Mature silica minerals have a basic structure of a polymeric network of tetrahedrally coordinated siloxane centers with different levels of hydroxylation (2,88). Silicic acid, the soluble form of silica at neutral pH, in nature is a weak acid with the chemical formula of Si(OH)$_4$. Soluble silicic acid will undergo polycondensation reactions to form amorphous silica gels or colloids once its concentration goes above 1mM, a concentration normally not seen under natural conditions (2,89). So the biomineralization of silica minerals normally involves multiple steps including silicic acid uptake and accumulation to high concentrations, polycondensation reactions, and regulation of mineral morphologies (2). Silica biominerals are commonly found in many organisms with various morphologies and biological functions, represented by diatoms cell walls, sponge spicules, radiolarians microskeletons, silica spines and nodules in higher plants (2,36). The most common silica minerals are rice husks, which are used for the industrial production of silicon nitrides (2).

Although chemically and structurally different from minerals of ionic salts, silica mineral producing organisms seem to use similar strategies as for ionic salts to control the process of silica mineralization. This involves the arrangement of amorphous silica building blocks into the final tightly packed silica biomineral by interactions between silica primary particles and
functional groups on biomacromolecules that is guided by self-assembled organic matrixes (2,90-92). For example, sponge spicules consist of amorphous silica nanoparticles deposited between the spaces formed by axial protein filaments and sclerocyte membranes with some additional proteins and polyamines (36). As the major component of the axial filaments of sponge spicules, the protein, silicatein, undergoes several steps of self-assembly to form a filamentous structure (93). The assembly of silicatein was believed to be driven by the hydrophobic patches on the surfaces of the silicatein subunits (93). The silicatein filaments and sclerocyte membranes make up the organic matrixes that delineate the spaces in which deposition of primary amorphous silica particles is guided (94,95). In addition to its self-assembly mediated structural guidance function, silicatein is a dehydrolase that catalyzes the condensation of silicon ethoxides (36,96). Silicateins catalyze this dehydration reaction with a mechanism similar to the enzymatic mechanism of serine-protease mediated proteolysis (36,96).

1.2.1.4 Biomineralization of iron oxides and sulfide minerals

Iron oxides and sulfides minerals are widespread in nature with different mineral forms, e.g., magnetite (Fe₃O₄), greigite (Fe₃S₄), geothiote (alpha-FeOOH), lepidocrocite (gamma-FeOOH), ferrihydrite (5Fe₂O₃-9H₂O). These iron minerals were synthesized by organism for a wide range of functions including magnetotaxis, grinding, magnetic navigation, mechanical strength, and iron storage. As well as the interest of basic scientists in the mechanism(s) by which iron minerals are produced by these organisms, engineers and applied scientists are interested in emulating these mechanisms in improved materials due to the many application
and extensive use of inorganic iron oxide materials like magnetic nanoparticles and magnetic
deVICES in industry and human society.

As an ancient synthetic process with a fossil record of 2 billion years, biomineralization of
magnetite nanocrystals ($\text{Fe}_3\text{O}_4$) has been found in all three kingdoms of living organisms
(97). Magnetic nanoparticles are believed to be involved in the geomagnetic navigations of a
group of migratory birds, fishes, honey bees, and other organisms (98,99). Single domain
magnetite nanocrystals were even found in the human brain (100) and suggested to be
involved in the human interaction with extremely low frequency (ELF) magnetic fields
(101). Transmission electron microscopic analysis of magnetite nanoparticles from the
human brain showed that these magnetites were morphologically similar to the single domain
MNPs produced by magnetotactic bacteria (101,102).

The most well studied biomineralization of iron oxide minerals is probably from
magnetotactic bacteria (MTBs), a family of aquatic prokaryotes that produce a chain or
chains of intracellular single-domain MNPs enveloped by lipid membranes, which is the first
known prokaryotic organelle, termed a “magnetosome.” MTBs produce these single domain
NPs with diameters of 35-120 nm, and arrange them into a chain along the long axis of cell
body, by which a strong single magnetic moment forms along the cell body (103). MTBs use
this magnetic dipole as a nano-scale “compass needle” to orient themselves against the
g geomagnetic field and swim toward environments with optimum nutrients and oxygen
concentrations (104-107). Biomineralization of MNPs in these bacteria is controlled by their
genetic network. A conserved genomic magnetosome island, the Magnetosome Associated
Island (MAI), is mainly responsible for the genetically controlled biomineralization of
magnetic nanocrystals in the cell, with most of the genes from this genomic island encoding magnetosome associated proteins, including the critical mms6 operon, mag operon, and mam operon (108-110).

With the characterization of genes from MAI and proteins associated with the magnetosome membrane, the field has come to the stage of unveiling the overall steps that the cells may take to synthesize the organelle and arrange them into a chain-like structure (111). The molecular mechanism(s) that MTBs use to control magnetosome biogenesis and crystallization control are discussed in Section 1.2.3 and Section 1.2.4 of this literature review. Similar strategies used by organisms making calcium and silica biominerals are also used by MTBs to control the biomineralization of magnetic nanocrystals, e.g., compartmentalization, and self-assembly of protein matrix proteins to form supramolecular structures.

In addition to the magnetic nanocrystals described above for magnetotaxis and navigation, iron oxides synthesized on the teeth of molluscs such as limpets and chitons also function to grind. Magnetite crystals are synthesized on the cutting edge of their sabre-like teeth with HA backbones. (2,112,113) The biomineralization of these magnetite-containing teeth is also believed to involve organic matrices formed by chitin fibers (113-116).

1.2.1.5 Summary

In this section, I reviewed the strategies that organisms commonly use during biomineralization to attain controlled crystal formation. As can be seen from examples of biomineralization in different minerals, living organisms rely on biomacromolecules
including proteins, lipid membranes, polysaccharides, and even cell body assemblies to exert fine control over every step crystal formation by employing an organic matrix to: enrich and accumulate ions, synthesize and assemble supramolecular structures, bind molecular clusters or cluster molecules to facilitate nucleation, template and guide structural growth.

Overall, biomineralization involves selective enrichment of elements from the environments and controlled incorporation of these elements into mineral structures for specific biological functions. The conservation of strategies used in nature for the controlled formation of crystals enables the application of previous knowledge about well-studied biomineralization systems to understanding unknown systems. The knowledge of these general strategies can be used to improve and devise new and novel materials.

1.2.2 Structural and functional properties of biomineralization proteins

In section 1.2.1, I already mentioned that biomineralization proteins with ion-binding and nucleation functions in early stage of biomineralization are often found to participate in further control of biomineral formation, for example crystal growth and morphology control. From biochemical structure-function perspective, each of these functions played by these proteins are determined by their structures. I will discuss several well studied biomineralization proteins as examples to show their structure-functional relations in biomineralization.

1.2.2.1 Biomineralization proteins are multifunctional proteins with flexible structures
The multifunctional properties of biomineralization proteins can be appreciated in the following perspectives. First, most biomineralization proteins with ion-binding and nucleation functions are identified at the protein/mineral interface, where they function to regulate mineral formation by interacting with crystallizing ions (117-119). One common feature of these proteins is the abundance of amino acid residues with hydroxyl/carboxyl-containing side chains in their primary sequences that are believed to be the structural basis for them to bind with metal ions and regulate the nucleation process (120-122).

Second, post-translational processing like phosphorylation, glycosylation or enzymatic cleavage is often observed for these acidic proteins. In fact, acidic proteins that are either highly phosphorylated, glycosylated, post-translationally processed, or rich in amino acid residues with hydroxyl/carboxyl side chains have been isolated and identified from various biomineralization systems, e.g., bones (79,123-125), tooth enamels (126,127), and mollusk shells (128-131). Remarkably, the maturation of amelogenin involves multiple post-translational modifications, and multiple steps of proteolytic cleavage of the extracellular matrix around enamel surface are also required for the maturation of this protein(126,132,133). Although there are no reports of phosphorylated proteins in magnetotactic bacteria for magnetite biomineralization, proteins with proteolytic activities were also identified on the magnetosome and are believed important for the biogenesis of magnetosomes (134,135).

Third, some biomineralization proteins have enzymatic activities. Biomineralization processes are often integrated with the metabolic networks of organisms. Myriads of proteins with enzymatic activities in these complex networks are directly or indirectly involved in the
biological control of crystallization in almost all known biomineralization categories (136). There are enzymes that are directly involved in mineral-organic matrix interactions in biologically controlled mineralization. For example, one of the well-known enzymes involved in biomineralization is carbonic anhydrase (CA), which catalyzes the dehydration of bicarbonate or carbonic acid to form carbon dioxide and water, or its reverse reaction. This chemical process is well known for its critical role in maintaining the oxygen/carbon dioxide homeostasis of human beings; and it is involved in calcium biomineralization in organisms at different evolutionary stages. Carbonic anhydrase was found to regulate the biomineralization speed of coral reefs by catalyzing the formation of carbonate ions. Inhibition of CA by Acetazolamide (Diamox) resulted in the inhibition of measurable calcification (137,138). CA was also found present in large amounts in the gland responsible for egg shell formation in birds, suggesting a function in the controlled mineralization of egg shells (139,140). Matrix protein nacrein, a soluble matrix protein directly involved in mollusk shell mineralization, was found to have a domain homologous with carbonic anhydrase, which is believed responsible for the carbonate crystal formation in nacreous layer of the mollusk shell (141). The significance of understanding how organisms use this enzyme as a regulator of calcium carbonate crystallization and to control the morphology of crystals is to learn from nature to control the properties of chemically synthesized materials.

Fourth, biomineralization proteins are capable of self-assembly and form intermolecular complexes. Self-assembly of proteins from monomeric small peptides into multimeric protein complexes have been described above, e.g., collagen and amelogenin. Crystal growth and morphology control are important part of biomineralization that are often mediated by proteins functioning to template and guide the growth of small grains of crystal nucleus at
specific lattice directions, or regulate the aggregation of prenucleation clusters (55,142-144). However, the size-scales of biominerals formed are often much larger than the sizes of biomineralization proteins that are involved in the templating and guidance of the crystallization process. This problem was solved by the proteins to form large inter-molecular complexes that can reach the size-scales that are comparable to the minerals formed(145).

Interestingly, a lot of biomineralization proteins can be classified as intrinsically disordered proteins (IDPs) (146). IDPs are group of proteins that are characterized by heterogeneously unorganized structures without defined three-dimensional arrangements as observed for common globular proteins, but the high flexibilities of their structure allows them to be involved in cellular activities that requires a lot of intermolecular interactions or multiple protein assemblies, e.g. cell cycle control, protein-DNA regulations, and cell signaling (146-148). Indeed, biomineralization also involves complex cellular and/or physiological activities that involve ion-protein, protein-protein, protein-polysaccharide, or protein-lipid interactions that structural flexibility is desired (147,148).

Examples of these proteins from biomineralization of bone, enamel, mollusk shell, and magnetic nanoparticles of magnetotactic bacteria will be discussed below focusing on their representative structural properties that determine their functions in biomineralization.

1.2.2.2 Bone-assemblies of proteins on a collagen matrix

Hydroxyapatite (HA) biomineralization in human skeletons involves type I collagen matrix and a number of non-collagenous proteins that coordinate to form bone minerals with
various morphologies and properties in different parts of the body (See section 1.2.1 for detailed information about collagen matrix) (149). These non-collagenous proteins play important roles in organizing assembly of collagen matrix and regulation of hydroxyapatite crystallization (150,151). One family of these non-collagenous proteins is well known as the small integrin-binding ligand N-linked glycoproteins (SIBLING). The SIBLING family proteins involved in hydroxyapatite mineralization include osteopontin, sialoproteins, dentin matrix protein, dentin phosphoprotein, and matrix extracellular phosphoglucomutase (146).

These proteins do not have homology in their primary peptide sequences, but their coding genes locate to the same 375kb region on the human genome. These proteins are rich in acidic residues, can bind with collagen matrix, and also interact with integrins on cell surfaces with their RGD motifs (152). Post-translational modification such as phosphorylation and glycosylation are commonly found within these proteins, which often involve regulation of these proteins’ self-assembly, interactions with collagen and integrins, hydroxyapatite binding, and nucleation (79,153,154).

The multifunctional structural-functional properties of these non-collagenous proteins can be exemplified by the dentin matrix protein (DMP1). Firstly identified from dentin by cDNA cloning, which gave it the name DMP1 (155), it was later found to be expressed in bone, cementum, and other tissues that do not even have minerals (156). DMP1 is an acidic protein with its primary sequence consisted of 21% Ser, 15% Glu, and 12% Asp, in which over 50% of the Ser residues are phosphorylated (155). While full length DMP1 was found in small amount from mineralized tissues (157), three post-translationally cleaved fragments of DMP1 (cleavage by matrix metalloproteinase-2 (158)) were found predominantly in both bone and
dentin tissues: the N-terminal 37kD fragment, the C-terminal 57kD fragment, and the glycosaminoglycan-containing N-terminal fragment (referred to as DMP1-PG) (159). The C-terminal domain of DMP1 contains the typical ASARM peptide (Serine Aspartate-Rich MEPE-associated Motif) that are found to be important for HA mineral formation, in which the MEPE stands for Matrix Extracellular Phosphoglycoproteins. Post-translational modification and cleavage play important roles in regulating the in vivo activities of DMP1. While phosphorylation of full-length DMP1 inhibits its HA nucleation functions, dephosphorylation of the full length protein and its cleaved fragments was shown to convert them into HA nucleators (159-162). DMP1 was also involved in cell signaling pathways in vivo by interacting with cell surface receptor αvβ3 integrin with its RGD motifs (163).

In vitro tests with different forms of DMP1 showed that full length DMP1 and DMP1-PG inhibits HA nucleation, whereas C-terminal DMP1 fragment and the N-terminal fragment can promote HA nucleation (159,161). Atomic force microscopic analysis of the C-terminal DMP1 fragment showed that it can self-assemble into oligomeric aggregates through intermolecular clustering after calcium binding (161). Four critical acidic clusters were identified from this C-terminal DMP1, which are believed to play a role in molecular recognition of mineral surfaces (155,161). The synthetic peptides of these acidic clusters can also oligomerize through clustering and form intermolecular β-sheet structures with calcium binding. Circular dichroism spectroscopy (CD) and fourier transform infrared spectroscopy (FITC) characterization of DMP1 showed that calcium can induce a structural change of the protein from random disordered structure into a more ordered structure (159). Small angle X-ray scattering and dynamic light scattering techniques also identified the oligomerization of
DMP1 with calcium binding (146,164). The self-assembly of DMP1 fragment induced by calcium binding was believed to provide a localized structural surface for initiation of the HA mineralization (161).

### 1.2.2.3 Enamel

Crystallization of enamel is primarily mediated by protein amelogenin, which was identified as the major matrix protein during enamel formation (165,166). Amelogenin is similar to DMP1 in the sense that it also goes through posttranslational splicing and interacts with other biomineralization proteins like enamelin, and it also participates in cell signaling pathways by interacting with other proteins like CD63 and LAMP1 (167,168). The primary sequence of amelogenin containing three domains and the role of each domain in the self-assembly of this protein during apatite mineralization was already discussed in section 1.2.1 of this literature review. Far ultraviolet circular dichroism spectroscopy (CD) (169), nuclear magnetic resonance (NMR) spectroscopy (170), and Trpophan fluorescence spectroscopy (171) analysis of amelogenin showed that its monomeric form was largely disordered without well-defined, continuous regions. The nanospheres of amelogenin oligomers were shown to have the central hydrophobic domain folded inside with the N and C-terminal exposed to the aqueous environment (171).

NMR spectroscopy and untracentrifugation analysis of amelogenin nanospheres identified a N-terminal α-helix-like segments and C-terminal PPII-helices in the oligomer, in which N-terminal domains interact in an ipsilateral manner (172). Recently, single molecule atomic force microscopic analysis together with molecular dynamic simulations of amelogenin and apatite interactions showed that the C-terminal carboxylic groups bind specifically to the
calcium ions on the (100) surface of crystallizing apatite (173,174). With the C-terminal domain having negatively charged residues exposed to the aqueous environment, the nanosphere can further interact with calcium phosphate precursors and further assemble into higher order structures that can serve as both nucleator and template for apatite crystals to grow (171).

1.2.2.4 Mollusk shells

As was discussed in section 1.2.1 of this review, some mollusks shells have two layers of calcium minerals: the calcite outer layer and the nacre layer of aragonite build on protein-polysaccharide matrixes (45). Acidic proteins have also been isolated from mollusk shells of different species by demineralizing the nacre layer and biochemical separation of the soluble proteins and silk-like-fibroin-polysaccharide (e.g., chitin) matrix (50,175,176). The nacre proteome n16 (n for the nacre) family proteins isolated from Pinctada fucata (the Akoya pearl oyster) is one such protein family that associates with the water-insoluble-matrix (176,177). More than twenty polymorphic forms of n16 family proteins have been identified and are shown to be actively expressed in recent years (176,178). These proteins can interact both with the protein-polysaccharide matrix and aragonite crystal surfaces (176,178). Taking the n16.1 protein identified by Samata et al as example (177), a highly acidic region containing a TDDD motif and other three acidic regions were identified from the primary sequence of this protein which was postulated to be potential calcium binders (177). Calcium carbonate modification domains were later identified from both the N and C-terminal domain of this protein (179). Interestingly, n16.1 itself inhibited the crystal formation when added at 10ug/ml in in vitro aragonite crystallization experiments, whereas it promotes the formation
of tabular aragonite crystals on the water-insoluble-matrix when the matrix was included together with n16.1 (177). This observation suggests interacts between n16.1 and the matrix that is crucial for it to function as a biomineralization protein. Recent studies showed that the N-terminal domain fragment of n16.1 termed n16N indeed can interact with both beta-chitin and nucleating aragonite mineral phase in vitro(180,181).

Secondary structural analysis of n16N showed that it takes random-coil conformation at lower concentration, and beta-sheet that is under equilibrium with the random-coil conformation at higher concentrations (179,182), which suggests that intermolecular interactions exists between monomers of n16N. Sequence analysis of 39 identified nacre proteins including the n16 family proteins by Dr. John Spencer Evans showed that all these proteins contain at least one or more regions known as intrinsically disordered peptides, and 95% of these proteins have interactive regions like amyloid-like motifs that enable them to either aggregate or bind with other proteins (181). Taken together, these observations suggests that n16 proteins are similar to amelogenin and non-collagenous proteins in the sense that they can interact with crystallizing ions, capable of self-assembly/oligomerization and structural rearrangement with either ion binding or intermolecular interactions.

1.2.2.5 Magnetotactic bacteria

Biomineralization of magnetic nanoparticles by MTBs is a genetically controlled process involving the coordinated functions of multiple proteins coded mainly by genes in mms, mag, and mam operons located on the MAI. Early biochemical analysis of magnetosomes from the model strain Magnetospirillum magneticum AMB-1 isolated three acidic proteins named Mms5, Mms6, Mms7 (MamD), and Mms13 (MamC) that were believed to tightly associate
with and regulate the morphology of the magnetite crystals. Although the primary sequences of these proteins are not homologous to known proteins in the protein data bank, they all have a LGLGLGLGAWGP motif in the hydrophobic N-terminal domain and an acidic C-terminal domain rich in hydroxyl or carboxyl containing amino acid residues (33).

Of particular interests is the Mms6 protein which was shown to promote the formation of magnetite in vitro (27,33,34,183). Mms6 have been shown to stoichiometrically bind Fe$^{3+}$ with a dissociation constant of $10^{-16}$M, which leads to conformational changes in the protein and allows it to bind more Fe$^{3+}$ (27). Mms6 self-assembles into multimeric micelles in aqueous solutions with its N-terminal hydrophobic domain buried inside and C-terminal domain exposed to solution (27). The C-terminal domain exposed outside the micelles may form small iron-complexing surfaces that can interact with crystallizing ions to initiate nucleation thus mediate crystal formation (27,183,184). However, the exact mechanisms of Mms6 self-assembly are still under investigation at this moment. It would be interesting to know how Mms6 changes structure with iron binding, and how iron binding at the exposed C-terminal domain affects the properties of the multimeric micelles (Chapter2 of this thesis).

In the meantime, Mms6 was isolated from magnetosome by dissolving the magnetosome membrane, and it co-purify with other magnetosome proteins (33). Although genetic analysis mms6 gene in magnetotactic bacteria showed that its deletion results in the missing of other magnetosome proteins like Mms5, Mms6, Mms7, and Mms13 from the magnetosomes (185,186), it is still unknown as of how Mms6 interacts with these proteins in vivo. Considering that there are also other magnetosome proteins involved in the formation of
magnetite nanoparticles, the *in vivo* network of multiple proteins for making these nano-sized magnets can be intimidating.

### 1.2.2.6 Multiple protein assemblies of biomineralization proteins

This complex systems of biomineralization are built on spatial and temporal control of protein-lipid and protein-protein assembly (187). The strategy of this kind of controlled multiple protein-protein assembly are commonly used in biomineralization systems. For example, the biomineralization of spicules in sea urchins involves the formation of a spicule matrix by various proteins, with functions ranging from membrane formation, mineralization, protein secretion control, signal transduction (188). Biomineralization of our bones or tooth enamels also involves similar spatial and temporal control of multiple protein assemblies (189-191). Due to the high complexity of these multi-protein assembly events that may occur during biomineralization, it is of great importance to understand how individual proteins function and interact with its counterparts in the biomineralization process. Indeed, the understanding of how a single protein collagen, which promotes the mineralization of bone, self-assembles into a supermolecular structure increases the understanding of biomineralization strategies like templating, structural guidance, and crystal growth inhibition at certain directions are used (192,193).

Despite the extensive genetic dissection of MAI on the magnetotactic bacterial genome, most of the structures and functions of magnetosome membrane proteins are largely unknown (9). Understanding how individual proteins are situated in the magnetosome membrane and how they function individually will allow us to learn basic principles of magnetosomal protein assembly thus guide our understanding of magnetosome biogenesis.
For example, it is of great interest to know how Mms6 interacts with lipid membranes and these magnetosome proteins \textit{in vitro}, which may provide some insights into the basic mechanism of how these proteins with multifunctional properties are coordinated into complex protein-lipid-mineral machinery. Hence, I have studied the mechanism of Mms6 self-assembly (chapter 2) and its biochemical activities on a membrane environment (chapter3, chapter4).

\subsection{1.2.3 Magnetotactic bacteria and magnetosomes}

Although the first observation and analysis of “magnetosensitive bacteria” were made by S. Bellini and documented in Italian early in 1963, this diverse group of bacteria only came to be realized by the scientific community 12 years later after Blakmore’s description of “magnetotactic bacteria” and their characteristic organelles termed “magnetosome” in 1975(194-196). Despite the fact that only a small number of MTB strains are available in the laboratory as axenic cultures due to their fastidious requirement for microaerophilic environments(104,197,198), it is now well established that they are taxonomically, physiologically and morphologically diverse(199-203).

With their diversity in more than one aspect, these magnetotactic organisms all contain the same “compass-like” organelles consisted of single-domain permanent magnetic crystals synthesized and arranged under precise biological control(199). Since late 1990s, with more than 20 years of technical improvements in microbiology(204), biochemistry, genetics and genomics (especially genomic sequencing strategies)(108,109,205-215), cell biology, and microscopy (e.g., cryo-EM and electron tomography)(216-224), the community has now
come to the stage of revealing the molecular and cellular mechanisms of how these organisms control this biomineralization process (217,225).

1.2.3.1 Biodiversity of magnetotactic bacteria

Magnetotactic organisms could be sampled and isolated from other microorganisms using simple equipments and a magnetic bar because of their magnetotactic properties. This allows the convenient identification of MTBs compared with other microbes, even the cultivation of most MTBs were not plausible because of their stringent requirement for microaerophilic environments(204). Due to the difficulty of getting enough axenic culture of MTBs comounded by the limited strength of traditional microbial separation and characterization techniques, the number and diversity of MTBs reported over the years represent no more than 1% of all magnetotactic bacteria in nature. Thanks to the progress of techniques like single cell isolation, genomic sequencing, culture-free genomic sequencing, metagenomic analysis, and comparative genomic analysis, it has been shown that MTBs are much more diverse than previously expected (201,226).

Known as fastidious microaerophilic creatures, MTBs are cosmic when talking about their distributions in different environments. They have been observed and identified from different geological and microbial habitats, ranging from fresh water ponds, stratified water columns, and brackish water to deep marine environments. Alkaliphilic MTBs were identified in aquatic environments with pH around 9.5 (227). Thermophilic MTBs were recently observed from hot springs with temperature up to 63oC(228). More other kinds of magnetotactic extremophiles are believed to exist waiting to be identified in the near future(229).
MTBs are also morphologically diverse. These gram-negative flagellated organisms include cell shapes of spirilla, vibrios, bacillus, cocci, bean-like, and even multicellular assemblages (230-232). As more and more various types of MTBs are identified and characterized through new techniques like culture-free metagenomic analysis of total MTBs populations in various environments, the origin and evolution of MTBs might be finally revealed as research efforts continues in this field(201).

Understanding of how these MTBs in different environments, alkaliphilic MTBs, control the biomineralization of magnetosomes with different morphologies may provide valuable insights into the chemistry of bio-controlled mineralization. For example, an exceptional strain of magnetotactic bacterium producing both greigite (Fe3S4) and magnetite (Fe3O4) crystals were discovered by Professor Bazylinski in 1995. The controlled crystallization and alignment of two chemically different crystals into a single magnetic dipole in one cell, together with the species specific morphologies of magnetic particles synthesized by various strains, lead to the early hypothesis that biological control of magnetosome biogenesis including the processes of crystallization and chain alignments (233).

Going with the biological diversity of these bacteria is the diversity of the magnetic crystals (magnetite or greigite) they create and the ways they arrange these crystals in the cell(225). Iron oxide crystals of various morphologies and chemical constituents have been identified in different groups of MTBs, most of which are single domain magnetic crystals with shapes, arrangements, and magnetic properties under precise genetic control (234,235).

Although majorities of magnetite particle shapes are octahedral and hexahedral from alpha-Proteobacteria, various shapes of magnetic crystals have been identified from other
branches of Proteobacteria (199). So far, magnetic crystals of octahedral, tooth-shaped, prismatic, bullet-shaped, octahedral, and hexahedral shapes have been identified from either cultivated or uncultivated MTBs (236). More interestingly, sulfate reducing bacterium Desulfovibrio magneticus sp. RS-1 (RS-1) were recent shown to have iron-phosphorus rich granules compartmentalized by cell membrane, whereas their bullet-shaped magnetosomes are actually not membrane-surrounded. This observation might lead to the finding of a new pathway of magnetosome biogenesis, and also add one more kind of bacterial organelle besides magnetosome (237). The various types of magnetic crystals biomineralized under genetic manipulation of crystallization process serve as great example of controlling crystallization chemistry for specific applications, which is the final goal of chemical synthesis.

1.2.3.2 Biogenesis of magnetosome: cell biology and physiological control

MTBs attract interests of researchers from biology, chemistry, material sciences, physics, geosciences, and more other fields because of the magnetic and physical properties of these magnetic crystals they synthesize. Great efforts have been focusing on the mechanism of how these bacteria exert precise control over the chemical process of iron oxide biomineralization. The idea that species specific properties of magnetites suggesting genetic control of magnetosome formation was proposed early, but experimental evidence were not enough until recently with the development and application of advanced biological and microscopic imaging techniques (233,238,239).

Due to the difficulty of culturing these fastidious microaerophiles and lack of tractable genetic system, genetic analysis of MTBs were mostly studied based on only two species,
*Magnetospirillum magneticum* and *M. gryphiswaldense*, the genomic sequences of which are all currently available for genomic analysis. It is now established that a conserved genomic magnetosome island (termed Magnetosome Associated Island, MAI) is mainly responsible for the genetically controlled biomineralization of magnetic nanocrystals in the cell, with most of the genes from this genomic island encoding magnetosome associated proteins, including the critical *mms6* operon, *mag* operon, and *mam* operon (108-110). With the characterization of genes from MAI and proteins associated with the magnetosome membrane, the field has come to the stage of unveiling the overall steps that the cells may take to synthesize the organelle and arrange them into a chain-like structure (111).

In Blakemore’s early reports of MTBs, it was shown that the iron-rich particles imposing permanent magnetic moments to these cells were actually enveloped within “intracytoplasmic membrane vesicles” (194), and that the chemical components of these particles are magnetite (Fe₃O₄) as shown by Mössbauer spectroscopic analysis (240,241). However, how MTBs produce these vesicles as an isolated compartment for magnetite synthesis in the cell remained unknown until the establishment of stable axenic culture of these organisms together with a tractable genetic system. Growing *M. magneticum* in iron limited medium results in empty magnetosome vesicles originating from invagination of the inner cell membrane (242). Cryo-electron tomographical analysis of these MTBs showed that these invaginated vesicles originating from cellular membranes were arranged into a chain by an actin-like protein named MamK (243). In *M gryphiswaldense* strain, a similar mechanism of membrane invagination and chain arrangement by cytoskeleton-like protein were observed (217,219). However, it is still under debate as for whether these vesicles stay attached with
the cell membrane or pinch off as detached vesicles with the maturation of magnetite crystals (111).

Invagination of cellular membrane is also accompanied by sorting of magnetosome specific proteins inside the magnetosome as shown by both biochemical and genetic analysis of magnetosomes. Firstly, being permanent nano-magnets, magnetosomes could be easily separated from the cell lysate with the intact magnetosome membrane (MM) surrounding the magnetite crystals, allowing for biochemical analysis of the MM separately from the cellular membrane (244). A number of proteins have been identified with this strategy from the MM that are unique for the magnetosome and not found in cytoplasmic membrane, including Mms6 which is capable of producing magnetite nanoparticles \textit{in vitro} (213,245,246).

With the development of site-directed mutagenesis methods and transformation system in MTBs (247,248), combined with the use of fluorescent proteins and microscopic techniques, by characterizing critical genes coded by the \textit{mms6}, \textit{mag}, and \textit{mam} operons, the field has been able to characterize a series of proteins associated with the magnetosome with various functions.

For example, MpsA and MpsB gene product might be involved in the membrane invagination, possibly with hydrolysis of GTP by Mms16 (249); MamA protein self-assembles into a protein network surrounding the cytoplasmic side of magnetosome, functioning to interact and recruit other magnetosome associated proteins (250); MamB and MamM protein are cation diffusion facilitators functioning to transport iron into the magnetosome chamber (10); Mms6 and MamGDCF might interact directly with iron and crystal surface to regulate the nucleation and crystal growth (251-253). Fig.1-1 shows a
model of a step-wised magnetosome biogenesis based on recent genetic analysis of genes from the MAI. Proteins involved in this stepwised process will be reviewed in detail in section 1.2.4.3 based on their structural and functional roles in each step, focusing on how the structural properties of these proteins determine their functional roles in magnetite biomineralization.

1.2.4 Membrane proteins in magnetosome biogenesis

Cellular cytoplasms are enveloped by lipid bilayer membranes that participate in various cellular activities (254). The diverse functions of cell membranes are determined by the various proteins that are either integrated into the membrane or just associated with either one leaflet of the bilayer membranes (255). Based on sequence analysis of multiple genomic sequences, ~20-30% of all genes in their analyzed genomes may encode integral membrane proteins (256). Membrane proteins play important roles in various cellular events, ranging from structural support, ion channel, molecular transporters, enzymes of various functions, and signal transduction (254,257).

Membrane proteins differ from other proteins in that they have specific structural properties that enable them to integrate into bilayer lipid membranes. Understanding the structural and functional properties of these important proteins and how they interact with their lipid environments is crucial for understanding the mechanisms of biological events governed by these protein-membrane complexes (258). Insights into membrane protein structural and functional properties also have important biomedical significances, since many human diseases are known to be caused by abnormalities of membrane proteins (255). Over recent decades, with progress in molecular biology, cell biology, genomics, biophysics,
biochemistry, bioinformatics, and various techniques for characterization of protein and membrane systems, more membrane proteins are being investigated in detail for their structural and functional properties. General properties of membrane proteins and the mechanisms by which they integrate into lipid bi-layers are becoming revealed and understood in depth (259). I will firstly review the common features of membrane proteins and model lipids for studying membrane proteins \textit{in vitro}. Then, I will explore what is known about the roles of important membrane proteins in biomineralization of magnetite in magnetotactic bacteria, and how these membrane proteins interact with other proteins and their lipid environments to regulate biomineralization.

\subsection*{1.2.4.1 Membrane proteins function optimally in the correct lipid environments}

The biological functions of cellular membranes depend on the proteins associated with them and the functions of membrane proteins depend on the lipid and protein environments surrounding them (260). Integral membrane proteins directly interact with lipid molecules, and also with other membrane proteins (258). These protein-lipid and protein-protein interactions are important for the folding of a membrane protein into its correct structure for specific biological functions (261,262).

\subsubsection*{1.2.4.1.1 Membrane proteins fold into their native structures in lipid bilayers}

The folding of membrane proteins in lipid membranes is driven primarily by hydrophobic interactions (254,263,264). Transmembrane domains of integral membrane proteins are rich in hydrophobic residues, and these transmembrane domains are embedded in lipid bilayers with secondary structures of either alpha-helices or beta-sheets, in which hydrophobic
residues are exposed to the hydrophobic portion of the membranes that is dominated by acyl chains (254,265,266). When they are removed from their membrane environment by the use of organic solvents, membrane proteins tend to aggregate and form insoluble precipitates because their extensive hydrophobic residues become exposed to an aqueous environment (254,267-269). Some membrane proteins of smaller molecular weight may not denature and precipitate, but instead aggregate into multimeric complexes with the hydrophobic residues buried inside the complex (268). Without the correct lipids or detergents to shield the hydrophobic domains, the structures and functions of membrane protein are likely to be adversely affected (270-272). Detergents or lipids are routinely included in the buffers for purification of membrane proteins to prevent aggregation (273).

The polar residues that also exist in trans membrane domains (258,265) are normally folded inside the protein core in lipid bilayers (265,274). Often these polar residues have specific functions such as forming ion channels, ligand binding pockets, activity center, structural stabilization, or other regulatory activities (275-277). Sometimes, polar residues of membrane proteins are exposed to the hydrophobic lipid-bilayers, and they are often involved in interactions with other membrane proteins or formation of multi transmembrane structures (276,278). Hydrophilic residues outside the transmembrane domain or at the lipid-protein interfaces can interact with the head groups of lipids through hydrogen bonding, water bridges, or electrostatic interactions (279,280).

The fluid mosaic model of membranes proposed by Singer and Nicolson in 1972 reasonably describes a typical cell membrane in which lipid bilayers forms a fluidic matrix that allows both integral and peripheral membrane proteins to interact with lipids of the
membrane (254,260). In this model, membrane proteins float in the membrane matrix, with their hydrophobic residues interacting with the hydrophobic acyl chains inside the lipid-bilayers and their polar residues exposed outside the membrane or interacting with head groups of lipids (254,259). The model also emphasizes the importance of protein-lipid, and protein-protein interactions in determining structure and functions of membrane proteins (254).

Structural characterizations of membrane proteins with and without lipids or detergent micelles showed that membrane proteins require the hydrophobic membrane environment, where hydrophobic acyl chains are abundant. The acyl chains shield the hydrophobic residues of the proteins for them to fold into the correct structures (258). Analysis of various membrane proteins with specific biological functions, such as ion transporters, signal transduction proteins, enzymes, and even membrane insertion peptides, showed that these transmembrane proteins all required the presence of a hydrophobic lipid or detergent environment in order to fold into the right structure to maintain their biological functions (281,282). Due to the difficulty of preparing membrane proteins for crystallographic studies, there is still only a limited number of membrane proteins that have been structurally characterized (283). Hence, a lot of effort is being devoted to the development of new detergents and preparation strategies for membrane proteins (255,284-286).

1.2.4.1.2 Membrane proteins require specific lipids for proper functioning

Simplifications of the fluid mosaic model tend to ignore the complexity and diversity of protein-lipid interactions in nature (259,287). Studies of various membrane proteins and their folding showed that the protein-lipid and protein-protein interactions on membranes of
cellular organisms are more complex than the traditional fluid mosaic model (261,288). Correct folding of membrane proteins from various cellular organisms into their functional conformations sometimes have specific requirements for certain lipid constituents in their membrane to fold into their active structure (264,273,274,289,290).

\textit{i. Head group specificity}

Membrane lipids can be categorized into nonionic, zwitterionic, anionic, and cationic based on the polarity of their head groups under physiological conditions (259,288). Polar residues can interact with the head groups of lipids through hydrogen bonding, water bridges, or electrostatic interactions (258). These specific interactions between membrane lipid can restrain the lateral movement of lipids on the membrane, and determine if the membrane protein can fold or remain folded in the native conformation after being embedded into membrane bilayer (291,292). Correct protein folding depends on the charges of amino acid side chains that interact with specific lipid head groups to form the correct constellation of hydrogen bonding, water bridge, or charge-charge interactions (292-294).

Head group sizes of membrane lipids vary and the proportion of different sized head groups in a membrane affects the self-assembly and packing of individual lipids (295). The packing of lipids on the membrane further affects the structure and function of the integrated membrane proteins (295). The effects of head group sizes on lipid membrane packing and morphologies can be demonstrated in artificial lipid systems like micelles, bicelles, and liposomes (295).

\textit{ii. Acyl chain specificity}
Properties of acyl chains of membrane lipids also affect the properties of membranes and the folding of membrane proteins (295). First, the saturation of acyl chains in lipid membranes affects the fluidity of membranes, which in turn affects the functionality of membrane proteins. Second, the length of acyl chains on each individual lipid determines the thickness of lipid bilayers formed with these lipids. Membrane proteins situated in lipid-bilayers whose thickness’ exceed or are less than the length of trans membrane domain will not fold correctly (296). Additionally, both the chain length and saturation of acyl chains of lipids will affect the phase transition temperatures of lipids (295,297), which determine the properties of membrane and membrane protein folding and function in the membrane (263,270,295).

1.2.4.1.3 Lipid rafts formed by protein-lipid complexes

Studies over the past two decades have revealed that membrane structures are more complex than described in the fluid mosaic model. One example of this additional complexity is the presence of lipid rafts and microdomains on eukaryotic cell membranes that are formed by specific protein-lipid and protein-protein interactions (298-301). Lipid rafts are transient but stable (originally identified as detergent resistant membranes) microdomains on eukaryotic cell membranes involved in diverse biological functions such as cell signaling and molecular trafficking across the cell membranes (302-304). They are rich in cholesterol, membrane proteins of specific functions (both integral and peripheral proteins), and specific types of lipids (e.g., sphingolipids and glycosylphosphatidylinositol) (300,304). Recently, lipid rafts were also found in prokaryotic cell membranes with similar structural and functional properties to their eukaryotic counterparts (305,306).
Lipid rafts and microdomains have been found to have extensive interactions with cytoplasm components and cytoskeleton elements (304,307-310). The lipid raft model revealed the complexity of protein-protein and protein-membrane interactions in cell membranes and expanded our view of cellular membrane from the two dimensional fluid mosaic models of membrane structure to a three dimensional membrane system that is integrated with cytoplasmic and cytoskeleton elements (311-313). This new view of membrane model, which takes into consideration lipid rafts and various functional microdomains on cell membrane, provides an intriguing basis for understanding how magnetosome membranes and membrane proteins are assembled to coordinate the controlled biomineralization of iron oxides. Considering that magnetosomes are membrane-enveloped prokaryotic organelles with direct interactions with the bacterial cytoskeleton (218,219,314-318), they might be better model organisms for studying of membrane assembly and the interaction of membrane structures with cytoskeletons than eukaryotic systems like yeast or mammalian cells. Their advantage lies in their simplicity as prokaryotic organisms, conserved MAI genomic island for magnetosome biogenesis that allows for easier molecular manipulation (109), well established cloning systems (318), and available genomic sequencing data for a number of model strains(206,209,211,212,319).

**1.2.4.2. Artificial model membranes for characterization of membrane proteins**

Considering the complexity and specificity of protein-lipid interactions on cellular membranes, great caution should be taken when designing experiments for characterizing membrane proteins (270). Thanks to the development of various model lipid membranes and detergents that can be utilized for studying of membrane proteins, our investigation of
membrane proteins can be conducted within a simpler environment than the cell membrane (272). Although these artificial lipid systems are much simpler than natural cellular membranes, structures of membrane proteins obtained with these artificial lipids still provide valuable information about structure-function relationships (320). Model membranes based on artificial phospholipids have been developed for characterizing membrane proteins, including lipid/detergent micelles, monolayers (321,322), planer bilayers (273,321,323,324), liposomes (325,326), bicelles (327-330), and nanodiscs (331,332). Depending on their dimensions, liposomes are subdivided into small unilamellar vesicles (SUVs, 20-50nm), large unilamellar vesicles (LUVs, 50nm-100um), and giant unilamellar vesicles (GUVs, 5-300um) (259,333). Here I will briefly discuss the properties of each model membrane and their application in studying membrane proteins.

1.2.4.2.1 Micelles

Micelles are formed by self-assembly of detergents above their critical micelle concentrations in aqueous environments. The formation of micelles is determined by multiple factors such as the chemical structure of the detergent, temperature, and ionic strength (especially for ionic detergents) (283). Detergent or lipid micelles are widely used for solubilization of membrane proteins in the early stage of protein extraction from native cellular membranes (283,324). Membrane proteins solubilized by detergents form mixed micelles with their hydrophobic transmembrane domains shielded inside micelles (324). However, micelles also have drawbacks for studying membrane proteins. First, micelles of protein-detergent complexes tend to have strong curvatures that may distort the structure of proteins. Second, membrane proteins reconstituted into detergent micelles are not ideal for
high resolution X-ray crystallography analysis since the micelle structure is more disordered than other model membranes (259). On the other hand, with the development of new detergents in recent decades, more novel detergents or detergent substitutes specifically designed for membrane protein characterizations are available commercially, which resulted in improved solubilization and characterization of membrane proteins (273,334-337).

1.2.4.2.2 Monolayers and planar bilayers

Monolayers and planar bilayers are advantageous for membrane protein study in that they are free of curvatures that may distort the protein structures (321). Monolayer lipid membranes are commonly generated by spreading lipids of interests on a liquid surface held in Langmuir trough (338). Membrane proteins with monolayer can be analyzed for their packing and assembly with specific lipids by measuring surface pressure isotherms (339). The interaction of membrane proteins with their ligands, substrates, or other membrane proteins can be studied by introducing these compounds in the aqueous phase underneath the monolayer (339). Planar bilayers were widely used for characterization of transporter or ion channel proteins when combined with patch clamps techniques (340-342). Supported bilayers are more suitable for direct observation of membrane proteins and membrane structures with microscopic and surface characterization techniques (343,344).

1.2.4.2.3 Lipid vesicles/liposomes of different sizes

Liposomes are sealed bilayer vesicles suspended in aqueous solutions. There are multiple ways of preparing liposomes with various sizes from 10nm up to1000nm in diameters (345) for different applications (326,346). In addition to their widespread application in drug
delivery and pharmaceutics (347), liposomes are useful model membranes for membrane protein studies (348). The complexes of membrane proteins reconstituted into liposomes are named proteoliposomes, and they are used for a wide range of biological studies, such as protein folding, membrane fusion, ion channel characterization, protein-lipid interaction studies (269,288,325). Worth noting are the giant unilamellar vesicles (> one micron) that are cell-size liposomal vesicles (345). These giant vesicles made of lipids of known chemical constituents are excellent candidates for studying membrane properties (e.g., microdomain formation, phase separation, membrane fusion, etc) (349-352), protein-protein & protein-lipid interactions (353-355) with the ongoing improving microscopic techniques.

1.2.4.2.4 Bicelles

Bicelles are prepared with a combination of long chain and short chain lipids. They are model bilayer membranes with the long chain lipids forming the planar bilayer portion and short chain lipids forming the rims to surround the discoid planar region (356-358). The microscopic morphologies of bicelles can be controlled by varying the molar ratio of long-chain lipid to short-chain lipid (q value) and the total lipid concentration (CL) (327,330,359). Bicelles at higher q value and concentrations (q>3, CL>15%) are commonly used in solid state NMR analysis of membrane lipids since they can be aligned by external magnetic field. At q value and CL (q<1, 5%<CL<15%), size of bicelles become smaller and they become isotropic free-tumbling discs that are not aligned by an external magnetic field (329). Isotropic bicelles are ideal for characterizations of membrane proteins by other than solid state NMR, e.g., solution state NMR, fluorescence measurements, circular dichroism spectroscopy, and enzymatic activity analysis (327-330,359).
1.2.4.2.5 Nano discs

Nano discs are lipid bilayers made of lipid and membrane scaffold proteins (360). Their sizes and compositions can be manipulated by engineering the membrane scaffold proteins and/or changing the lipid composition (361). The planar lipid bilayer in the middle of nanodiscs and their stability and high solubility in aqueous solutions make them good model membranes for studying membrane proteins by NMR, crystallography, and spectroscopic techniques (362).

1.2.4.3 Biomineralization mediated by membrane proteins in magnetotactic bacteria

Magnetotactic bacteria may be good model microbes for studying membrane biology and protein-lipid interactions due to their uniqueness as prokaryotic organisms with complex organelle-like structures. Magnetotactic bacteria are well-known for producing magnetosomes, unique prokaryotic organelles that consist of magnetic crystals enclosed within membrane vesicles that are aligned as intracellular chains along the cells (194, 199, 217). Magnetosomes enable these bacteria to passively orient in the geomagnetic field and help with the path-finding, a process named as magnetotaxis (363, 364). What make these organisms intriguing are the magnetic nanoparticles they produce in the magnetosomes under ambient conditions with precise control of particle morphologies and magnetic properties (316, 365-367). A lot of research effort has been devoted to these bacteria to understand the biochemical and molecular mechanisms of biomineralization in these nanosized magnetosome vesicles. One of the critical goals of this research is to learn the strategies by which magnetite nanocrystals are synthesized naturally at lower energy cost and better control of particle properties (368, 369).
The fact that magnetosomes are membrane-enveloped structures was firstly revealed by transmission electron microscopy (195). Biochemical analysis of the magnetosomes separated from other cell contents showed that the magnetosome membrane, similar to the cell membrane, contains three major groups of lipids (neutral lipids and free fatty acids, glycolipids and sulfolipids, phospholipids in a weight ratio of 1:4:6), with phosphatidylethanolamine (PE) and phosphatidylinerine (PS) identified as the major components in the phospholipid groups (245). Two proteins, unique to the magnetosome membrane with molecular sizes of 15 kD and 16.5kD, were identified in this study and were proposed to function specifically in magnetosomes. They were believed to be negatively charged proteins since they migrate anodically in the first dimension of two-dimensional PAGE analysis (245). Later efforts to isolate proteins from magnetosomes resulted in the identification of more proteins that are specific to magnetosome membranes, e.g., magnetosome associate protein MpsA (246), a small GTPase Mms16 (249), magnetite binding proteins Mms5, Mms6, Mms7, and Mms13 (33). As for the lipid profile of magnetosomes, beside the confirmation of previous observations that the magnetosome membrane is rich in PS and PE and is not much different from the cell membrane, several amide-linked fatty acids that were normally found in outer cell membranes were missing in the magnetosome membrane (370). How the magnetosome membrane is formed and the proteins assemble and coordinate functionally on the membrane for iron oxide biomineralization has been clarified by genetic analysis of these bacteria (109,199,205,242,318,371).

The conservation of magnetic nanoparticle properties in specific strains of magnetotactic bacteria was indicative of a biomineralization mechanism that is controlled by genetics
This was confirmed by the identification of a conserved MAI genomic island from magnetotactic bacteria that encodes most of the genes responsible for magnetosome biogenesis (109). The development of gene transfer, mutagenesis, and related molecular cloning techniques in magnetotactic bacteria over the recent decades allow characterization of genes encoded by the MAI genomic islands (207,374-377). Thorough genetic analysis of operons on the MAI genomic island identified *mamAB, mamGFDC, mms6*, and *mamXY* operons, which are conserved among most magnetotactic bacteria, as involved in the stepwised magnetosome biogenesis (378-380). As represented in Fig.1-1 magnetosome formation is under genetic control, with more than one protein involved in each step (381). In the following paragraphs, membrane proteins that have been identified and characterized from magnetosomes will each be reviewed for their roles in the stepwise biomineralization process with a focus on their structural self-assembly and functional properties on the magnetosomal membranes.

**1.2.4.3.1 Magnetosome membrane formation: protein sorting, vesicle invagination, and chain formation**

The generation of magnetosome vesicles from the inner cell membrane precedes the crystallization of iron oxides in magnetotactic bacteria (242). Proteins in *M. gryphiswaldense* involved in vesicle biogenesis and protein sorting are encoded mostly (MamY as the only exception) by the *mamAB* operon (Fig.1-1), which is viewed as the most essential operon for magnetosome formation due to the lack of magnetosome vesicles in the deletion mutants (382).
The mamL gene product is a 123aa protein whose deletion by mutagenesis resulted in cells missing magnetosome vesicles (213). Structural prediction identified transmembrane alpha-helices in MamL but no experimental data yet is available for its structural characterization (383). mamQ deletion also results in a lack of magnetosomes (213). The function of MamQ is unknown, but structural prediction suggests it is an integral membrane protein with a transmembrane N-terminal domain and a C-terminal helix-turn-helix domain that forms a negatively charged surface in the magnetosome lumen (383). The MamY-GFP fusion protein localizes close to the magnetosome membrane in early stage of magnetosome formation, which suggests its close interaction with either the magnetosome membrane or proteins on the magnetosome membrane facing the cytoplasm (384). MamY mutant cells can still form magnetosomes but the vesicles are enlarged and the magnetic particles are smaller (384). It was predicted to be a BAR domain containing protein, and this is consistent with its potential role of interacting with magnetosome membranes during vesicle invagination (383,384). MamB is a multifunctional transmembrane protein. It is a putative iron transporter belonging to the cation diffusion facilitator (CDF) family with the typical six transmembrane domain helices forming the transmembrane domain. It is also believed to be involved in magnetosome membrane formation since its deletion mutant did not produce magnetosome vesicles (10,213). MamB forms a dimer that is stabilized by MamM (another CDF transporter on magnetosome membrane) and also interacts with the PDZ domain of MamE by its putative TPR recognition signature in the C-terminal domain (10,135).

Following the invagination of inner cell membranes mediated by the proteins mentioned above and possibly others as-yet unidentified, more proteins are believed to be recruited onto the magnetosome membrane for the biomineralization of magnetites (381). MamE is
essential for magnetosome biogenesis and was proposed to be involved in protein sorting because the deletion mutant of MamE only produced empty magnetosome vesicles without magnetite synthesis. Dislocation of other magnetosomal proteins like MamA and MamI was also observed in MamE deletion cells (213,385). MamE has a single transmembrane domain in the N-terminal region, which is believed to be responsible for its anchoring onto the magnetosome membrane (200). Following the transmembrane domain is a putative serine protease domain with a conserved catalytic His-Asp-Ser center (383,385). The protease domain is believed to be involved in its processing of magnetosomal proteins involved in later stage of magnetite biomineralization since mutation of the protease motif did not affect the earlier stage of magnetite particle formation in the magnetosome (135) and site-directed mutagenesis of the catalytic center for protease domain in MamE resulted in the production of smaller magnetites without affecting the cellular localization of other proteins (135). Downstream of the protease domain is a C-type cytochrome domain with a heme-binding motif, the mutation of which results in the production of smaller magnetite particles (135). The cytochrome domain is believed to participate in the regulation of oxido-reductive potential of magnetosome vesicles during magnetite crystallization (135,200,386,387). The C-terminal end of MamE contains one or two putative PDZ domains (134,135,200), which are known to be involved in inter-protein interactions, functional regulation of proteins and protein cellular localization (383). However, like most other magnetosome membrane proteins, the means by which MamE interacts with the membrane and how its protease, cytochrome, and PDZ domains coordinate to interact with other proteins and regulate multiple aspects of magnetite production are still unknown.
As a highly conserved magnetosome protein among all identified magnetotactic bacteria, MamA assembles into a “coat” surrounding the magnetosome vesicles and also attaches to the magnetosome chain (223,388). Although it is not an integral membrane protein, MamA self-assembles by way of its TPR domain into a shell like structure surrounding the magnetosome membrane, which creates a charged surface facing the magnetite that is believed to provide an assembly platform for other magnetosome proteins (242,379,388,389).

Although only MamE and MamA are shown in Fig.1-1 as proteins involved in protein sorting on the magnetosome, this process also involves other proteins like MamO, MamN, MamE and perhaps other proteins (9,135). Studies of these proteins clearly showed that the assembly of proteins and membrane structures start playing their roles at the first step of magnetosome biogenesis. But, how the cell controls the expression of these genes, how each protein is expressed and relocated to the magnetosome membrane, and how they assemble into the complex of magnetosome vesicles remains unknown.

Although the roles of MamJ and MamK in magnetosome biogenesis are associated with the last step of magnetosome biogenesis (Fig.1-1), the arrangement of magnetosome vesicles into a chain was actually observed even without mature magnetites (317). MamJ and MamK are not integral membrane proteins, but they interact with other magnetosome-associated proteins to help arrange the magnetosome vesicles into the chain structure (317,390).

1.2.4.3.2 Membrane proteins for iron uptake and nucleation

Magnetosome vesicles provide compartmentalization for magnetite crystallization, which means that iron ions (Fe$^{3+}$ and (or) Fe$^{2+}$) need to be transported into the vesicle by specialized
proteins for the biomineralization to proceed (9). MamH is also encoded by the *mamAB* operon, and its deletion mutant produces fewer magnetosomes with smaller magnetite crystals (213,380). Structural prediction of MamH reveals its homology to the Major Facilitator Superfamily (MFS) proteins, which are known to be transmembrane transporters (109,380,383,391). MamH was believed to be an integral membrane protein functioning either to transport iron across the magnetosome membrane for magnetite formation (380), or transport other ions such as phosphate or protons for regulating the electron redox potentials in the magnetosome vesicles (383,392). MamZ shares a similar MFS domain with MamH, and the mutant of MamZ produces a limited number of wild-type-like magnetites in magnetosomes close to the center of cell body and also smaller magnetites with needle shaped or flake-like structures in magnetosomes close to the ends of cell body (380). Interestingly, a *mamHZ* double mutation has more severe effects on magnetite formation, the biomineralization of which is greatly jeopardized with very few cells able to make regular magnetites (380). It was proposed that the MFS domains on MamH and MamZ may have redundant functions for regulating ion flow in magnetosome vesicles (380), and this hypothesis is consistent with the phenotype of *mamH* and *mamZ* mutants. In addition to the MFS domain, MamZ has a C-terminal ferric oxidoreductase domain (YedZ-like), and it is believed to be involved in the regulation of redox potential on the magnetosome membrane by interacting with other magnetosome membrane proteins including MamX and MamH (380). The deletion of the YedZ-like oxidoreductase domain leads to the same effects as deleting the full length *mamZ* gene in the mutant cells, which suggests that this domain is important for the protein’s function to regulate biomineralization (380). The C-terminal domain of mamZ was also predicted to be similar to cytochrome bc1 (383). However, more
biochemical experimental evidences are needed to test if the predicted functions of these domains are correct and also to show the assembly of mamHXZ multiple-protein complex on the magnetosomal membrane.

In addition to the two putative MSF proteins mentioned above, MamM and MamB were also shown to be ion transporters on the magnetosome membrane belong to the Cation Diffusion Facilitator (CDF) protein family (10). The deletion mutant of MamM only produces empty magnetosomes without magnetite biomineralization (10). CDF domains of both MamM and MamB are believed to be involved in iron transport across the magnetosome membrane via a proton-coupled antiport mechanism since the mutation of both genes individually results in impaired biomineralization of magnetite but not the accumulation of iron in cytoplasm (10). Structural analyses of MamM showed that the cytoplasmic C-terminal domain of MamM, which forms a V-shaped dimeric structure that changes with binding of cationic ions. This conformational change is proposed as important for the protein to adapt the right conformation as a transporter (393). However, the transporter activity of MamB and its role in biomineralization is still not characterized. Structural predictions identified a negative pocket in C-terminal domain of MamM that is not observed in MamB. This difference may explain why they function differently in vivo as shown by the phenotypic differences of their mutants (383). However, more experimental data is needed to confirm the predictions. Besides their proposed function as iron transporters, MamM and MamB interact with other magnetosome proteins and these interactions also appear to be important for the controlled biomineralization (10). This again brings the question of how these many proteins are expressed and located onto the
magnetosome membrane and how they interact with each other to assemble into the functional membrane-protein complex that controls the biomineralization of magnetites.

MamO is believed to be involved in the nucleation of nanoparticles in magnetosome because *mamO* deletion cells only produce empty magnetosome vesicles (134,213). Structural prediction showed that MamO has a trypsin-like peptidase N-terminal domain (135). A mutant form of MamO generated by site-directed mutagenesis of the predicted peptidase active site triad residues into alanine can still complement the *mamO* deletion mutant (135). The identification of other functionally equivalent peptidase genes named *LimO* (Like MamO) in the MAI suggested that the peptidase mutation effect of MamO might be complemented in trans by LimO. Indeed, the *mamO* deletion strain without the *limO* gene can only be restored by wild type MamO but not by peptidase mutant of MamO (135). Although biochemical evidence is still needed to prove that this N-terminal domain has peptidase activity, the above observation indicated that MamO might have *in vivo* protease activities (135). MamO also has a C-terminal transmembrane domain of unknown function (DUF18) (134,135), which was suggested to be an anion transporter or localization determinant to recruit other magnetosome proteins (135). MamI from *M. gryphiswaldense* was proposed to be involved in the early nucleation of magnetites because the smaller iron-rich, but poorly crystalline particles, synthesized in the mutant cells were found to be hematites, a possible precursor for the mature magnetite in wild-type cells (394). However, MamI from *M. magneticum* AMB1 was proposed to be involved in magnetosome membrane invagination because GFP-MamI localizes close to the magnetosome membrane and its deletion mutant cells of *M. magneticum* AMB1 fails to produce magnetosome vesicles (9).
Despite extensive genetic analysis of the in vivo functions of these potential transporters and nucleation proteins on the magnetosome membrane, most of these proteins need more detailed biochemical characterization to confirm their proposed biological functions. It is also important to understand the biochemical and cellular mechanisms of how these proteins assemble on the invaginated inner cell membrane and coordinate to regulate the early stages of biomineralization.

1.2.4.3 Membrane proteins for crystal growth and maturation

MamE, a multifunctional protein with a role in protein sorting and interaction with other proteins (135) with a putative CXXCH heme binding domain is believed to coordinate with other CXXH motif containing proteins including MamP, MamT, and MamX to regulate the electrochemical potential of magnetosome membrane during the growth of magnetic nanoparticles (200,386,387). The structural profile of MamP is similar to that of MamE (200). MamP also has an N-terminal transmembrane domain, which was termed as magnetochrome domain because it is a “c-type cytochrome domain specific to magnetotactic bacteria”, and a PDZ domain. MamP was initially thought to regulate crystal number and size in the bacteria because its deletion from the M. magneticum AMB1 strain resulted in nonmagnetic cells with fewer magnetosomes but larger magnetite crystals (200). MamP without the transmembrane domain from Magnetotactic ovoidal bacterium MO1 strain was crystallized and structurally characterized, which revealed that the PDZ domain of MamP may be involved in its dimerization (386). The structural analysis identified a crucible-like acidic pocket in dimeric MamP. When the acidic residues were changed to alanine the resulting cells showed magnetite size defects and reduced magnetic responses (386). The
structure of MamP also revealed a highly solvent-exposed heme-binding domain characteristic of magnetochromes (386). Further in vitro mineralization experiment with MamP suggested that the protein may have ferrous oxidase activity; hence it was proposed that the biomineralization of magnetite starts with oxidation of ferrous iron in the magnetosome, contrary to the partial reduction of ferrihydrite mechanism proposed by Dr. Frankel and Blakmore (386,395). However, this model fails to explain the predominant presence of ferric irons in the magnetosome that was observed in the early stage of magnetite biomineralization (396-398), and direct evidence that MamP was not localized to the magnetosome membrane at that early time. Since then, MamP from M. magneticum AMB1 has been found in cell membrane fractions instead of in magnetosome membrane fractions. As a result, it was proposed that MamP might play a role in the synthesis of iron storage minerals or in the conversion of storage irons into irons that can be used in magnetite biomineralization (399). The localization of MamP on the cell membrane also brings up questions of whether MamP interacts with MamE and how they form the proposed electron transfer complex on magnetosome vesicles.

The structural and biochemical properties of MamT and MamX as magnetochrome proteins are still unknown, but they share a similar structural profile with MamE and MamP having an N-terminal transmembrane domain and a cytochrome domain (200,213,383,386). In MamT deletion cells, the magnetosomes contain smaller particles than wild-type (213); whereas MamX deletion cells are not significantly different from wild type cells except the superparamagnetic nanoparticles produced by them are of irregular shapes (380,400). More experiments are needed to understand how these magnetochrome proteins assemble on the
magnetosome membrane into an electron transfer complex that can regulate the oxidoreductive states of iron.

MamN is homologous to a sodium/proton antiporter and its deletion results in empty magnetosome vesicles, which indicated that MamN’s involvement in biomineralization might relate to ion transporters, nucleation, or maintenance of correct microenvironment in magnetosomes that is crucial for controlled biomineralization (213).

MamR was believed to be involved in the control of magnetite crystal size and numbers in magnetotactic bacteria since its mutation (with simultaneous mutation of a functional redundant gene of mamR named amb1006) resulted in the formation of smaller particles and shorter magnetosome chains (213). mamS deletion cells synthesize magnetosome vesicles with multiple magnetic particles clustered together; hence it is suggested to be involved in the post-nucleation steps of magnetosome formation (213).

For the proteins discussed above, which are believed to be involved in the crystal growth and maturation steps of biomineralization, the evidence is mostly based on the genetic and phonotypical characterizations of their deletion strains. The various phenotypes of these deletion strains suggest that the Mam proteins may function at different steps or aspects of the biomineralization process. Cell biology studies on how magnetotactic bacteria exert spatial and temporal control over the in vivo synthesis and localization of these proteins may help elucidate how the crystal growth and maturations are controlled by these proteins. In the meantime, biochemical and biophysical characterization of each protein will provide more information about how these proteins interact and biochemically function in vivo on
magnetosome vesicle and assemble into the structure that allows for controlled biomineralization.

1.2.4.3.4 Crystal maturation and morphological control

With nucleation and crystal growth in magnetosome mediated by the aforementioned proteins, the fine control of nanoparticle morphologies are finally achieved by proteins encoded by the *mms6* and *mamGFDC* operons (Fig. 1-1). The *in vivo* functions of the *mms6* and *mamGFDC* operons were also subjected to genetic analysis. Despite the discrepancy of phenotypes observed for *mms6* deletion strains under different experimental conditions (185,401), proteins encoded by these two operons are shown to be involved in regulating different aspects of magnetite’s size and morphology (185,186,394,401).

Interactions among the *mms6* and *mamGFDC* encoded proteins seemed to be important for their proper functions, as indicated by the loss of Mms7 (MamD) and Mms13 (MamC) from magnetosome membranes when Mms6 was absent due to a non-polar gene deletion (185). Based on analyses of crystal morphologies of individual mutants of *mms* genes, it was proposed that Mms proteins (including Mms5, Mms6, Mms7, and Mms13) may assemble on the magnetosome membrane with specific locations for each protein coordinated so as to constrain the crystal growth directions in the vesicle to produce magnetites of the typical cubo-octahedral shape for this organism (186). However, this model awaits further tests for the following reasons. First, the biochemical functions of these proteins are still unknown. Second, there is no direct evidence to show their physical interactions either *in vivo* or *in vitro*. Third, the coordinated assembly of Mms proteins to regulate crystal formation requires precise control of protein localization on the magnetosome membrane and there is yet no
experimental evidence to establish the distribution of each protein on the magnetosome membrane.

Isolated from the magnetosome of *M. magneticum* AMB1 as a magnetite surface binding protein (33), Mms6 was shown to bind iron and promote the formation of magnetic nanoparticles in *in vitro* synthesis (34,183,184,402,403). Biochemical characterization of Mms6 from *M. magneticum* AMB1 showed that it self-assembles into multimeric micelles and can bind ferric iron with a unique two phase binding curve (27). The observation of Mms6 integrating into liposome membranes is the first direct evidence that Mms6 interacts with membrane lipids (27). However, the exact mechanism of how Mms6 interacts with lipid membrane and how it assembles on the membrane with other proteins *in vivo* are still under investigation.

**1.2.4.4 Summary**

As is evident from the above discussions of magnetosome proteins, stepwise biomineralization of magnetosome is an intimidatingly complex system. But this complex system is built on spatial and temporal control of protein-lipid and protein-protein assembly (187). Despite the genetic dissection of MAI on the magnetotactic bacterial genome, most of the structures and functions of magnetosome membrane proteins are largely unknown (9). Understanding how individual proteins are situated in the magnetosome membrane and how they function individually will allow us to learn the basic principles of magnetosomal protein assembly and guide our understanding of magnetosome biogenesis.
As one of the biomineralization proteins identified on the magnetosome membrane, Mms6 functions both \textit{in vitro} and \textit{in vivo} in regulating the formation of magnetite nanoparticles (183, 185, 402). Although previous work showed that Mms6 self-assembles into a multimeric complex and it interacts with membrane lipids (404), how Mms6 interacts with lipids and self-assemblies in a membrane environment is yet unknown. Hence, I have studied the mechanism of Mms6 assembly and its biochemical activities on a membrane environment in experiments that are discussed in this thesis.

1.2.5 Magnetic nanoparticles: fabrication and application

1.2.5.1 Properties and application of magnetic nanoparticles

1.2.5.1.1 Magnetism and magnetic materials

Our first impression of magnetism may be the magnet on the refrigerator door, or the small round magnet you disassemble from a radio player. However, all matters are magnetic in a broad sense, with most of them having low levels of magnetism (405). Magnetism originates from movement of electrons around orbits of atoms and is generally classified into five categories: diamagnetism, paramagnetism, ferromagnetism, ferrimagnetism, and antiferromagnetism (406).

Magnetic properties can be distinguished by how materials respond to an external field as shown in Fig.1-2. Under normal temperature and moderate field strength, diamagnetic materials exhibit no magnetic moment and are not magnetically ordered in a magnetic field due to the lack of unpaired electrons in their constituent atoms (407). Diamagnetic materials
are actually expelled to some extent by a magnetic field, which is shown by reverse values of magnetization against the applied field in the magnetic responsive curve in Fig.1-2.

Paramagnetic materials (e.g. magnesium, molybdenum, lithium, and tantalum) have a limited number of atoms with unpaired electrons and these individual atomic magnetic moments in the material don’t interact magnetically (408). Thus paramagnetic materials also don’t exhibit net magnetic moments as whole entities. When an external magnetic field is applied to paramagnetic materials, individual atomic moments in paramagnetic materials can be realigned temporarily, producing a weak net magnetic moment in the material with same direction as the external field. These materials readily become nonmagnetic on withdrawal of the external field. This property is shown by the magnetic responsive curve as a straight line in two directions from the xy intercept in Fig. 1-2 (409).

Compared with diamagnetic and paramagnetic materials, ferromagnetic and ferrimagnetic materials (FM) are stronger magnetic entities (e.g., oxides of iron, cobalt, and nickle) and are capable of strong magnetic interactions with magnetic fields due to the parallel or antiparallel alignment of strong atomic moments in the materials’ crystal structure(406). The difference between ferromagnetism and ferrimagnetism is illustrated in Fig.1-3.

Ferro/ferri- magnetic materials retain their magnetic moments in the absence of the external magnetic field as shown by the hysteresis loop on the magnetic responsive curve in Fig.1-2. Ferromagnetic and ferrimagnetic materials larger in diameter than micrometers can form permanent magnets like the refrigerator magnets used in our daily lives. When less than 20nm in diameter (for magnetite, Fe$_3$O$_4$), ferro/ferrimagnetic materials demonstrate superparamagnetism (SPM), a similar property to paramagnetism except the magnetic
moment is much stronger in the presence of an external field. The magnetic responsive curve is shown for SPM in Fig.1-2 (410). Antiferromagnetic materials are also formed by s- and d-block elements like iron and nickel, but they don’t have net magnetic moments due to the cancellation of electronic exchange force inside the bulk materials (411).

1.2.5.1.2 Magnetic nanoparticles (MNPs)

MNPs refer to various ferro/ferrimagnetic nanoparticles (typically with diameter of 5-500nm) that can be manipulated through an external magnetic field. In this size range, the particle size has large effect on the magnetic properties of the materials (408). The magnetic moments of bulky ferromagnetic materials tend to be reduced due to the formation of individual magnetic domains, inside which a uniform magnetization direction forms (412). Domains inside the particle are separated by domain walls consistent with the minimization of total internal magnetostatic energy. Magnetic particles below a critical diameter cannot support more than one domain, and are thus described as “single domain” (413). As shown in Fig.1-4A, with sizes of magnetic materials decreasing, multi-domained particle becomes single domain particle once it passed the critical size of $r_c$ (407). However, the critical size $r_c$ is complex and hard to be generalized since it is affected by multiple factors, e.g., the chemical composition and structure of material, shape and morphology of particles, coating and modifications, interactions among particles like coupling and aggregation (408). Thus, as shown in Fig.1-4A, the coercivity of non-interacting single domain particles changes with size following the curve of solid line, whereas the coercivity of magnetic nanoparticles with coupling between each other change with size following the curve of dashed line (407,414). The concept of “pseudo single-domain” has been used to describe nanoparticles that fall in
the size range of small multigrain MNPs that is bigger than well-defined single domain MNPs but smaller than true multidomain MNPs (413,415,416). The hysteresis loops of typical single domain (solid line), multidomain (dashed line) are shown in Fig.1-4B. As the sizes of magnetic nanoparticles gets smaller to \( r_0 \), the thermal dynamical flipping of atomic magnetization dominates, and MNPs become superparamagnetic with no coercivity (as shown in Fig.1-4B by the dash-dotted sigmoidal curve without hysteresis) (407).

Magnetic materials in nanometer size range have distinctive properties from their larger magnetic entities due to the increasing importance of crystalline and shape anisotropy (412,417). Single domain MNPs above the superparamagnetic threshold are desirable for applications that require a permanent and strong magnetic moment under ambient temperatures due to their high coercivity and maximum spontaneous magnetic moment (418,419). MNPs in the superparamagnetic family, on the other hand, are ideal for applications that need a reversible magnetic moment that can be controlled by an external field(410,420).

1.2.5.1.3 Applications (potentials) of MNPs

Because of their unique magnetic properties, MNPs have attracted attention for their technical and medical applications. In the technical area, MNPs are useful for applications in \( in \textit{vitro} \) magnetic separation, biosensor development, and magnetic tagging of cells and other biological entities (421). MNPs are also widely studied for pharmaceutical and biomedical applications including delivery of drugs, genes, antibodies, radionuclides, and various treatment moieties (410,421). MNPs can resonantly respond to an external time-varying magnetic field, resulting in a transfer of energy from the exciting field to the nanoparticle,
which leads to the heating up of MNPs(422). Noninvasive hyperthermic treatment of solid
tumors like breast cancer, and prostate cancers using this property of MNPs is in clinical trial
(423,424). MNPs are also commonly used as an enhancers of image contrast for medical
magnetic resonance imaging (425).

For industrial applications, suspended MNP colloids are critical components of
ferrofluids, which are important materials for both traditional industrial products like liquid
O-ring in rotary and exclusion seals, heat transfer in loudspeakers, dampers in stepper
motors, computer disk drive seals (426) and modern micro/nanoelectromechanical system
devices (427). Magnetic tape cassettes and discs may have faded from our daily lives, but
magnetic tapes are still widely used and under further development for high capacity and
long-term storage of important documents and information (428). In this information age, the
explosion of multimedia information and the ongoing miniaturization of electronic devices
calls for increasingly higher data storage capacity within minimized spaces, which requires
stricter control of the sizes and magnetic properties of MNPs in order to push the storage
capacity to the limit (428-430).

Different applications require different magnetic and functional properties of MNPs. The
structural and functional controls on the fabricated MNPs are also application specific and
require controlled fabrication of MNPs with varied sizes, shapes, and often further
functionalization. For example, single domain MNPs with sizes above the
superparamagnetism threshold are favored for targeting and magnetic separation purposes
because they have maximum magnetic moment within the grain volume and their magnetic
anisotropy are more controllable (431). For ferrofluids, intracellular or intravenous targeting,
and magnetic resonance imaging applications, superparamagnetic nanoparticles are favored because the presence and absence of their magnetic moment could be easily controlled by an external field due to their smaller sizes (432,433). MNPs for application in biological systems usually need to be further modified with lipids, proteins or coatings of specific structures and properties for target recognition, localization, or protection from degradation and host immunoresponses (426,434,435). These requirements compounded by the difficulty of controlling size, shape, and crystalline properties of naked MNPs, presents a significant challenge for material chemists.

1.2.5.2 Chemical synthesis of MNPs

Traditionally, two strategies have been used to obtain MNPs: the top-down and bottom-up strategies (421,436). The bottom-up strategy, which involves chemical synthesis of MNPs from small molecule precursors, is favored for applications in the biomedical and technical areas because it allows for better control over particle constituents, size, morphology, and modifications (421,426). Over the last few decades, considerable effort has been devoted to the chemical synthesis of MNPs with desired sizes, morphologies, narrow size distributions and controlled magnetic properties. MNPs are usually obtained by one of four routes capable of producing high quality particles, which are: thermal decomposition, hydrothermal synthesis, microemulsion, and co-precipitation (419,431).

Although these methods successfully result in a majority of MNPs of narrow size distributions and controlled morphologies, they all have drawbacks such as high energy consumption due to extreme reaction conditions or polydisperse particle sizes (431,437). For example, thermal decomposition works by high temperature decomposition of
organometallic compounds rapidly injected into hot surfactant solutions, yielding iron-based MNPs with excellent size control and distribution. However, as can be seen from the name of this method, high temperature treatment involves larger energy consumption and expensive organometallic precursors (419).

The hydrothermal method similarly requires faced high energy consumption due to the high pressures and high temperatures required in particle synthesis. In contrast, the microemulsion method, which is conducted at ambient temperatures, involves the confinement of reactant salts emulsified in separate cavities stabilized by surfactants. The cavities confine the nucleation, growth, and agglomeration of particles of ion precursors, promote the formation of magnetic particles that can be separated from the emulsion by centrifugation after adding ethanol to extract surfactant and organic solvent. This strategy is problematic because of its low yield and wide distribution of particle sizes (419,438).

The co-precipitation method involves aging ion precursors in aqueous media at stoichiometric molar ratio (e.g. Fe$^{3+}$: Fe$^{2+}$=2:1, Co$^{3+}$:Fe$^{3+}$.Fe$^{2+}$=1:1:1). This method produces MNPs with particle sizes that can be adjusted by controlling the pH and ionic strength of the precipitation reaction. It is widely used for preparation of MNPs for biological system-related applications for two reasons. Firstly, particles produced in this way can be readily encapsulated by organic polymers immediately after or even during the synthesis process. Secondly, this method has great potentiality to be scaled up for mass production (419,425,431). However, the co-precipitation method at ambient temperature normally creates with superparamagnetic nanoparticles with a wide size distribution (diameters varying from 4 nm to 15 nm) and poor crystalline structures. These structural characteristics
are accompanied by poor magnetic susceptibility and low saturation magnetic moments. Heat
treatment and the incorporation of di-block copolymers in the co-precipitation reactions were
shown to effectively create larger particles that fall in the single domain range, but the
synthesis of desirable MNPs at ambient temperature is still a big challenge (439-441).

1.2.5.3 Biomineralization of MNPs--Bioinspired synthesis of MNPs

Contrary to the extensive efforts of chemists in tackling the problems of creating magnetic
nanoparticles, organisms from nature (e.g. bacteria, protocists, and fish) seem to produce
MNPs at ease with precise control of size, shape, and crystalline properties. As an ancient
synthetic process with a fossil record of 2 billion years, biomineralization of magnetite
nanocrystals (Fe₃O₄) is found in all three kingdoms of living organisms (97). Progress over
the past two decades in understanding the molecular and cellular mechanism of
magnetosome biogenesis in magnetotactic bacteria (MTBs) has increasingly attracted the
attention of material scientists (316,442,443). Inspired by how these organisms build these
intracellular magnetic nanocrystals, a lot of research has been conducted to develop ways of
mimicing the “tricks” of biomineralization used by these microbes. In the following part of
this section, I will briefly review the MNPs that have been fabricated and/or functionalized
for different application purposes by methods learned from MTBs.

1.2.5.3.1 Engineering and functionalization of MNPs from bacterial cells—in vivo
synthesis

The preparation of MNPs directly from bacterial cells has evolved from crude extraction
of bacterial cells to targeted engineering, isolation, and post-modifications for specific
application purposes (403,444). Before the “tricks” of MTBs for fabricating MNPs in their cell bodies were known, it was realized that MNPs from these microbes are single domain magnetic nanoparticles (372,445-447) with promising magnetic properties. The MNPs in MTBs cells are themselves ideal magnetic materials with great application potentials. For example, utilizing MTBs and their MNPs for domain configuration analysis of SiFe magnetic sheets was proposed early in 1989 and later implemented (448-450). Bioremediation of heavy metal pollutions using MTBs was proposed by Mergeay (451), and later implemented by Bahaj for magnetic separation of radionuclides (452), heavy metal accumulation, and waste water treatment (453-457).

As the understanding of MTBs progress, more “tricks” for bio-controlled MNPs synthesis were revealed to material scientists. For example, the discovery of incorporation of titanium (458), copper (459), and other metal elements’ into MNPs of different species of MTB, led to the finding that other transitional metal elements could be included in the cell culture to produce MNPs with different compositions and improved magnetic properties for specific application purposes (460,461). The realization that Fe$^{3+}$ reduction is involved in magnetite synthesis in MTBs (462) inspired Zhang et al to produce single domain magnetites by adding high concentration of ferric oxihydroxide (70mM) in the fermentation medium of an thermophilic iron-reducing bacterium TOR-39 (463). The magnetosome associated protein MagA was cloned as a fusion protein with protein A. The protein A-MagA fusion protein becomes integrated into the magnetosome membrane and provides a tag for isolating MTBs. The isolated MTBs were used as the basis of a chemiluminescence enzyme immunoassay to quantify IgG (464,465). MNPs isolated from bacterial cells have also been coated with
biopolymers including chitosan, N-trimethylchitosan, carboxymethylchitosan and dextran to provide biocompatible surfaces for medical applications (466).

MNPs are commonly used as contrast enhancers of magnetic resonance imaging (MRI) for medical and diagnostic purposes (467-469). MNPs from MTBs and the bacterial cells were shown to be easily controllable and detectable by MRI and they were proposed as potential enhancers for MRI imaging and carriers of treatment medications for targeted delivery (418,470). Later it was reported that viable MTBs cells can be directly used as MRI contrast enhancers (420,471-473). MTBs cells have been employed with MRI to construct nanorobotic platforms for therapeutic and diagnostic applications (472,474,475). Magnetosomes isolated from MTBs can also be injected into mouse tail veins to enhance in vivo MRI imaging of brain vasculatures (476).

MNPs from MTBs have also been explored for use in hyperthermia treatments of tumors due to their remarkable magnetic properties (422,477-481). Production of MNPs by the bacterial cells was optimized for fermentation conditions (478,482), particle properties control (466,481,483), biocompatibility (478,484), and tumor treatment conditions (478,482,485). MNPs isolated from Magnetospirillum gryphiswaldense increased the thermal treatment efficacy of colon carcinoma in the mouse (486). Arranging the MNPs in a linear array to mimic the magnetosome chain was demonstrated, both theoretically and experimentally, to significantly improve their heating performance (487). With the increasing needs of MNPs for various research and technical requirements over this decade, a method for large scale fermentation of MTBs for production of MNPs has been developed (461). The photosynthetic and biotechnologically-compatible bacterial strain, Rhodospirillum rubrum,
has been genetically modified for the production of magnetic magnetosome organelles by the insertion of a set of magnetosome genes from *Magnetospirillum gryphiswaldense* (488). These research efforts not only opened up more routes that MNPs from microbes can be applied for, but also results in more insights into the mechanism of how bacterial biomineralization works(489,490).

1.2.5.3.2 *In vitro synthesis of MNPs as inspired by MTBs*

Despite the progress in developing applications for MNPs synthesized by MTBs, the low yields and high cost of making MNPs from cultured cells still limit their usefulness. Hence it is desirable to optimize a cost-effective chemical synthesis strategy that is easier to scale up. Understanding how microbes achieve the controlled biomineralization *in vivo* has greatly helped chemists optimize their chemical synthesis of MNPs with better size and morphology control by mimicking the strategies of these microbes (491,492). The known strategies used by MTBs for controlled biomineralization are discussed in section 1.2.2 and section 1.2.4 of the literature review, and the corresponding chemical synthesis methods that tried to mimic these strategies are reviewed here accordingly.

MNPs from MTBs are compartmentalized with the magnetosome membrane that originates from an invagination of the cytoplasmic membrane (242). The magnetosome membrane firstly provides a spatial confinement for the crystallizing MNP inside the vesicle. Secondly, the membrane envelope allows for control of Fe ions flow across the membrane by specific iron transporters (10,11,493,494). Furthermore, the magnetosome membrane protects MNPs from oxidation (495). Stephan Mann proposed the utilization of artificial phospholipid vesicles as a model system to mimic biomineralization in living organisms.
Encapsulation of the crystallization reactions was proposed to help control biomineralization due to the effects of chemical regulation, organic-interface effect, and spatial organization on size and morphologies (496,497). The realization that magnetosome specific proteins may play important roles in MNP synthesis lead to the hypothesis that including these proteins in the lipid vesicles may further control the MNP synthesis in vitro (245,498). The encapsulation of chemical reactions for process control is only one of the most commonly used strategies for MNPs and the engineering of functionalized MNPs for specific application purposes (499-501). To develop MNPs for different applications (e.g. molecular recognition, targeting, fluorescence, or protection), the lipids or polymers enveloping the MNPs cores are ideal targets for functionalization (502-504).

Magnetosome proteins like Mms6 (183,184,505,506), MmsF (507), MamC (443,508), which are known to be involved in magnetite nucleation and morphological control in vivo, have been explored as additives in chemical MNP synthesis. Added into the in vitro synthesis reaction of magnetite with partial oxidation method, Mms6 was able to promote the formation of cubo-octahedral magnetic nanoparticles similar to what was observed in magnetotactic bacterial cells (34,183,505). Acidic C-terminal peptides from the Mms6 sequence conjugated to a diblock copolymer F127 micelles (35), or peptides with residues from both the C-terminal domain Mms6 rich in acidic residues (506) also displayed the ability to regulate formation of magnetic nanoparticles during in vitro synthesis. MmsF and MamC were similar to Mms6 in that they all contain acidic regions that are believed to interact with irons during biomineralization of iron. Indeed, both MamC and MmsF are able to promote the formation of magnetic nanoparticles of bigger sizes and narrower size distribution for in vitro synthesis of magnetic nanoparticles than no-protein controls.
In situ synthesis of magnetic nanoparticles on patterned surfaces by either conjugating Mms6 onto surfaces through covalent cross-linking (509) or hydrophobic interactions with self-assembled monolayers of octadecanethiol on gold surface (510) were also achieved recently. However, the molecular mechanism of how these biomineralization proteins regulate in vitro biomineralization reactions and how their in vitro functions relate to their in vivo functions are still under investigation.

1.2.5.4 Summary

Due to the unique properties of magnetic nanoparticles and their application (potentials) in many aspects of society, magnetic nano materials have attracted researchers from various fields of disciplines. Traditional methods of fabricating magnetic nanoparticles, however, are limited by drawbacks such as high energy consumption under extreme reaction conditions or uncontrolled material size and morphologies for chemical synthesis. On the contrary, nature seems to have better strategies of controlling the synthesis of magnetic nanoparticles with excellent crystallinity and tight size and morphological control under ambient conditions. Efforts to directly produce MNPs from living organisms are limited by the difficulties of scaling up to industrial levels and further isolation and cleaning up of the desired magnetic materials. The most promising strategies seemed to be learning the strategies utilized by organisms to make magnetic nano materials, and using these strategies to improve the chemical synthesis procedures.
1.2.6 Tables and Figures

**Fig. 1-1** Hypothetical model for magnetosome biosynthesis in *M. gryphiswaldense*. Magnetosome biosynthesis depends on stepwise protein assembly on membrane vesicles. Reproduced with permission from Lohsse et al. (394).

**Fig. 1-2** Magnetic behaviors of materials under the influence of external magnetic field. Positive and negative values in both M and H stand for magnetic moments in two opposite directions. Reproduced with permission from Arruebo et al. (417).
Fig.1-3: Sketch of individual atomic magnetic spins in ferromagnetism, ferrimagnetism, anti-ferromagnetism without external magnetic field. Reproduced with permission from Jeong et al. (407).

Fig.1-4. Dependence of magnetic properties on particle sizes. A: coercivity (Hc) vary with the sizes of MNPs: in the single-domain regime, the coercivity can follow either the solid curve for non-interacting particles or the dashed line for particles that have coupling between them; MNPs in superparamagnetic regime has no coercivity. Reproduced with permission from Jeong et al. (407). B: hysteresis loop of single domain (solid line), multi-domain (dashed line), and superparamagnetic MNPs (dash-dot line).

1.3 References


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protein phases that introduce nanopatterning and nanoporosities into mineral crystals. 

Biochemistry 53, 4317-4319


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CHAPTER 2: INTEGRATED SELF-ASSEMBLY OF THE MMS6 MAGNETOSOME PROTEIN TO FORM AND IRON-RESPONSIVE STRUCTURE

Modified from a paper published in *The Journal of International Molecular Sciences*

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**Abstract**

A common feature of biomineralization proteins is their self-assembly to produce a surface consistent in size with the inorganic crystals that they produce. Mms6, a small protein of 60 amino acids from *Magnetospirillum magneticum* strain AMB-1 that promotes the *in vitro* growth of superparamagnetic magnetite nanocrystals, assembles in aqueous solution to form spherical micelles that could be visualized by TEM and AFM. The results reported here...
are consistent with the view that the \( N \) and \( C \)-terminal domains interact with each other within one polypeptide chain and across protein units in the assembly. From studies to determine the amino acid residues important for self-assembly, we identified the unique GL repeat in the N-terminal domain with additional contributions from amino acids in other positions, throughout the molecule. Analysis by CD spectroscopy identified a structural change in the iron-binding C-terminal domain in the presence of \( \text{Fe}^{3+} \). A change in the intrinsic fluorescence of tryptophan in the N-terminal domain showed that this structural change is transmitted through the protein. Thus, self-assembly of Mms6 involves an interlaced structure of intra- and inter-molecular interactions that results in a coordinated structural change in the protein assembly with iron binding.

**Keywords:** Mms6; micelle; structural rearrangement

### 2.1 Introduction

Many organisms have developed the ability of creating highly ordered inorganic structures that they use for a variety of purposes ranging from structural support to magnetic guidance. Explorations of the mechanisms by which these biomineralization processes are controlled led to the identification of several biomineralization proteins with a common feature that they self-assemble into multi-molecular structures (1). These larger structures are believed to be the functional units for biomineralization. In addition, the growth of crystals, such as occurs during biomineralization, is believed to involve movement and subsequent fusion of “islands” of minerals (2). Being mediators of crystal growth, biomineralization proteins could be reasonably postulated to drive the movement of such mineral islands.
Mms6 is a biomineralization protein isolated from \textit{Magnetospirillum magneticum} AMB-1 that promotes the formation of superparamagnetic magnetite particles \textit{in vitro} \textsuperscript{(3,4)} and is found associated with the magnetites of magnetosomes when isolated from these bacteria \textsuperscript{(5)}. How Mms6 promotes magnetite crystal growth and the role(s) that it plays in the production of magnetosomes \textit{in vivo} are unclear, although it has been proposed to form a scaffold in the magnetosome membrane that brings together proteins responsible for forming the magnetic particles \textit{in vivo} \textsuperscript{(6)}. In view of the frequent association with biomineralization proteins of the ability to self-assemble, it is likely that self-assembly contributes to the function of Mms6. The importance of Mms6 self-assembly is also suggested by the fact that this protein, which promotes the formation of particles of about 50 nm in diameter, is only 6000 Daltons as a monomer.

We have previously reported that Mms6 self-assembles as a micelle \textsuperscript{(7)}. Mms6 is an amphiphilic protein with a hydrophobic N-terminal domain and a hydrophilic C-terminal domain. Amphiphillic proteins have been demonstrated to self-assemble into a variety of suprastructures, some of which are micelles \textsuperscript{(8-11)}. Here we explore the nature of the self-assembly of Mms6 to better understand this protein assemblage that actively promotes the formation of magnetite nanoparticles and to determine if Mms6 is capable of movement within the larger assembly. The underlying hypothesis is that Mms6 self-assembles to form a larger structure in which individual proteins or groups of proteins are mobile with the potential of driving the fusion of mineral islands to form nanocrystals. We show that both domains contribute to stability of the micelle formed by the wild-type Mms6. By contrast, two Mms6 mutants that do not promote formation of superparamagnetic nanoparticles form less stable micelles. In addition, iron binding by the C-terminal domain of the wild-type, but
not that of the control m3Mms6 mutant, results in a structural change that is conveyed from the C-terminal to the N-terminal domain.

2.2 Results and Discussion

2.2.1 Visualization of the self-assembled Mms6

Previous biochemical and biophysical analyses of Mms6, including analytical ultracentrifugation, size exclusion chromatography and protease probing suggested that Mms6 self-assembles as a micelle that can fuse with liposomes without the aid of detergents (7). The propensity to form micelles was supported by the observation that Mms6 spreads readily on an air-water interface (12). However, none of these methods provided information regarding the shape of the micelle and if the micelles interact. We visualized the assembled structure of Mms6 by transmission electron microscopy (TEM) using negative staining and atomic force microscopy (AFM) combined with surface immobilization (Figure 2-1). For this latter approach we used a mutant Mms6(A133C) (numbering of the amino acids in Mms6 is relative to the first amino acid in the ORF found in the gene encoding this protein. The mature protein is a truncated version of the translated protein due to the removal of a significant length of the N-terminal region of the initially translated polypeptide) in which the terminal Ala was replaced with Cys by which the protein was attached to the gold surface. Our initial premise was that the exchange of the C-terminal Ala for Cys would be unlikely to alter the structure or function of this protein as one strain of magnetotactic bacteria has been found to contain a sequence of Mms6 with a C-terminal Cys (13). However, this expectation was not borne out as we could clearly see from the TEM images that each protein assembly is different from the others.
Whereas the wild-type Mms6 appeared as spherical aggregates of a variety of sizes (Figure 2-1B), the Mms6-A131C appeared as a lattice of protein (Figure 2-1C,G) and Mms6-A133C appeared as a combination of worm-like structures and spheres (Figure 2-1D–F). Changing the percent hydrophobic amino acids in oleosin has been shown to alter self-assembly in anionic strength dependent manner (8). Here the variation in protein sequence is more subtle, being a change of one hydrophobic amino acid residue for a polar residue. The possibility that the replaced cysteine might form inter-protein disulfide linkages was shown unlikely because the same images were obtained when 20 mM β-mercaptoethanol was included with each protein. The AFM images gave similar interpretations of the assembled structures of the two mutant proteins (Figure 2-1E–H). By TEM and dynamic light scattering (DLS) (7), we obtained two independent measures of the diameters of the wild-type protein spheres, which were 21 and 26 nm respectively. From these results we can estimate that each micelle contains 500–1000 protein molecules if they are not hollow spheres. This measured diameter is of interest with respect to the size of magnetite particle formed in vitro by Mms6, which is reported as ~30 nm in diameter (4).

The diameters of the Mms6(A133C) spheres were also measured by TEM and AFM as 27 and 39 nm respectively. Although apparently slightly larger, the difference between the Mms6 and Mms6(A133C) micelles was not significant as the coefficients of variation for these measurements (N > 80) were 14% and 18% for TEM and AFM respectively. Finally, dimensions of the Mms6(A133C) worms and the Mms6(A131C) lattice pieces were found to be the same by TEM and AFM, with the measurements being 15 × 92 nm and 16 × 86 nm respectively. Careful examination of the TEM images of Mms6 showed a number of very small particles, which had dimensions the same (14 nm) as the lattice and worm structures of
the two A to C mutants. Previous DLS measurements also identified this population of smaller particles that were more numerous (94% of the particles) than the larger ones, but involved less of the total protein mass (7). This observation brings up the possibility that these smaller Mms6 particles represent a minimal Mms6 assembly and the difference between the mutants and wild-type protein is how these structures come together to form a larger structure, which for Mms6 results in larger aggregates and for the C-terminal mutants results in longitudinal fusions. Regardless of the molecular relations between the observed structures, these results clearly show that the C-terminal domain of Mms6 is involved in determining its assembled structure.

2.2.2 Role of the N-terminal domain in promoting Mms6 self-assembly

To understand the structural contributions to Mms6 self-assembly, we first examined the role of the N-terminal hydrophobic domain, which contains two prominent features that might contribute to the intermolecular interactions that maintain the micellar structure. These features are the two tryptophans and the GLGLGLGLGL motif that is reminiscent of the repeated motifs found in the silk proteins that mediate self-assembly (14). However, the GL repeat is unique to a subset of magnetosome-associated proteins including Mms6, Mms7, MAM-G, MAM-D, and AMB0956.

To determine if Mms6 self-assembly involves the GL repeat and the tryptophans, we prepared substitution mutants of each and tested them for their respective abilities to self-assemble. The Leu in the GL repeat was replaced with Ala to produce a protein with a GA repeat replacing the GL repeat. Each Trp was replaced separately with Ala or Phe, the latter
expected to have less impact on protein structure. Self-assembly was assessed by the relative sizes of the particles as measured by size exclusion chromatography. The results showed that replacing the GL repeat with a GA repeat greatly disrupted self-assembly. Whereas, replacing either Trp with Phe, another bulky hydrophobic group, did not affect self-assembly, replacement of either Trp with Ala resulted in less stable complexes with the size distributions including smaller protein multimers and monomers (Figure 2-2; Table 2-1).

2.2.3 The C-terminal domain contributes to the stability of the Mms6 micelles and assembles in multimeric forms independently of the N-terminal domain

For studying the ability of Mms6 to bind iron, we created two mutant proteins in which either the positions of the hydroxyl and carboxyl groups in the C-terminal domain of Mms6 were shuffled (m2Mms6) or the amino acid sequence of the C-terminal domain was scrambled (m3Mms6) (7). Both mutants were designed to have similar hydropathy profiles as Mms6. Although all three proteins have a similar amphiphilic character only Mms6 binds iron and promotes the formation superparamagnetic nanoparticles (7). In this work, we investigated Mms6 and its m2 and m3 mutants for their abilities to self-assemble and undergo a structural change in the presence of iron and for correlations with their observed abilities to biomineralize magnetite in vitro. We examined the self-assembly properties of these two mutants and found that a significant percent of the protein in each case traveled as monomers and trimers compared with Mms6, which runs entirely in the void volume of the size exclusion column (Figure 2-3A). To determine if these forms of the protein are in equilibrium, we took individual peaks from the column and either re-ran the protein through the column (Vo sample) or concentrated the protein (trimer, peak 2) and re-ran the
concentrated protein through the column. The results demonstrated that these forms of the protein are in equilibrium as expected from a protein that forms a micelle with a defined CMC (Figure 2-3B).

Destabilization of the Mms6 assembly with C-terminal mutations suggested that the C-terminal domain might interact with itself. The synthetic 21 amino acid peptide C-terminal domain was resolved by size exclusion chromatography and found to distribute with a profile consistent with multimers. Peptides consisting of the C-terminal 21 amino acid sequences of the m2- or m3Mms6 distributed in less homogeneous profiles, suggesting disruption of the multimeric structure (Figure 2-3C). In a number of experiments and under different conditions, we determined that the C21Mms6 peptide can form multimers that range from dimers to octamers, including trimers, tetramers, heptamers and octamers. The distribution between these forms depended on the concentration of salt in the buffer and the pH and was not affected by the presence of iron (Table 2-2). The results clearly show that the C-terminal domain of Mms6 self-assembles in the absence of the N-terminal domain.

**2.2.4 An iron-dependent change in C-terminal domain structure is transmitted to the N-terminal domain of Mms6**

At pH 3, Mms6 binds iron to a high saturating stoichiometry of ~18:1 (Fe$^{3+}$:Mms6) at saturation (7). We examined the possibility that this binding results in a change in structure of Mms6. To determine if the C-terminal domain alters in conformation when it binds iron, we monitored its CD spectrum as a function of the molar ratio of iron:protein (Figure 2-4A,B). The results showed a change in the CD spectrum over the molar ratio of 1:1 to 8:1 (Fe$^{3+}$:Mms6). This spectral change was not observed for the control peptide, m3C21Mms6,
that does not bind iron. Thus, it appears that the C-terminal domain of Mms6 changes in structure upon interaction with iron. Interestingly, a similar change in CD spectrum was reported for a protein fragment from abalone shell during calcium biomineralization, which was interpreted to reflect structural re-organization of the protein upon interaction with calcium (15).

To determine if the structural change due to C-terminal domain iron binding is transmitted to the N-terminal domain we took advantage of intrinsic fluorescence in the N-terminal domain, which changed for Mms6 in the presence compared with the absence of Fe$^{3+}$ (Figure 2-4C). The control mutant protein, m2Mms6, which does not bind iron, showed no change in intrinsic Trp fluorescence.

Mms6, and the two mutant proteins that do not bind iron (m2- and m3Mms6) were examined by small angle neutron scattering (SANS) for evidence of a structural change due to the binding of Fe$^{3+}$ (Figure 2-4D–F). The SANS plots show differences in the low $q$ region for Mms6 in the presence compared with the absence of iron, but not for m2- and m3Mms6. The SANS plots also show some differences for the m2Mms6 in the plateau area in the high $q$ region, which may be due to local changes in a small length scale of m2Mms6 in the presence of iron. This study provided evidence for a shape change in the wild-type Mms6 protein assembly due to iron that was not seen for the m2- and m3Mms6 mutants. However, it should be noted that these mutations also disrupt Mms6 self-assembly, with a significant proportion of the protein appearing as monomers and trimers.

To identify amino acids in the C-terminal domain that might be involved in maintenance of the Mms6 assembled structure and also in mediating transmission of the structural change
from C- to N-terminal domains, the Mms6 sequence was submitted to I-TASSER for a prediction of its structure (Fig. 5 (16,17)). From this predicted structure, it appeared that the Leu128, Leu132 and I117 in the C-terminal domain may interact with the N-terminal domain. The contributions of these amino acid residues to Mms6 self-assembly were tested by exchanging each independently with Gly and determining the effect of these mutations on the micellar integrity by size exclusion chromatography (Table 2-1). Whereas in many measurements under different conditions, the wild-type Mms6 remains as a large assembly that passes through the column with the void volume, mutants in which either of the two Leu was replaced with Gly resulted in a less stable structure with a significant portion of these mutant proteins were found in smaller multimers in most tests. By contrast, replacement of Ile117 with Gly did not destabilize the micelle.

2.3 Experimental Section

2.3.1 Protein reagents and preparation of mutants

Expression vectors for mutant Mms6 proteins were prepared by site-directed mutagenesis of the Mms6 sequence using the Quick Change II kit from Agilent Technologies and according to the manufacturer’s instructions. Mature forms of Mms6 and its mutants as fusion proteins with histidine tags were expressed in E. coli, purified from inclusion bodies and refolded by dialysis (7). Synthetic C21 peptides and peptide mutants were ordered from GenScript.
2.3.2 Atomic force microscopy (AFM)

Mms6(A133C) or Mms6(A131C) on a flat gold surface was scanned using AFM to explore the morphology of the protein on surface. Briefly, template-stripped gold served as the substrate, which was prepared by resistively evaporating 250 nm of gold onto a 4-in. silicon wafer with an Edwards 306A resistive evaporator. Glass microscope slides were cut into 1 × 1 cm squares and sonicated in diluted 5% Contrad 70, deionized water, and ethanol (twice), each for 30 min, and dried under a nitrogen stream. The clean glass chips were glued to the gold-coated wafer with two-part Epotek 377 (Epoxy Technology, Billerica, MA USA) and heated at 150 °C for 1.75 h. The glass chips were then gently detached from the silicon wafer. The sandwiched gold film remained on the topside of the glass chip to yield a smooth gold surface. Three µL of 0.2 mg/mL Mms6(A133C) or Mms6(A131C) in buffer BC100 (20 mM Tris, 100 mM KCl, pH 7.5) was dropped on the gold substrate and incubated overnight at room temperature in a humid chamber created by a water-moistened filter in a sealed petri dish. The surface was then washed twice with BC100, 0.5% Tween 20 followed by two washes with 0.5% Tween 20 then dried under a nitrogen stream. AFM images were acquired using a Nanoscope III Digital Instruments/Veeco (Santa Barbara, CA, USA) in tapping mode. The diameter and length of the micelles on the reported image were obtained by measuring ~100 randomly chosen micelles.

2.3.3 Circular dichroism (CD) spectroscopy

Spectra were obtained with a Jasco J-710 spectropolarimeter (JASCO Corporation, Tokyo, Japan) in a 0.1 cm path-length quartz cell at 25 °C with scanning speed of 50 nm/min, resolution of 0.2 nm, bandwidth of 1.0 nm, sensitivity of 20 millidegree, time response of 8 s
and average of 2 scans. Data was analyzed using JFIT (written by Bernhard Rupp, 1997 http://www.findthatzipfile.com/search-38652539-hZIP/winrar-winzip-download-cdfit.zip.html).

### 2.3.4 Fluorescence spectroscopy

Five micro molar Mms6 or m2Mms6 in 50 mM sodium formate, 100 mM KCl, pH 3.0 were used for tryptophan fluorescence quenching measurements. Fluorescence readings (Ex: 290 nm, Em: 340 nm) were taken using a Cary Eclipse fluorescence spectrophotometer immediately after adding 40 µM FeCl₃ (0 time) or after 2 h incubation with FeCl₃ at 25 °C. The fluorescence values from samples of buffer under each condition with or without iron were subtracted from the values of equivalent samples containing protein. With the background thereby subtracted, these values were normalized against the “0 time” values to obtain the relative fluorescence quenching due to the interaction of each protein with Fe³⁺. The experiment was repeated 8 times and the average quenching and the standard deviation were calculated.

### 2.3.5 Size exclusion chromatography

Size exclusion chromatography was performed in an AKTA FPLC system (GE healthcare, Uppsala, Sweden) through a prepacked Superose 12 10/300GL (separation range: 1 kDa to 300 kDa), Superdex G 75 10/300GL (optimal separation range: 3 kDa to 70 kDa) and Superdex Peptide 10/300GL (optimal separation range: 7 Da to 100 kDa) columns at 4 °C. Flow rates were 0.4–0.5 mL/min. The inner dimensions of all columns were 10x300-310 mm (inner diameter x length) with bed volumes of 24 mL. All column samples were prepared by
centrifugation at 14,000× g at 4 °C for 1 h. Blue dextran was used to determine the void column volume \( (V_o) \) of all columns. The elution volumes \( (V_e) \) of cytochrome c (MM 10.37 kDa), aprotinin (MM 6.5 kDa), insulin B chain oxidized form (MM 3495 Da) and B12 (MM 1355 Da) (all from Sigma, St. Louis MO, USA) from a Superdex Peptide 10/300GL column were used to generate the standard curve for the apparent molecular mass estimations of C21Mms6, m2C21Mms6 and m3C21Mms6. The C21Mms6, m2C21Mms6 and m3C21Mms6 were identified using o-phtalaldehyde (OPA, Thermo Scientific, Waltham MA, USA) by adding 200 μL of OPA to 20 μL of column fraction and measuring fluorescence (Ex: 350 nm, Em: 450 nm).

2.3.6 Small-angle neutron scattering (SANS)

The SANS measurements were performed on the Low-Q Diffractometer (LQD, Lujan Center, Los Alamos National Laboratory, Los Alamos, NM, USA) of the Lujan Center at Los Alamos National Laboratory (LANL). All the solutions were made with D₂O. One mL of 0.1 mg/mL protein was mixed with 250 μL each of 0.25 M FeCl₂ and 0.5 M FeCl₃ in D₂O or with D₂O alone. The samples were sealed in quartz banjo cells with 2 mm path lengths. The scattering vector, \( q \), was varied between 0.003 and 0.3 Å⁻¹, where \( q = (4\pi/\lambda)\sin(\theta/2) \) with the neutron wavelength \( \lambda \) and the scattering angle \( \theta \). The scattered intensity \( I(q) \) was placed on an absolute scale in the units of cm⁻¹. SANS data were analyzed by software provided at the Lujan Center and corrected for empty-cell and background scattering.
2.3.7 Transmission electron microscopy (TEM)

Proteins were examined by transmission electron microscopy with negative staining achieved by using the single droplet procedure (18). Briefly, 10 µL of 0.2 mg/mL protein in 2 mM Tris-HCl (pH 7.5) were individually applied to carbon coated 200 mesh copper grids. After 3 min most of the protein solution was wicked off with a filter paper and the spot covered by a droplet of fresh 2% uranyl acetate. Excess uranyl acetate was removed after 30 s and the grids were air-dried at room temperature. TEM imaging was performed using a Tecnai G2 F20 Scanning Transmission Electron Microscope (STEM; FEI, Hillsboro, OR, USA) at an operating voltage of 200 kV. Multiple fields of each sample were randomly chosen and examined. Measurements of particle sizes were determined manually from electron micrograph images with at least 80 different particles measured for each determination.

2.4 Conclusions

In summary, our data is consistent with a model in which Mms6 self-assembles in micelles in a fashion that involves independent intermolecular interactions between N-terminal domains and between C-terminal domains. In addition, intramolecular interaction(s) between N-terminal and C-terminal domains are evident when the C-terminal domain binds iron. Thus, the Mms6 micelle can be viewed as an integrated multimolecular structure that is responsive to iron. Future studies will be directed to determine if the structural changes observed on iron binding are integral to the ability of this protein to promote the formation of magnetite crystals that are much larger in dimension than Mms6 itself but similar in size to the Mms6 protein assemblies.
Acknowledgments

This work was supported by the U.S. Department of Energy, Office of Basic Energy Science, Division of Materials Sciences and Engineering. The research was performed at the Ames Laboratory. Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract No. DE-AC02-07CH11358. Thanks to the Lujan Center and their scientific staff, Monika Hartl and Rex Hjelm, at Los Alamos National Lab for the use of the SANS facilities and help on the LQD beam line. The Lujan Center is sponsored by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy. Thanks also to the Microscopy and NanoImaging Facility of Iowa State University and Tracey M. Pepper for her help with initial TEM studies.

Conflict of Interest

The authors declare no conflict of interest.

2.5 References


2.6. Tables and Figures

Table 2-1. The integrity of Mms6 assemblies is compromised by mutations in the N-terminal domain. All protein samples were loaded onto the Superose 12 column at 0.2 mg/mL protein with the exception of one sample of the GL repeat (*), which was loaded at 1 mg/mL. For each mutant protein, the distribution of protein resolved on the column and in the void volume (Vo) was determined by estimating the area under the peaks using the peak integration function in the UNICORN™ software. The percent of total protein in the void volume was then calculated and is shown in the table (% protein in Vo).

<table>
<thead>
<tr>
<th>Mms6 protein</th>
<th>% protein in Vo</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100 (15 repeats)</td>
</tr>
<tr>
<td>W79F</td>
<td>96, 96</td>
</tr>
<tr>
<td>W79A</td>
<td>79, 83</td>
</tr>
<tr>
<td>W95F</td>
<td>97, 97</td>
</tr>
<tr>
<td>W95A</td>
<td>49, 70</td>
</tr>
<tr>
<td>W79F, W95F</td>
<td>75, 82</td>
</tr>
</tbody>
</table>
| L84A, L86A, L88A, L90A, L92A | 46, 54, 55 |*
| I117G                 | 94, 95          |
| L128G                 | 63, 86          |
| L132G                 | 29, 37          |

Table 2-2. Self-assembly of the C-terminal domain of Mms6. Eighty micrograms C21Mms6 (C-terminal 21 amino acids) was resolved through a Superdex Peptide 10/300 column in the presence of the buffers and other constituents as shown in the table. The sizes of the multimers, as determined from a standard curve, are reported as the average ± standard deviation with the number of independent replicates shown in parentheses.

<table>
<thead>
<tr>
<th>Buffer content</th>
<th># C21Mms6 units/multimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>water, pH 7.1</td>
<td>&gt;75 K (2)</td>
</tr>
<tr>
<td>10 mM Pi, pH 7.5</td>
<td>7.3 ± 0.58 (3)</td>
</tr>
<tr>
<td>10 mM Pi, 100 mM KCl, pH 7.5</td>
<td>4.0 (1)</td>
</tr>
<tr>
<td>20 mM Tris, pH 7.5</td>
<td>2.0 ± 0.00 (3)</td>
</tr>
<tr>
<td>20 mM Tris, 100 mM KCl, pH 7.5</td>
<td>4.1 ± 0.14 (2)</td>
</tr>
<tr>
<td>20 mM Tris, 1.5 or 3 M KCl, pH 7.5</td>
<td>1.8 ± 0.38 (5)</td>
</tr>
<tr>
<td>20 mM Tris, 6 M GnHCl, pH 7.5</td>
<td>1.1 (1)</td>
</tr>
<tr>
<td>50 mM Formate or Citrate, pH 3</td>
<td>2.0 ± 0.08 (3)</td>
</tr>
<tr>
<td>50 mM Formate or Citrate, pH 3 with FeCl₃:protein = 8:1</td>
<td>2.1 ± 0.15 (4)</td>
</tr>
</tbody>
</table>
Figure 2-1. Mms6 and mutants visualized by atomic force microscopy (AFM) and transmission electron microscopy (TEM). (A–D) Negatively stained samples at 50 nm resolution of (A) Buffer, (B) Mms6, (C) Mms6(A131C), and (D) Mms6(A133C) were imaged by TEM. (E-H) AFM images of proteins immobilized on gold surfaces by a C-terminal cysteine. (E) Mms6 (A133C) amplitude image, scan area 5 µm × 5 µm, (F) Mms6 (A133C) height image, scan area 5 µm × 5 µm, and (G) Mms6(A131C) height image with two maximum scale settings (3 µm above and 2 µm below the gray line); (H) Scale relevant to AFM height images in F and G.
Figure 2-2. *N*-terminal domain residues important for Mms6 self-assembly. The identified Mms6 mutant proteins, all at 0.2 mg/mL, were resolved through a Superose 12 column in BC100 buffer. The molecular masses of the protein in last two peaks in panel (A) were estimated as 91 kDa and 36 kDa, whereas the last two peaks in panel (B) were estimated as 91 kDa and 20 kDa. The molecular mass of Mms6 including its His tag is 10,298 Da.

Figure 2-3. The effects of C-terminal domain mutations on Mms6 self-assembly. (A) Mms6 and two C-terminal domain mutants (m2- and m3Mms6), each at 1 mg/mL in BC100 buffer, were resolved by size exclusion chromatography through a Sephadex G75 column; (B) The void volume (first peak) and the middle peak (peak 2) of the m3Mms6 separation in (A) were separately concentrated to 0.2 mg/mL and each resolved again through the same column; (C) The synthetic C-terminal peptides of wild-type Mms6, m2- and m3Mms6 (0.2 mg/mL) were passed through a Superdex Peptide 10/300 column and quantified in each sample by o-phtalaldehyde (OPA) fluorescence. The red dashed profile for Mms6 in panel A is a repeat of a previously reported experiment (7).
Figure 2–4. Interaction between C-terminal and N-terminal domains results in the transmission of a C-terminal domain structural change that occurs on iron binding. (A, B) CD spectra were determined for 100 µM C21Mms6 or m3C21Mms6 in 50 µM sodium formate, 100 mM KCl, pH 3.0 that had been incubated with increasing Fe$^{3+}$:protein molar ratios for 2 h. Two independently collected data sets are included. In panel A the datasets are distinguished by one set being represented as lines and the other as markers. Due to the contribution of salts to the CD spectra below 205 nm, only the portion of the spectra above this wavelength is shown; (C) Intrinsic fluorescence changes measured at two molar ratios of iron:protein for Mms6 and m2Mms6, both at 5 µM; (D–F) SANS Intensity profiles with and without iron for Mms6, m2Mms6 and m3Mms6. The SANS experiment was performed only once whereas all other experiments were performed at least twice.
Figure 2-5. Predicted Mms6 Monomer Structure. The mature Mms6 primary sequence was entered into I-TASSER using the default parameters from the server without additional restraints. One of the predicted structures is shown as a stick model with the N-terminal domain as green and the C-terminal domain as red. This structural prediction is based on the crystal structure of chain A of D-β-hydroxybutyrate dehydrogenase (*Sinorhizobium Meliloti*; PDB 3v2Ha) which is 29% identical to Mms6 Three amino acids in the C-terminal domain are identified in space-filled mode with Leu128 (cyan) and Leu132 (gold) in the upper segment of the image and Ile117 (purple) in the lower portion of the image.

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CHAPTER 3: DYNAMIC STRUCTURAL CHANGE OF MMS6 WITH IRON BINDING:

STRUCTURAL PREPARATION FOR BIOMINERALIZATION

Manuscript in preparation for submission to *The Journal of Biological Chemistry*

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**Abstract**

The magnetosome-associated protein, Mms6, can alone promote the formation of magnetic nanoparticles in vitro. This in vitro biomineralization function of Mms6 is believed to be related to its self-assembly into multimeric micelles with iron binding properties. Here we report that Mms6 undergoes structural rearrangements on binding iron that can be measured by intrinsic trp fluorescence. Both phases of iron binding (high affinity stoichiometric and low affinity, high capacity) were linked to the fluorescence changes. The high affinity and stoichiometric binding
measured at pH 7 demonstrated the same high affinity as was determined by direct iron binding with $^{55}$Fe filter capture assays. This fluorescence change is proposed to be an intramolecular structural change as it is not temperature-dependent. The high capacity and low affinity binding of iron is cold sensitive as is the fluorescence change that could be measured at low pH with high molar ratios of iron to protein. Trp119 was identified as the residue for which the signal was measured. Thus intrinsic fluorescence spectroscopy reveals a complex combination of structural changes in Mms6 that probably involve both intra and inter-molecular interactions.

3.1 Introduction

Biomineralization is the process by which living organisms fabricate inorganic materials with well-defined and regular properties under ambient conditions. Magnetotactic bacteria (MTBs) are a diverse family of organisms that can fabricate magnetic nanoparticles in magnetosomes, which are prokaryotic organelles enclosed by lipid bilayer membranes (1-4). Magnetosomes are aligned along the long axis of cell body to form a nano-sized magnetic compass that can be used by MTBs to sense the geomagnetic field, thereby facilitating their path-finding process in nature (5,6). The sizes and shapes of the single domain nanoparticles in magnetosomes are controlled genetically with characteristic morphologies for each species of MTBs (4,7,8).

A magnetosome-associated protein Mms6 was originally isolated from the magnetite surface protein and shown to promote the formation of magnetic nanoparticles in vitro in co-precipitation synthesis reactions (9-11). The abilities of Mms6 to bind iron and self-assemble contribute to its in vitro function of promoting magnetite crystallization (12-14). As an amphiphilic protein with a hydrophobic N-terminal domain and hydrophilic C-terminal domain, Mms6 self-assembles as a micelle (12), forms a monolayer at the buffer-air interface (15) and
binds monolayers of hydrophobic octadecanethiol (ODT)-coated gold surfaces where it promotes the formation of magnetic nanoparticles (16). A hydrophathy profile of the Mms6 primary sequence showed that N-terminal domain of Mms6 has a region that could integrate into a membrane.

Multimeric assembly seems a reasonable means by which a small protein can position itself to create a much larger inorganic crystal. Consistent with this view, self-assembly is a commonly observed characteristic of biomineralization proteins that, like Mms6, are often small as monomers (17). The question then arises as to the structural and functional independence of the Mms6 monomers. We have previously demonstrated that the interaction of Mms6 monomers in the micellar multimer and the binding of iron involve both C- and N-terminal domains (12,13). Here we demonstrate that iron binding results in a slow change in protein structure that can be observed in micelles. We have also explored the contribution of selected amino acid residues to multimeric stability. From these results we conclude that Mms6 self-assembles into micelles by way of its N-terminal domain and that the C-terminal domain is important for maintaining intermolecular interactions between monomers. Furthermore, the binding of iron results in a structural rearrangement in each monomer that is transmitted from the C-terminal to the N-terminal domain.

3.2 Materials and Methods

3.2.1 Reagents, proteins, and preparation of mutants

Other common chemical reagents used in this study were of analytical grade or higher purity and obtained from Sigma-Aldrich. Mms6 mutants were generated using the Quick Change II site-directed mutagenesis kit from Agilent Technologies following the manufacturer’s
instructions. Mms6, m2Mms6, m3Mms6, Mms6(W103A), Mms6(W103F), Mms6(W119A),
Mms6(W119F), and Mms6(L108A,L110A,L112A,L114A,L116A) have been previously reported (11-13).

3.2.2 Size exclusion chromatography

The stability of micelles formed by Mms6 and mutants were tested by size exclusion chromatography through a prepacked Superose 12 10/300GL (separation range: 1 kDa to 300 kDa) in an AKTA FPLC system (GE healthcare, Uppsala, Sweden). The inner dimensions of all columns were 10 x 300 mm (inner diameter x length) with bed volumes of 24 mL. All column samples were prepared by centrifugation at 14,000× g at 4°C for 1 h before loading on the column. The chromatography was conducted with a flow rate of 0.5 mL/min at 4°C.

3.2.3 Intrinsic fluorescence measurements

Unless otherwise stated, all measurements of intrinsic fluorescence were made at 25°C with samples in Corning Costar White 96 well plates (Cat#3912) using a Cary Eclipse fluorimeter (Varian) with settings of λ^ex, 290 nm; λ^em, 300-400 nm. Fluorescence of samples with all ingredients except the protein taken under the same conditions was used as background to subtract the effect of buffer and components other than protein.

Spectral analyses of the trp-containing Mms6 and mutants were performed after subtracting the fluorescence readings from equimolar samples of the trp-less Mms6 (W103F, W119F). The fluorescence spectrum of 40 μM tryptophan was obtained in buffer A. For measuring intrinsic fluorescence at 4°C, the bottom chamber of the 96 well plates was filled with refrigerant, sealed with water proof tape and precooled at 4°C overnight. Intrinsic fluorescence spectra of 5 μM
Mms6 in buffer B were taken on the precooled plate after 0~1min and every 15 min of incubation with or without 30 µM ferric chloride at 4°C over a period of 6 h.

3.2.3 $^{55}\text{Fe}^{3+}$ binding assay

Binding of Mms6 to $\text{Fe}^{3+}$ was measured using $^{55}\text{FeCl}_3$ with the filter capture assay as previously reported (12). Mms6 (100 nM) in 100 mM KCl, 20 mM Tris-Cl, pH 3 was incubated with $^{55}\text{Fe}$ (PerkinElmer) as ferric chloride (pH 3) for 2 h at 25°C or 4°C. The samples were captured on nitrocellulose filters and washed, and the $^{55}\text{Fe}$ was quantified by liquid scintillation spectrometry.

3.3 Results

3.3.1 Residues in Mms6 that are important for micelle stability

To identify amino acids that are important for maintaining Mms6 structure, we examined the effect of alanine replacements on the Mms6 multimeric structure, which can be assessed by size exclusion chromatography. The wild-type Mms6 exists as a micelle that travels with the void volume and the monomer is included in the column volume. Mutant recombinant proteins were created and prepared, each with one exchange to alanine or glycine, and tested by size exclusion chromatography to determine if the micelle structure were disrupted by the mutation (Fig.3-1A). The structure of Mms6 is not yet known, so we used a recent iTASSER-created (13,18-20) model of Mms6 to display the positions of the amino acids responsible for maintaining Mms6 micelle structure and those for which alanine exchange has no effect. Although we do not yet have a validated structure for Mms6, the amino acids distributing along the proposed structure in a coherent way with respect to their effects on micellar stability does suggest that the amino
acids in one of the two proposed loop in the C-terminal domain and the hydrophobic trps and the GLGL motif in the N-terminal domain (residues shown as red sticks in Fig.3-1B) are important for maintaining structural integrity. Substitution of amino acids in the two proposed helices (residues shown as green sticks in Fig.3-1B) does not disrupt the protein structure (Fig.3-1A).

The interaction between C- and N-terminal domains appears to be important for Mms6 structural integrity. Three hydrophobic amino acids (I141, L152 and L156) in the C-terminal domain are the most likely candidates for interaction with the hydrophobic N-terminal domain and are situated appropriately in the model to mediate C to N-terminal domain contact. However, at the relatively high protein concentrations tested for the effect of amino acid disruption, the loss of either of these side chains alone did not disrupt the assembled structure. To further test a possible role for one or more of these amino acid residues in maintaining Mms6 structural integrity, we then determined the abilities of lower concentrations of these mutants to maintain their assembled structure compared with the wild-type protein at the same concentration (Fig.3-1C,D). If one or more of these amino acids contributes to Mms6 stability, their substitution might raise the CMC of the Mms6 micelle resulting in the assembled structures being more unstable than the WT when tested at lower protein concentrations. Of these residues, only the substitution of Leu156 with gly destabilized the micelles at 200 µg/mL protein. However, stability is restored when the Leu152 side-chain was also removed. This suggests that, rather than being necessary for protein stability, the loss of Leu156 perturbs the surrounding structure, which perturbation is reversed by removing Leu152.
3.3.2 Intrinsic fluorescence and iron binding activity are correlated

We have previously shown that Mms6 binds iron at pH 7 stoichiometrically and with high affinity. Here we show that Mms6 undergoes a change in intrinsic fluorescence upon binding iron, which is correlated with the iron binding (Fig.3-2A). The $K_{\text{d app}}$ of Mms6 was determined to be $22 \pm 8.6 \, \mu \text{M}$ by Scatchard analysis (Fig.3-2B) and thus the $K_d$ to be $\sim 6 \times 10^{-17} \, \text{M}$, which matches the value that we determined previously for mms6 by using a filter capture assay with $^{55}\text{Fe}$ (12).

3.3.3 Periodic fluorescence change of Mms6 with iron binding

Mms6 binds iron with a two phase binding pattern at pH 3, the pH that is close to the conditions involved in in vitro magnetite synthesis (11). The first binding phase is stoichiometric and high affinity, and second phase is cooperative with a Hill value of three (12). The apparent cooperativity of this process may be due to the binding by Mms6 of prenucleation clusters of iron-hydroxide, which are the prominent form of iron in solution (21-23). The number of iron molecules per iron-hydroxide prenucleation cluster has been estimated at between 2.3 and 2.5 (24) and to be independent of anion (25). Another reason for cooperative binding of iron might be a structural change in the protein upon binding iron.

We looked for evidence of a structural change in Mms6 by measuring the intrinsic trp fluorescence, which we observed to change as a function of time of incubation with iron and iron concentration (Fig 3-3). The change of fluorescence intensity reached a maximum two hours after iron binding (Fig.3-3A). The relevance of the change in intrinsic fluorescence as a function of time after adding iron is demonstrated by the lack of change in structure exhibited by m2Mms6, a mutant protein in which the -OH and –COOH groups are shuffled and that does not
bind iron. Mms6 binds iron to high capacity in groups of 3 to a stoichiometry of ~18 (11). We examined the dependence of intrinsic fluorescence on the Fe\(^{3+}/\)protein molar ratio and observed a pattern with periods of about 12 (Fig.3-3B). The m2Mms6 mutant did not show the same periodicity of Trp fluorescence with significantly different results from Mms6 as evaluated by a paired T test over the range of molar ratios of 5-9 (p=6x10\(^{-5}\)). These results are consistent with our observations to date that Mms6 undergoes a structural change with iron binding (12,13).

3.3.4 W119 is responsible for the fluorescence change of Mms6 with iron binding at pH 3

Mms6 has two Trp residues, both or one of which might be signaling the changes in intrinsic fluorescence. Signaling by both might help to explain the unusual periodicity with Fe\(^{3+}/\)protein ratio. To identify which Trp residue contributes to the change in intrinsic fluorescence, we exchanged each trp for phe to create two mutant proteins, each with only one trp. Only Mms6(W103F) and not Mms6(W119F) demonstrated the periodicity of intrinsic fluorescence (Fig.3-3C), which identifies W119 as the single Trp that is signaling structural changes in Mms6 with iron binding.

3.3.5 Periodic changes in intrinsic fluorescence are observed in independent fluorimeter platforms

The unusual fluorescence transition in fluorescence Mms6 with varying molar ratios of iron was obtained with samples present in a 96-well plastic plate. To verify that the periodic changes observed in this context were independent of the means of gathering the data, we also examined the fluorescence intensity of Mms6 as a function of iron/protein molar ratio in a quartz cuvette (Fig.3-4A). Although a similar periodicity was observed, it was less obvious in the cuvette
format due to a significant decrease in signal with increasing iron concentration. This decrease is consistent with the reported ability of iron to quench the trp signal with increasing iron concentration (26) as also shown here (Fig.3-4B).

3.3.6 Mms6 adsorption to plastic

We investigated the possibility that the Trp is in a different environment when the protein is in plastic compared with in quartz. Many proteins adsorb to plastic and this may be true for the amphiphilic Mms6. To test this hypothesis, a fluorescence scan of Mms6 at pH 3 was taken with varying molar ratios of iron/protein (Fig.3-5, before). The liquid was then decanted immediately after taking the fluorescence reading, and buffer (free of protein and iron) were added back to each sample well. Fluorescence readings were again taken (Fig.3-5, after). The same periodic fluorescence pattern was observed after replacing the protein solution with fresh buffer as in the initial reading, showing that the protein adsorbs to the plastic surface. Thus, the fluorescence reading comes from a planar arrangement of Mms6 in the plastic wells compared with a suspension of micelles in the quartz cuvette. Although artificial, the planar array is closer in aspect to what is expected to be its natural environment in a magnetosome membrane.

3.3.7 Temperature dependence of the intrinsic fluorescence change in Mms6 with iron binding

The change in intrinsic fluorescence of W119 as a function of time and iron/protein molar ratio suggests that Mms6 structure changes slowly over time after binding iron and achieves different structures with iron load. It is not known if these structural transitions reflect intramolecular or intermolecular rearrangements. To investigate if the molecular assembly of monomers were likely responsible for the observed changes, we evaluated the effect of temperature on the fluorescence change. Enzymes that rely on their molecular assemblies for
activity are frequently inactivated at low temperature due to either their dissociation or their denaturation (27). Mms6 did not exhibit intrinsic fluorescence changes with time after the addition of iron when incubated at 4°C (Fig.3-6A) nor did it bind iron at high capacity (Fig.3-6B). However, the stoichiometric iron binding of Mms6 at 25°C (Fig.3-6B, black cycle) that can also be observed at low temperature (Fig.3-6B, cube) suggests that the high-affinity stoichiometric iron binding is not sensitive to temperature (Fig.3-6B). These results are consistent with the fluorescence change and iron binding at ratios of iron greater than 1 is associated with a multimeric structure of Mms6.

3.4 Discussion
Mms6 forms a multimer in solution that behaves as a micelle (12,28). This is a reasonable expectation of a small amphipathic protein. Here we have examined the residues in Mms6 that are important for maintaining micellar stability. These residues can be mapped on a proposed model for Mms6 that was obtained through the application of i-TASSER software (13). The model predicts that the C-terminal domain consists of two alpha helices separated by a flexible loop. We show that the residues in the C-terminal domain that reduce Mms6 micellar stability can all be mapped to this C-terminal domain. The hydrophobic residues in the C-terminal domain are predicted to lie at the interface of C-terminal and N-terminal domains. Although replacement of these residues with alanine does not destabilize the Mms6 micelles when tests are performed at high protein concentrations, the leu156 replacement results in a higher CMC, which is evident when the protein is diluted. This leucine is predicted to sit close to the tyr 119, whose fluorescence emission changes with iron binding. Although this structure of the monomer is predicted by homology folding and is not a demonstrated structure for this protein, it provides a useful scaffold for displaying residues that destabilize the multimer and it supports hypotheses to
explain the structural changes that might be occurring in this protein within the monomer and between monomers in the micelles.

Mms6 binds iron with two phases, which are 1) high affinity and stoichiometric and 2) low affinity and high capacity (12). The high affinity binding is also observed with a C-terminal domain peptide but not with mutant forms of the protein or peptide in which the C-terminal domain oxygen-containing side chains are exchanged for alanine (12). Thus, it seems that the high affinity binding activity is a feature of the monomer. This conclusion is supported here by the observation that the high affinity binding activity is not cold-sensitive and a monomeric form of Mms6 binds with the same affinity for iron as the multimeric Mms6 (data currently not shown).

Unlike the high affinity iron binding, which is assayed at pH 7 in the presence of ferric citrate to chelate the iron, high capacity iron binding requires that the free iron concentration be increased to µM values as the affinity is in the low µM range (12). For that measurement, the pH of the solution must be low. In these studies, we have used pH 3, which is the initial pH at which the protein was demonstrated to promote iron crystal formation. At this pH, we can observe high capacity binding by using a $^{55}$Fe-filter capture assay. The presentation of iron to the protein at low pH also results in a change in trp fluorescence. This change is slow, taking 2 h to reach a maximum. We demonstrated that the protein adsorbs to the plastic and remains in the wells when the buffer is removed. The slow change in fluorescence with iron binding may reflect a large number of entropy-driven rearrangements required within the multimer for a complete change in structure with the binding of iron.

Cold denaturation has been observed in globular proteins in which the transition is exothermic, involves a decrease in $\Delta H$ and $\Delta S$, which suggests that the driving force is dehydration of
nonpolar groups (27). Yeast glyceraldehyde-3-phosphate dehydrogenase is similar example to Mms6 of a cold-sensitive enzyme for which the monomer is active and binds NAD with high affinity but the multimer dissociates at low temperature and loses enzymatic activity. For Mms6, the monomer binds a single iron molecule per mole of protein and remains active at low temperature. In analogy to other cold-sensitive proteins, the multimer may dissociate as the primary response to low temperature, with subsequent re-association to bury revealed hydrophobic groups resulting in an irregular structure no longer displays high capacity iron-binding activity. Thus, our results show that the Mms6 monomer binds iron with high affinity to a stoichiometry of 1. We propose that the iron-bound C-terminal domain then sets the stage for the intermolecular interactions in the multimer that enable high capacity binding of prenucleation iron cultures such that the protein can build the magnetite crystal lattice.

ACKNOWLEDGEMENTS

This work was supported by the U.S. Department of Energy, Office of Basic Energy Science, Division of Materials Sciences and Engineering. The research was performed at the Ames Laboratory. Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract No. DE-AC02-07CH11358.

3.5 References


### 3.6 Figure Legends

**Figure 3-1.** Residues in Mms6 that are important for micelle stability. A,C) Mms6 mutants with Ala substitutions of C-terminal oxygen-containing (A) or hydrophobic side chains (C) were analyzed by size exclusion chromatography through a Superose12 10/300 column at 1 mg/ml protein in 20 mM Tris, 100 mM KCl, pH7.5 (buffer A) at 4°C. B,D) The oxygen-containing residues that stabilize the micelles are shown in red and those that do not affect stability are in green. The oxygen-containing side chains are represented as stick models (B) and the hydrophobic residues are shown in space-filling mode (D).

**Figure 3-2.** Intrinsic fluorescence of Mms6 at pH7.5. Mms6 (5 µM) was incubated with various molar ratios of ferric citrate in 20 mM Tris, 100 mM KCl, pH7.5 for 2 h in brown test tubes, followed by centrifugation at 14,000g for 30min, after which the supernatants were transferred to a quartz cuvette for intrinsic fluorescence measurements. All steps above were conducted at 25°C. A) The relative fluorescence change (fraction maximum value) of Mms6 as a function of ferric citrate concentration (●). The insets shows the spectra of Mms6 with
increasing concentrations of iron. **B)** The data from (A) are represented as Scatchard plot for calculation of $K_d$ based on the relative fluorescence changes at 340 nm with iron binding.

**Figure 3-3. Intrinsic fluorescence of Mms6 changes with iron binding. A,B)** Intrinsic fluorescence of 5 µM Mms6 or m2Mms6 was monitored at 340 nm in buffer B at various times after the addition of iron (A) or at 2 h after adding iron and at various protein/iron molar ratios (B). The data (with background subtracted) was normalized to the 0 min time point (A) or the 0 iron condition (B). The results show an average of 8 (A) and 5 (B) independently performed experiments with standard deviations as error bars. **C)** Intrinsic fluorescence of Mms6 (WT) or Mms6 with phe exchanged for trp at positions 103 (W103F) or 119 (W119F) was measured at iron/protein molar ratios of 0, 6, 12, and 18 as in B and normalized to the results normalized to the 0-iron condition.

**Figure 3-4. Fluorescence intensity of Mms6 in the presence of a range of molar ratios of iron/protein measured in a quartz cuvette. A.** Intrinsic fluorescence of Mms6 was determined in buffer B after incubation for 2 h in the presence of various molar ratios of FeCl$_3$. Arrows show the positions of inflection of the observed periodicity. **B.** Tryptophan (40 µM) was incubated with various concentrations of ferric citrate in buffer A and fluorescence emission measured at the peak OD for each emission scan (355-360 nm).

**Figure 3-5. Mms6 adsorption to plastic.** Mms6 (5 µM) was incubated with various molar ratios of FeCl$_3$ for 2 h in 96 well plates before fluorescence readings were taken for samples with mms6 (gray triangles) and with buffer only (black circles). The wells were decanted, fresh buffer B was added and the wells again read for fluorescence at 340 nm. Wells to which protein had been added originally (empty triangles) and to which no protein had been added originally (empty circles). The resulting fluorescence readings were normalized to the no iron samples.
**Figure 3-6. Temperature dependence of the Mms6 interaction with iron.** A) Normalized fluorescence was measured at 25°C and 4°C as described for Fig.3-2 B) Iron binding by Mms6 was determined using the filter capture assay as described in Materials and Methods with 100 nM Mms6 in 20 mM Tris-HCl, 100 mM KCl, pH 3. All data are the average of duplicate values. Error bars represent the high and low values of the duplicates. The range of duplicate values for the 4°C measurements are within the size of the symbols. The dashed line identifies the molar ratio of 1 mole of iron/mole Mms6.

### 3.7 Tables and Figures

**Figure 3-1**
CHAPTER 4 THE BIOMINERALIZATION PROTEIN MMS6 IS A LIPID-ACTIVATED FERRIC REDUCTASE

Manuscript in preparation for submission to The Journal of Biological Chemistry

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Running head: Biomineralization protein Mms6 is a lipid-activated ferric reductase

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Abstract

The magnetosomes of magnetotactic bacteria consist of magnetic nanocrystals with defined morphologies and magnetic properties enclosed in vesicles that originate from cytoplasmic membrane invaginations. Although many proteins are involved in creating magnetosomes, a single protein, Mms6, from these bacteria can direct the crystallization of magnetite nanoparticles from iron solutions in vitro. The in vivo role of Mms6 in magnetite crystal formation is debated and the observation that Mms6 binds ferric and not ferrous iron raises the question of how Mms6 could promote the crystallization of magnetite, which contains both ferric
and ferrous iron. Here we show that Mms6 is a ferric reductase that reduces ferric to ferrous iron using NADH and FAD as electron donor and cofactor, respectively. Much higher reductase activity was observed when Mms6 was integrated into bicelles. The ferric reductase activity is correlated with iron binding activity in a series of mutant proteins with alanine exchanges in the C-terminal domain. These results are consistent with a hypothesis that Mms6, a membrane protein, promotes the formation of magnetite by a mechanism that involves reducing iron.

4.1 Introduction

Since first reported in 1975 (1) magnetotactic bacteria (MTB) have attracted interest because of their ability to synthesize magnetite crystals in specialized organelles called “magnetosomes”. Superparamagnetic magnetite crystals of similar size and shape to the bacterial magnetites are formed in vitro due to the presence of recombinant Mms6, a magnetosome associated protein (2,3). Although Mms6 alone is not responsible for the formation of magnetic nanoparticles in vivo (4), its in vitro activity provides us an opportunity of understanding the mechanism by which this biomineralization protein functions.

We have previously demonstrated that Mms6 forms a micellar quaternary structure in vitro that may provide a surface for magnetite nanoparticle formation (5). Mms6 consists of two subdomains, with the N-terminal domain responsible for anchoring the C-terminal domain in the micelle from which the C-terminus binds iron and forms magnetic nanoparticles. Analysis of Mms6 and its C-terminal domain by fluorescence, and CD spectroscopy provides evidence that the protein undergoes a structural change upon binding iron and exhibits two modes of interaction with iron (5,6).
The magnetite crystal lattice contains \( \text{Fe}^{2+}(\text{Fe}^{3+})_2\text{O}_4 \). Whereas Mms6 binds Fe\(^{3+}\), it does not bind Fe\(^{2+}\) (7). Although this deficiency can be circumvented *in vitro* by making available a high concentration of Fe\(^{2+}\), the ratio of Fe\(^{3+}\)/Fe\(^{2+}\) *in vivo* is unlikely to be 2:1. Rather, Fe\(^{3+}\) is proposed as the predominant form of iron in magnetosomes (8-12). Thus, if Mms6 were to be involved in initiating or promoting the growth of magnetite crystals in vivo, it would need to cooperate with a protein that could reduce the available Fe\(^{3+}\) or it could itself be a reductase. Here we show that Mms6 is a ferric reductase, capable of producing the Fe\(^{2+}\) required for placement in the magnetite crystal lattice. Mutational analysis suggests that the reductase activity is located in the C-terminal domain and probably overlaps with the iron-binding domain of this protein. We also show that the reductase activity of Mms6 is greatly enhanced when the protein is integrated in a membrane as it is believed to be localized *in vivo* (2,4,13).

4.2 Materials and Methods

4.2.1 Reagents, proteins, and preparation of mutants

Phospholipids used to make bicelles and liposomes were purchased as stocks dissolved in 100% chloroform from Avanti Polar Lipids. Bio-Beads™ SM-2 Resin used for removing detergents was purchased from Bio-Rad. Other chemical reagents were of analytical grade or higher purity and were obtained from Sigma-Aldrich. Site-directed mutagenesis of Mms6 was conducted using the Quick Change II mutagenesis kit from Agilent Technologies following the manufacturer’s instructions. The mature forms of Mms6 and its mutants were expressed and purified as described previously (5,6,14). The C-terminal domain of Mms6 (C21Mms6: KSRDIESAQSDEEVELRDALA) and its mutants (m2C21Mms6: KDRSIDEAQESDSVELREALA;
m3C21Mms6: QSLERAE DEDADISAVEKLSR) were chemically synthesized by Genscript (Genscript Corp., www.genscript.com).

4.2.2 Bicelle and Mms6-bicelle preparation

Bicelle stocks consisting of 1,2-dimyristoyl-sn-glycerol-3-phosphocholine (DMPC) and 1,2-dihexyl-sn-glycero-3-phosphocholine (DHPC) (MDMPC:MDHPC=1:1, q=1) with total lipid concentration of 250mM in buffer A (20 mM Tris, 100 mM KCl, pH7.5 at 25˚C) was prepared as described in (15) with minor modifications. Chloroform was removed from an equimolar mixture of DMPC and DHPC in 100% chloroform in a glass vial on ice under a mild stream of argon in a ventilated hood. This lipid mixture was dessicated overnight under constant vacuum at 4 °C overnight. The dessicated lipids were re-suspended in the appropriate volume of buffer A to achieve a total lipid concentration of 250 mM. The re-suspended bicelle lipids were subjected to repeated warm (45 °C)/cool (ice) cycles until the solution became non-viscous and transparent. The bicelles stocks were used immediately or aliquoted and stored at -20 °C until used.

The Mms6-bicelle complex was prepared as described in (16). Eighty micromolar Mms6 or a mutant Mms6 was mixed with 100 mM bicelle (q=1) in buffer A. The test tubes were sealed with screw caps and the protein-micelle mixtures were treated with four cycles of freeze (liquid nitrogen) and thaw (25 °C). The protein-micelle mixtures were stored in 4 °C for up to three weeks or maintained at -20 °C before use. The Mms6-bicelles were incubated at room temperature for one hour until used for experiments.

4.2.3 Liposome and Mms6-liposome preparation

Five individual liposome stocks of 100 mM DMPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-
glycero-3-phospho-(1'-rac- glycerol) (DOPG), or 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) in buffer A were prepared by extrusion through polycarbonate filters (17). The Mms6-liposome stocks were prepared by mixing Mms6 and liposomes at final concentration of 80uM Mms6 and 8mM liposome in buffer A with 0.5% Triton X-100, and incubating at 25 °C for 2 h with constant inversion. The Triton X-100 was removed by incubating with Bio-Beads™ SM-2 Resin pre-hydrated in buffer A at 25 °C with constant inversion for 3 h using a ratio of 35 µg Triton X-100 per mg of resin. The Mms6-liposome sample was harvested by removing the supernatant after the beads were allowed to settle by gravity.

4.2.4 Ferric reductase activity

Ferric reductase activity was monitored by the spectral change in ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p-disulfonic acid monosodium salt hydrate), which binds Fe^{2+} to form a complex with maximum absorbance at 562nm and molar extinction coefficient of 27,900M^{-1}cm^{-1} (18,19). The assay mixture for C21Mms6 contained 0.1 mM NADH, 25 µM or 75 µM ferric citrate, 1 µM flavin mononucleotide (FMN), 1mM ferrozine (all from Sigma) and 85 µM C21Mms6 in 20 mM Tris-HCl, 100 mM KCl, pH 7.5 in a total volume of 250 µL. The reaction was initiated by addition of C21Mms6. The assay mixture for the full-length protein contained 20 µM Mms6 with or without 25 mM bicelles, 100 µM NADH or NADPH, 800 µM ferrozine, 100 µM FAD or FMN in buffer A. The reaction was started by the addition of ferric citrate and monitored by reading A_{562} each 1 or 2 minute for a period of 6 hours.

The background absorptions from samples lacking Mms6 read over the same time periods were subtracted from samples with Mms6 before calculating the rate of reductase activity. The V_{max}, K_m, and K_{cat} of Mms6 as a ferric reductase were calculated by the Lineweaver-Burk plot.
The effect of pH on activity was tested by using 100 mM sodium phosphate buffers with pH’s ranging from 5.9 and 7.8. All spectrophotometric measurements were performed at room temperature in 96-well clear plate (Falcon, Catalog#351172) and read with a Synergy II plate reader (full-length protein) or a Biotek, Model: Ceres 900 plate reader (C21Mms6). A mixture containing the same components (exclude peptide or protein) as the assay mixture was used as blank. The concentration of ferrous iron was determined by $A_{562}$ using extinction coefficient of 27,900M$^{-1}$cm$^{-1}$. The activity is expressed as nmol Fe$^{2+}$ formed/min/mmol protein.

**4.2.5 Intrinsic fluorescence spectroscopy**

Twenty micromolar Mms6 or a mutant Mms6 with or without 25mM bicelles or 2mM liposomes in buffer A were incubated at 25°C for 2 h before collecting fluorescence spectra. Spectral analyses of the trp-containing Mms6 and mutants were performed after subtracting the fluorescence readings of equimolar samples of the trp-less Mms6 (W103F, W119F). The fluorescence spectrum of 40 µM tryptophan was obtained with and without bicelles in buffer A for correction of Mms6 spectra during decomposition of Mms6 fluorescence spectrum by the Protein Fluorescence and Structure Toolkit (20).

**4.2.6 Dynamic Light Scattering (DLS)**

Twenty micro molar of Mms6 and its mutants with or without bicelles or liposomes in buffer A were analyzed at 25°C with a Zetasizer Nanoparticle analyzer (Model: ZEN3690, Malvern Instrument Ltd., Southborough, MA). All samples were centrifuged at 14,000g at 25°C for one hour to remove particulates prior to taking DLS measurements.
4.3 Results

4.3.1 Mms6 is a ferric reductase with specificity for FAD over FMN

The results of our previous studies suggest that Mms6 binds Fe\(^{3+}\) cooperatively in groups of 3 (5) and does not bind Fe\(^{2+}\) (21). But because the crystal lattice of magnetite contains Fe\(^{3+}\):Fe\(^{2+}\) at a ratio of 2:1, these binding characteristics would not be compatible with crystal building unless Mms6 is capable of reducing Fe\(^{3+}\). Reduction of Fe\(^{3+}\) by Mms6 was monitored by the increase of A\(_{562}\) from the Fe\(^{2+}\)-ferrozine complex. Our results show that Mms6 is a ferric reductase (Fig. 4-1A-C). The two mutant forms of this protein that do not bind iron to high affinity or high capacity (m2Mms6 and m3Mms6) also do not show reductase activity (Fig.4-1A).

To determine its requirements for electron donor and co-factor, Mms6 was tested for reductase activity in the presence of combinations of electron donor and cofactors. The results clearly show that Mms6 prefers NADH, but can also use NADPH as electron donor, but exclusively uses FAD over FMN as cofactor (Fig.4-2A). The V\(_{\text{max}}\) and K\(_m\) values for ferric citrate were 36 ± 18 nmole of ferrozine-Fe\(^{2+}\)/min and 236 ± 248 μM, respectively. Since 20uM Mms6 were used for the reductase test, the K\(_{\text{cat}}\) was calculated to be 3 x10\(^{-5}\) sec\(^{-1}\). This is much lower than the K\(_{\text{cat}}\) of 0.9 sec\(^{-1}\) reported for a ferric iron reductase isolated previously from *Magnetospirillum magnetotacticum* (22).

4.3.2 The C-terminal domain of Mms6 contains the catalytic site

The C-terminal domain mutations (m2 and m3) of Mms6 eliminated reductase activity (Fig.4-1A), which suggested that the C-terminal domain contains the catalytic site for Fe\(^{3+}\) reduction. The C-terminal domain is functionally independent of the N-terminal domain as demonstrated by the reductase activity of a synthetic C-terminal domain peptide (C21Mms6) (Fig.4-1D). Even
slower than the full-length protein, the specific reductase activity of C21Mms6 was determined to be $49 \pm 3.0 \text{ nmol Fe}^{2+}/\text{min/mmol C21Mms6}$. Again, the peptides containing the same mutant sequences as in the full length Mms6, m2C21Mms6 and m3C21Mms6 showed no reductase activity at 25 µM ferric-citrate.

In these assays the cofactor for Mms6 reductase was FAD with NADH as the electron donor. The specificity of Mms6 for cofactor and donor was explored by testing its ability to use NADPH and FMN as electron donor and cofactor respectively (Fig.4-2A). The full-length protein showed a slight preference for NADH over NADPH and a requirement for FAD that could not be substituted by FMN. By contrast, the C-terminal domain peptide showed no preference for FAD over FMN (Fig.4-2B).

4.3.3 Mms6 interacts with lipid membranes

4.3.3.1 Mms6 integrates into lipid membranes

Numerous observations suggest that Mms6 is a membrane protein (2,4,5,7,13). Thus, we investigated the effect of a lipid environment in its reductase activity. The N-terminal domain of mature Mms6, an amphipathic protein, is largely hydrophobic and might integrate into a membrane. Our previous studies to show that Mms6 spontaneously integrates into liposomes (5) and orients at the surface of a Langmuir trough (21) supports this supposition. Mms6, which exists as micelles in the absence of lipid, was incorporated into bicelles and the hydrodynamic diameters of the resulting particles were determined by dynamic light scattering techniques. Mms6 (20 µM) exists as micelles in aqueous solution with diameters of ~12-15nm (Fig.4-3, black solid line). After incubation with 0.5% of Triton-X100 at 25°C followed by detergent removal with hydrophobic beads, the Mms6 micelles were larger in hydrodynamic diameter.
(averaged ~45 nm), possibly due to the fusion of micelles induced by the detergent (Fig.4-3, gray solid line). DMPC/DHPC bicelles (25 mM) had hydrodynamic diameters of ~10 nm (Fig.4-3, □), which is consistent with their expected size (15). Mms6-bicelle complexes also have a hydrodynamic diameter of ~10 nm (Fig.4-3, ■). The incorporation of Mms6 into bicelle is supported by the disappearance of the larger Mms6 micelles (d=12-15nm) in the Mms6-bicelle sample. A similar size of the bicelle and Mms6-bicelle may be because incorporation of Mms6 into the bicelles results in a change in shape of the complex, which may not be observed by DLS in which a spherical shape is assumed for all particles. Bicelles are discoidal with diameters of 10 nm and thickness ~4-5 nm. Incorporation of Mms6 into POPC or DOPC liposomes (~100 nm, Fig.4-3, ○) is also accompanied by the absence of Mms6 micelles in the Mms6-liposome samples (Fig.4-3, ●).

4.3.3.2 Mms6 changes structure when it is integrated in lipid membranes

Integration of Mms6 into lipid membranes was also confirmed by measuring its intrinsic fluorescence before and after membrane integration to see if there are structural rearrangements after Mms6 is placed in lipid environment. As shown in Fig.4-5A and B, significant increase of fluorescence intensity and blue shift of spectrum were both observed when Mms6 was integrated in bicelle, which suggested that the Trp residues in Mms6 are switched to a less polar local environment. Since there are two Trp residues in Mms6 primary sequence, single-Trp mutants Mms6(W103F) and Mms6(W119F) were also tested for intrinsic fluorescence before and after membrane integration to see if any one or both of the residues are contributing to the fluorescence change observed during membrane integration. As can be seen from Fig.4-5C and D, Mms6(W119F) did not show as much fluorescence intensity increase and no spectral shift
was observed as the wildtype Mms6. Whereas Mms6(W103F) displayed similar increase of fluorescence intensity and blue shift of spectrum after membrane integration (data not shown here). This result suggests that the local environment of Trp119 was changed from a hydrophilic environment into a less polar environment when Mms6 was integrated in membranes.

Since hydrophobic resides are known to be involved in the interactions between membrane-associated domains and lipid bilayers, it is interesting to know if the hydrophobic GL-repeat is involved in Mms6’s interactions with lipids. As can be seen from Fig.4-5E, no fluorescence spectral shift was observed for Mms6(5G1-to-5GA) mutant when the mutant was prepared with the same method for bicelle integration. This suggests that that Mms6 interacts with membrane lipids at least partially by hydrophobic interactions between the N-terminal domain and lipid bilayers.

Protein fluorescence and structural toolkit (PFAST) were utilized for decompositional analysis of Mms6 spectrum when it is integrated in bicelles (23). Fluorescence Correlation Analysis Tool from the PFST tool kit decomposed Mms6 spectrum into two subcomponents (Fig.4-5F), with component1 constitute 90% of the total spectrum and component 2 constitutes only 10% of the spectrum. Component1 was classified as classII Trp fluorophores, which correspond to Trp residues that are exposed to bound water with long dipole relaxation time. This assignment of fluorescence spectral components is consistent with single-Trp mutational studies of fluorescence spectra since we have shown that Trp119 is the only residue that contributes to the fluorescence signal change of Mms6 in bicelles. These results clearly showed that Mms6 interacts with lipid membranes with a structural rearrangement.
4.3.4 Lipids promote higher ferric reductase activity of Mms6

When incorporated into bicelles the ferric reductase activity of Mms6 was significant increased (Fig.4-4A). The $V_{\text{max}}$, $K_{\text{m}}$ and $K_{\text{cat}}$ for Mms6 in bicelle lipids were 200 nmole ferrozine- Fe$^{2+}$/min. 76μM, and 3.3x10$^{-2}$ sec$^{-1}$, respectively. This represents about 60-100-fold increase in activity of Mms6 compared with in the absence lipids. The pH profile of the Mms6 ferric reductase activity while in lipid bicelle was very sharp with optimal activity at pH 6.9, but not at pH 6.5 or pH 7.5 (Fig.4-4B).

4.3.5 The C-terminal residues involved in reductase and iron binding activities of Mms6

Iron chelation is expected to be achieved by way of -OH or -COOH containing side-chains in the C-terminal domain and these might also be involved in reductase activity. Consequently, we created a series of alanine replacement mutants, each with a different amino acid (S, D, or E) replaced by alanine. These mutants were tested for iron binding and ferric reductase activities. For both assays, the activities of the mutants were normalized to that of the wild-type protein (Fig.4-6, 4-7). The effect of mutations on reductase and iron binding activity were highly correlated with a small group of mutants (S138A, S143A, S146A) showing a large decrease in iron binding and reductase activity (Fig. 8A). The concordance in the results from these two assays strongly suggests that these residues are important for the function of Mms6. However, although suggestive of a requirement of these amino acid residues for the iron chelation and reductase activities, this data may also reflect their requirement in a structural role.

To further investigate the likelihood of the involvement of the triad (S138A, S143A, S146A) as iron chelators compared with structural components, we determined the positions of these residues on a model of Mms6 created by ITASSER (6,24,25). It is of interest to this discussion that the program identified the di-iron nitric oxide reductase fragment of FprA from Moorella
thermoacetica (PDB ID:1ycfA) (26) as the most likely structural equivalent to Mms6 on which to build the Mms6 model (6). The model for Mms6 predicts that the Ser, Asp, and Glu amino acids that are potential sites for iron binding and reductase reside in a flexible loop region that links two helical regions in the C-terminal domain. This location is consistent with a role in chelation and could also explain the observed effect of iron-binding on the protein’s structure (26).

4.4 Discussions

In this study, we report the characterization of Mms6 as a ferric reductase. The ferric reductase activity is observed in the synthetic C-terminal peptide of Mms6, which itself has structure (6), and can promote the formation of magnetic nanoparticles when conjugated to the di-block copolymer Pluronic F-127(5,27). Mms6 prefers NADH over NADPH as electron donor and is specific for FAD as co-factor. By contrast, the C-terminal domain does not discriminate between FAD and FMN, being capable of using both as co-factors in iron reduction. This observation suggests that the N-terminal domain coordinates with the C-terminal domain in the full-length mature protein to reduce iron, perhaps by providing a binding site for FAD. Of interest in this regard is the fact that the protein chosen from the database for modeling Mms6 by ITASSER is an oxido-reductase for which the FAD-binding domain is in the structural equivalent of the N-terminal domain of Mms6.

Many results from our studies and those of others identify Mms6 as a likely membrane integrated protein (2,4,5,7). This prediction is consistent with the amphipathic nature of this protein. Here we observed that, when integrated into lipid bilayers, the reductase activity of Mms6 is ~100-fold higher than in the absence of lipids. The pH optimum of ferric reductase
activity for Mms6 is pH7, similar to that shown for the iron reductase of *Magnetospirillum* (formerly *Aquaspirillum*) *magnetotacticum* (22).

Magnetite (Fe$^{2+}$[Fe$^{3+}$]$_2$O$_4$) and greigite (Fe$^{2+}$[Fe$^{3+}$]$_2$S$_4$) crystals contain a combination of Fe$^{3+}$ and Fe$^{2+}$ ions in the crystal lattice (22,28). On the basis of Mössbauer spectroscopic analysis of magnetite synthesis in *M. magnetotacticum* a mechanism of magnetite precipitation involving partial reduction of ferrihydrite precursors has been proposed to precede magnetite formation. This stepwise model includes uptake of siderophore-complexed Fe$^{3+}$ (29,30), periplasmic or cytoplasmic reduction and release of Fe$^{3+}$ from siderophores as Fe$^{2+}$, transport and re-oxidation of Fe$^{2+}$ into magnetosome chamber in the form of ferrihydrite ([Fe$^{3+}$]$_2$O$_3$•0.5H$_2$O), and finally crystallization of magnetite through partial reduction and dehydration reactions (10,11). More recently, genes have been identified that encode proteins involved in iron and proton transport, ferric iron reduction, ferrous iron oxidation, and redox controls that could coordinate the precipitation of irons of multiple valence into crystalline structures in magnetosomes (28,31-38). Although some aspects of the original model have been updated in molecular detail, the stepwise biological control over iron flow during magnetosome formation is still the basic framework of the current model for magnetosome biogenesis (39). The dominance of ferrihydrite in isolated magnetosomes suggests extensive oxidation of ferrous iron in the magnetosome (10). Several findings, including the identification of an hematite (alpha-Fe$_2$O$_3$, ferric oxide) phase in early magnetosomes, evidence that magnetite forms by phase transformation from a highly disordered phosphate-rich ferric hydroxide phase that may be complexed with ferritins, the identification of ferrihydrite from ferritin as precursors of magnetite suggest rapid oxidation of iron after it enters the magnetosome (8) (9,12). This model is also supported by the presence of
ferrous iron oxidase MamP (28,35) and redox control proteins (MamX, MamZ, and MamH) on the magnetosome membranes (36-38).

With Fe$^{3+}$ in magnetosome as the majority precursor for magnetite crystallization, the control of the 2:1 stoichiometry of Fe$^{3+}$:Fe$^{2+}$ is presumably controlled by a ferric reductases (22). Ferric reductases have been identified in *M. magnetotacticum* (22) and MSR-1(40). However, these enzymes were isolated from the cytoplasmic fractions of the bacteria and not the magnetosomes (22,40,41). Six other ferric reductases were also identified recently from MSR-1, two of which were further characterized (34). Although these later two proteins show both *in vivo* and *in vitro* reductase activities and deletion both of the genes together reduced iron absorption by the cells and eliminated magnetite production, evidence is lacking to show that they are specifically associated with the magnetosome (34,41). MamZ was also predicted to have a C-terminal ferric reductase domain that faces the periplasmic (internal) magnetosomal side and cells expressing a mutant MamZ with deletion of the C-terminal reductase-like domain produced regular magnetite crystals in the cell flanked by flake-like defected particles (33,42). As one of the redox-control proteins that can interact with MamX and MamH, MamZ is believed to form an iron oxidoreductase and transport complex on the magentosome membrane with a C-terminal reductase catalytic site (33,42). However, direct evidence of the C-terminal domain being a ferric reductase is lacking.

Identified from the isolated magnetosome membrane of AMB-1 as a magnetite-associated protein (43), Mms6 promotes the formation of magnetic nanoparticles *in vitro* when included in co-precipitation synthesis reactions (43,44). Genetic evidence suggests that Mms6 regulates the morphology of magnetites in the later stage of crystallization *in vivo* and alternatively that it is an
accessory protein that is not essential for magnetite formation (4,45). Mms6 has also been proposed to function together with Mms5, Mms7, and Mms13 to control the cubooctahedral shape of magnetite crystals in AMB-1 and has been specifically assigned the role of promoting crystal growth on the 110 face of magnetite (13).

Recombinant Mms6 binds iron with high affinity and high capacity and also self-assembles into multimeric micelles that are important for its in vitro function of promoting magnetite formation (5,6). Mms6 binds ferric iron, but not ferrous iron (21). This brings up the question of how Mms6 interacts with both ferric and ferrous ions in the magnetosome in which the crystallization of ions are expected to be stringently controlled as both ferric and ferrous ions are needed for crystal growth at the 110 crystal surface.

Even by including Mms6 into lipid membrane, the activity observed for Mms6 as a ferric reductase is still not as high as would be expected for a robust reductase that catalyzes the reduction of ferric iron during magnetite biogenesis in vivo. This may be because the experimental conditions were not optimum for Mms6 to function as a reductase or that Mms6 may require intermolecular interactions with other magnetosome proteins like Mms5, Mms7, and Mms13 that co-purify with Mms6 from the magnetosome membrane (43). However, our results are consistent with the view that Mms6 may function as a ferric reductase in vivo to regulate the crystallization of magnetites.

ACKNOWLEDGEMENT

This work was supported by the U.S. Department of Energy, Office of Basic Energy Science, Division of Materials Sciences and Engineering. The research was performed at the Ames Laboratory. Ames Laboratory is operated for the U.S. Department of Energy by Iowa State
University under Contract No. DE-AC02-07CH11358. We thank Samir Mehanovic for help in the development of the reductase.

4.5 References


4.6 Figure Legends

**Figure 4-1.** Reductase activity of Mms6 and C21Mms6. Ferric reductase activity was measured as described in Materials and Methods. A) The reduction of Fe3+ presented as Fe3+-citrate for 20 µM Mms6, m2Mms6 and m3Mms6 in ambient air. B) The initial velocity of Mms6 reductase activity as a function of Fe3+-citrate concentration under anaerobic conditions. C) A double reciprocal plot of the initial velocity and Fe3+-citrate concentration for 20 µM Mms6 in air. D) Reductase activity of C-terminal peptides, C21, m2C21 and m3C21 in air.

**Figure 4-2.** Mms6 ferric reductase specificity for cofactor and electron donor. Mms6 reductase activity was tested with 100 µM NADH or NADPH as electron donors combined with either 100 µM FAD or FMN as cofactors in 20 mM Tris, 100 mM KCl, 1 mM ferrozine, 100 µM ferric citrate, pH7.5. Reaction mixes run in parallel without Mms6 were used as the background to subtract from the results of incubation with 20 µM Mms6 with the combinations of cofactor and electron donor shown.

**Figure 4-3.** Integration of Mms6 into liposomes and bicelles. Samples (500 µL in 20 mM Tris, 100 mM KCl, pH7.5) of 20 µM Mms6, 20 µM Mms6 with transient 0.5% Triton X-100
treatment, 25mM bicelles, 2mM liposomes, 20 µM Mms6/25mM bicelle complex, or 20 µM Mms6/2mM liposome complex were tested by dynamic light scattering. Size distributions for each sample are shown as a volume percentage.

**Figure 4-4. Effect of lipid environment and pH on Mms6 ferric reductase activity.** A) Twenty µM Mms6 alone or incorporated into 25mM q=1 DMPC/DHPC bicelles were tested as described in Materials and Methods. The Vi is expressed as µM ferrozine-Fe2+/min. The Vmax, Km and Kcat for Mms6 in bicelle lipids were 200 nmole ferrozine- Fe2+/min, 76 µM, and 1.7x10-3 sec-1, respectively. O, no bicelles; ■, with bicelles. B) The effect of pH on activity was tested in 100 mM sodium phosphate buffers with pH’s ranging from 5.9 and 7.8. The assay mixture contained 20 µM Mms6 incorporated in 25 mM q=1 DMPC/DHPC bicelle lipids with 800 µM ferrozine, 100 µM NADH, 100 µM FAD, 100 mM sodium phosphate buffer with or without 100 µM ferric citrate at the specified pH values.

**Figure 4-5. Effect of lipid integration on its intrinsic tryptophan fluorescence of Mms6.** Fluorescence spectra were collected ((λex=290 nm) and analyzed as described in Materials and Methods. Smoothed curves for fluorescence intensity of Mms6 (A) or W119F Mms6 (B). The same scans of Mms6 (C) or W119F Mms6 (D) but normalized to the maximum value and not smoothed. F: The Mms6 fluorescence spectra in the presence of lipid bicelles was decomposed as described in Materials and Methods to resolve sub-component 1 that contributes to 90% of experimentally observed spectra and belongs to Class II trp fluorophores from sub-component 2 that contributes to 10% of the experimentally observed spectra and belongs to Class III trp fluorophores.
Figure 4-6. **Ferric reductase activities of Mms6 and mutants with Ala-substitutions in the C-terminal domain.** The ferric reductase activity of each recombinant protein was determined as described in Materials and Methods with 20 µM protein with 0, 40, 80, 160 µM ferric-citrate.

Figure 4-7. **Iron binding activities of Mms6 and mutants with Ala-substitutions in the C-terminal domain.** The iron binding activity of each recombinant protein was determined as described in Materials and Methods with 1 µM protein and 20 µM ferric-citrate.

Figure 4-8. **Correlation between Mms6 iron binding and ferric reductase activity.** A. The data shown in Figs 6 and 7 are plotted to show the correlation in activities relative to the wild-type Mms6 controls. B. The model of Mms6 tertiary structure predicted by i-TASSER with the residues identified in A as important for Mms6 ferric reductase and iron binding activities represented in the space filling mode. Residues in green do not destabilize micelle (measured by size exclusion chromatography). Side chains in red destabilize micelle formation.
4.7 Figures

Figure 4-1

Figure 4-2
Figure 4-3

Figure 4-4
Figure 4-7

Figure 4-8

VGGTWGKGLGLGLGLGAWGPILGVVGAGAVYAMKSRDIESSAQSDDEELRD\n
Red: destabilizes  Green: no effect on structure  Space-filled: decreased reductase and iron binding
CHAPTER 5 CONCLUSIONS

5.1. Conclusions

Structural and functional studies of Mms6, a biomineralization protein from magnetotactic bacteria that can promote the in vitro crystallization of magnetic nanoparticles, are reported in this thesis. The results from initial studies suggested that self-assembly of Mms6 into multimeric micellar structures in aqueous solution might be relevant to its biphasic iron binding properties and its biomineralization functions(1). We found that the self-assembly of Mms6 involves both the hydrophobic N-terminal domain and the highly hydrophilic C-terminal domain.

The self-assembled micelles of Mms6 were visualized by TEM and AFM, the sizes of which, measured under both conditions, are consistent with previous measurements in aqueous solutions(1). Mutants of Mms6 were prepared with the same overall hydropathy profiles but with either the positions of OH/COOH containing residues in the C-terminal domain shuffled (m2Mms6) or all the residues in C-terminal domain scrambled (m3Mms6). These mutations destabilized Mms6 micelles. Site-directed mutagenesis in the C-terminal hydrophobic residues (Ile141, Leu152, and Leu156) also destabilized the micelles showing that these residues contribute to stability of the Mms6 micelles. We also found that the C-terminal domain alone can assemble into multimers in the absence of the hydrophobic N-terminal domain. This opens the possibility for involvement of the hydrophobic residues in the C-terminal domain in either intermolecular or intramolecular hydrophobic interactions in the Mms6 multimer.

By protein mutational studies and gel filtration chromatography, we identified a unique GL repeat in the N-terminal domain that is important for Mms6 micelle formation. The trp residues
in the N-terminal domain can be concluded as important for stability of the Mms6 multimers because micelles formed of Mms6 with these trp residues changed to ala were unstable, whereas micelles made of Mms6 with phe in place of trp retained micellar integrity. The nature of the amino acid substitutions suggests that the hydrophobicity of the amino acids in this position are important for protein structure and multimer stability.

We observed conformational changes in both C-terminal and N-terminal domains of Mms6 upon binding iron. A structural change in the C-terminal peptide upon iron binding was observed by circular dichroism spectroscopy. The C-terminal domain contains the iron-binding site of Mms6. The structural change in the C-terminal domain due to iron binding was also shown to be transmitted to the N-terminal domain by an observed change in intrinsic fluorescence in the presence of ferric iron. By examining mutants of Mms6 in which W103 and W119 were exchanged for phe, the intrinsic fluorescence change was pinpointed to the second trp residue, W119. An overall shape change of Mms6 assembly upon iron binding was also observed by small angle neutron scattering spectroscopy and confirmed by small angle X-ray scattering in our later studies by our collaborators(2).

A periodic change in intrinsic fluorescence with increasing molar ratios of iron to Mms6 was observed at pH 3 in 96 well plates, but not in quartz cuvettes. Analysis of the intrinsic fluorescence spectra revealed that Mms6 displays distinct trp fluorescence spectra when tested in quartz cuvettes and plastic 96 well plates. We showed that Mms6 adsorbs onto the hydrophobic surfaces of 96 well plates, which may result from hydrophobic interactions between the N-terminal domain and the plastic surfaces. Such interaction of Mms6 with the hydrophobic surface is expected to alter the conformation of the hydrophobic N-terminal domain. The red-shifted and
quenched spectrum of the Mms6 trp(s) on the plastic suggest that, in this environment, the N-terminal domain may be extended to expose the trp to an aqueous environment in which it is readily quenched by the dissolved oxygen.

The observations that Mms6 adsorbs on plastic surfaces, inserts spontaneously into liposome bilayers(1), and assembles into a monolayer at air-water interfaces(1,3) support the hypothesis that Mms6 is a membrane protein. For the first time, we have characterized the structural properties of Mms6 in the presence of model membrane bilayers. The integration of Mms6 into lipid bilayers results in a structural rearrangement of the protein as shown by an increase of trp fluorescence intensity and a blue shift of the trp fluorescence spectra. By substituting each the trp residue independently in Mms6 and performing spectral decomposition analysis, we showed that the trp119 residue is the major trp residue that contributes to 90% of the fluorescence signal.

We also showed that Mms6 has ferric reductase activity, with NADH and FAD as preferred electron donors and cofactors, respectively. Consistent with the view that Mms6 is an integral membrane protein, higher reductase activity was observed when Mms6 was integrated into either lipid bicelles or liposomes compared with the protein assembled as a micelle in the absence of lipid. Although the reductase activity is not expected to play a role in the in vitro synthesis of magnetic nanoparticles in which both ferric and ferrous ions are provided, this activity may be involved in its function in vivo to regulate the crystallization of magnetite.
5.2 References

