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Lipoprotein MtsA of MtsABC in *Streptococcus pyogenes* primarily binds ferrous ion with bicarbonate as a synergistic anion

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Abstract **Lipoprotein MtsA is a critical component of MtsABC responsible for iron binding and transport in the Gram-positive bacterium *Streptococcus pyogenes*. The present collective experimental data establish that Fe^{2+} is the primary binding ion for MtsA under optimal physiologically relevant conditions. The binding affinities of MtsA to metal ions are $Fe^{2+} > Fe^{3+} > Cu^{2+} > Mn^{2+} > Zn^{2+}$. We report for the first time that bicarbonate is required as a synergistic anion for stable ferrous binding to MtsA, similar to the iron binding in human transferrin. This work provides valuable information, which helps to understand iron metabolism in bacteria, and creates a basis for developing strategies to suppress bacterial infection.**

Keywords: MtsA; Ferrous ion; Bicarbonate; Metal binding; *Streptococcus pyogenes*

1. Introduction

Iron is essential for the survival of most of the bacterial pathogens, which have developed high-affinity iron-uptake systems to acquire iron directly from host iron-containing proteins including transferrin, ferritin, and heme-containing protein complexes [1]. Extensive work has been carried out to study the iron-uptake mechanisms in Gram-negative bacteria, whereas there is a relative paucity of information concerning iron acquisition in Gram-positive bacteria. *Streptococcus pyogenes* is a Gram-positive human pathogen that causes common pharynx or skin diseases and invasive deep-tissue infections [2]. A typical ABC transporter, MtsABC, consisting of a lipoprotein MtsA, an ATP-binding protein and a hydrophobic integral membrane protein, was found to be important for iron-uptake in this bacterium in vivo [3,4]. As an extracellular membrane-anchored protein with high percentage of homology to periplasmic ferric-binding proteins in Gram-negative bacteria [5], MtsA was proposed to function as a metal ion chelator and transporter. With the ATP-binding protein providing energy, MtsA interacts with the integral membrane component, which leads to iron release and subsequent transmembrane uptake [3].

As a critical component in MtsABC, MtsA has recently been cloned, expressed and briefly characterized. However, divergent results from cellular and biochemical studies were reported regarding the metal binding capabilities and properties of this protein [3,4,6]. MtsA was shown to bind Fe^{3+}, Cu^{2+} and Zn^{2+}, with the first at a different site from the other two [3]. In the bacterial metal competition uptake experiments, the accumulation of ^{55}Fe and ^{54}Mn were substantially reduced by either Fe^{3+} or Mn^{2+} ions, whereas Zn^{2+} reduced the iron accumulation to only 50% and Cu^{2+} completely had no effects, suggesting that Fe^{3+} and Mn^{2+} are preferably transported by MtsA [4]. In a separate study, MtsA at 100 μM was shown to bind 57.1 μM Zn, 6.4 μM Fe, and 2.9 μM Mn upon the purification of the recombinant protein [6]. Apart from these results, little is known about the biochemical and physiological details of metal binding to MtsA.

To understand the chemistry of metal binding to MtsA, we purified and characterized MtsA using various biochemical and biophysical methods. The present data collectively established that Fe^{2+} is the principal binding ion for MtsA under physiologically relevant conditions. Our experiments also revealed that bicarbonate is required as a synergistic anion for stable Fe^{2+} binding in MtsA.

2. Materials and methods

2.1. Expression and purification of MtsA

MtsA-GST plasmid, in which the gene of *S. pyogenes mtsA* (AF180521, without the signal peptide and the N-terminal cysteine residue) was cloned into GST vector, was kindly provided by Dr. Lars Björck (Lund University). MtsA was expressed in *Escherichia coli* BL21 (DE3) star cells and purified according to the Amersham Biosciences protocol. In the conditions used for the expression and purification of MtsA, no evidence indicated that the protein was lipidated. Protein identity was confirmed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI–TOF MS). Briefly, the protein bands were excised from gels and subjected to in-gel tryptic digestion for overnight [7]. Peptide mass spectra were recorded on a Voyage-DE STR MALDI–TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Peptide-mass-fingerprinting was carried out by searching against NCBInr protein database with MS-Fit (http://prospector.ucsf.edu/) [8,9].

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Abbreviations: ABC, ATP binding cassette; *E. coli*, *Escherichia coli*; ICP-MS, inductively coupled plasma mass spectroscopy; IPTG, isopropyl β-D-thiogalactoside; MALDI, matrix-assisted laser-desorption ionization; NMR, nuclear magnetic resonance; PMF, peptide-mass-fingerprinting; *S. pneumoniae*, *Streptococcus pneumonia*; *S. pyogenes*, *Streptococcus pyogenes*

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2.2. Electronic spectra of metal binding

UV–vis spectra were recorded on a Cary 3E spectrometer (Varian) at 25 °C. Hepes (50 mM pH 7.4) buffer served as the reference for full-range spectra. Anion solutions (1 mM) including $NaHCO_3$, Na_2NTA, Na_2SO_4, KCl, KH_2PO_4, and $Na_3C_6H_5O_7$ were examined for the ability to enhance the binding capability of MtsA for Fe^{2+}. Nitrogen gas (HK Oxygen & Acetylene Co.) was flushed into the protein solutions and the freshly prepared $Fe(NH_4)_2(SO_4)_2$ solution to maintain Fe^{2+} form during the experiments. pH dependent ferrous binding of MtsA was also examined in buffers containing 50 mM Hepes, 10 μM $NaHCO_3$ with pH adjusted to 4.5–9.0 with 1 M NaOH or 1 M HCl.

2.3. Equilibrium dialysis

Binding of Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} to MtsA was determined with equilibrium dialysis and inductively coupled plasma mass spectroscopy (ICP-MS), as previously described [10]. The 200 μl of purified proteins (1 μM) was dialyzed (1 kDa cut-off membrane) against 1.0 l of 50 mM Hepes, 10 μM $NaHCO_3$ (pH 7.4) supplemented with a series of concentrations of Fe^{2+} (as $Fe(NH_4)_2(SO_4)_2$), Fe^{3+} (as $FeNH_4(SO_4)_2$), Cu^{2+} (as $CuSO_4$), Zn^{2+} (as $ZnCl_2$), and Mn^{2+} (as $MnCl_2$) from 0 to 150 μM overnight at 4 °C. Measures were taken to minimize carbonate concentration by vacuuming the solution for 20 min with constant mixing just before the experiments, and sealing the dialysis beaker with parafilm and constantly flushing with N_2 in the case of Fe^{2+} dialysis. The concentrations of metal ions were determined by ICP-MS upon centrifugation at 12000 × *g* for 20 min to remove any possible insoluble species. The data were subjected to Hill analysis for the determination of the stoichiometry and the binding affinity.

2.4. ^{13}C NMR

^1H-decoupled ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX500 spectrometer equipped with a 5 mm broad-band probe as described previously [11,12]. ^{13}C NMR experiments were performed under 298 K, using 1 mM MtsA protein in 0.1 M KCl solution (10% D_2O and 90% H_2O) containing 40 mM $NaH^{13}CO_3$. Iron release experiment was carried out with 20 mM EDTA being directly added to the MtsA-Fe^{2+} solution to remove bound iron.

3. Results and discussion

3.1. Expression and purification of MtsA

It has been illustrated that in bacteria, iron is taken up as Fe^{3+}/Fe^{2+} or heme into cells through systems associated with siderophores, hemephores, or specific receptors [13,14]. For *S. pyogenes*, three putative iron transporters have been identified so far, including systems containing lipoproteins HtsA, FtsB and MtsA [3,6,15]. Under the conditions of iron limitation, these proteins are up-regulated and these iron-uptake systems have high affinity to sequester iron or iron complexes. Among the three putative iron transporters, MtsA is the only free iron transporter identified so far in *S. pyogenes*, as HtsA and FtsB prefer binding to heme [6] and ferrichrome [15], respectively.

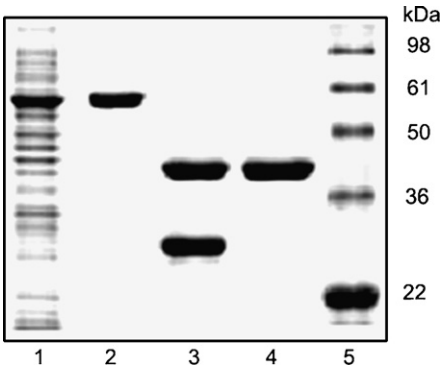

Fig. 1. Expression and purification of MtsA. Twelve percent SDS–PAGE analysis of recombinantly expressed MtsA. *lane 1*, whole cell lysate; *lane 2*, fusion protein; *lane 3*, factor Xa cleavage products, MtsA (~38 kD) + GST(~26 kD); *lane 4*, pure MtsA; *lane 5*, molecular marker.

Expression and purification of MtsA basically followed the procedure described previously [3]. To improve the yield of the protein, modifications of the experimental conditions were made: when OD_{595} reached 0.8, IPTG was added to a final concentration of 0.4 mM to induce MtsA expression. Recombinant proteins were purified following the manual of GST recombinant protein kit of Amersham Biosciences. Typically, around 10 mg recombinant proteins were obtained per liter culture media. On the SDS–PAGE gel, the purified MtsA migrates with an apparent molecular weight of ~38 kDa (Fig. 1), slightly higher than the calculated value of 32 kDa and consistent with previously reported results [3,6]. MALDI–TOF MS analysis (data not shown) confirmed the identity of MtsA.

3.2. MtsA-Fe^{2+} binding facilitated by bicarbonate

Electronic spectroscopy is commonly used to monitor the binding between transition metal ions and proteins as such interactions often induce characteristic UV–vis electronic absorptions. MtsA (apo-form) alone showed no characteristic electronic absorbance in 310–600 nm (Fig. 2A, black line). Addition of Fe^{2+} produced a positive broad absorbance peak at around 365 nm (Fig. 2A, red line), suggesting possible weak ferrous binding to the protein [23]. In the presence of 40 mol equiv. of bicarbonate, the minor peak at 365 nm was developed to a pronounced shoulder peak upon ferrous titration (Fig. 2A, green line), indicating that bicarbonate can facilitate and stabilize ferrous binding to MtsA. The requirement of bicarbonate as a synergistic anion in ferrous binding is quite specific, as several other anions, including NTA^-, Cl^-, SO_4^{2-}, $H_2PO_4^-$ and citrate, showed minimal effects when examined under identical conditions (data not shown).

In addition, by measuring the metal-binding constants with equilibrium dialysis and ICP-MS [10], the pH dependence of Fe^{2+} binding affinity of MtsA was examined between pH 4.5 and 9.0. The affinities of Fe^{2+} binding to MtsA in the presence of $NaHCO_3$ was pH dependent and maximal at pH 7.4, as reflected in a plot of Fe^{2+}-binding affinities at different pH values (Fig. 2B). Therefore, pH 7.4 buffer containing $NaHCO_3$ was used in subsequent experiments. At pH 4.5 or 9.0, the binding was completely abolished.

^{13}C NMR spectroscopy was applied to further verify that the binding of Fe^{2+} in MtsA involves concomitant binding of bicarbonate as a synergistic anion. In a buffer containing $NaH^{13}CO_3$, the ^{13}C NMR spectrum of apo-MtsA showed a sharp peak at around 160 ppm, apparently assigned to free $H^{13}CO_3^-$ (Fig. 3; the broad peaks within the range of 170–180 ppm correspond to the signals of natural abundance of ^{13}C in the protein). When excess Fe^{2+} was added into the solution, the bicarbonate resonance peak was intensified and broadened, suggesting simultaneous ferrous bindings to MtsA. When free bicarbonate and free or weak non-specifically bound ferrous ions were washed out by a gel filtration column with 0.1 M KCl, a new sharp peak at around 163 ppm appeared while the free bicarbonate peak at 160 ppm disappeared in ^{13}C NMR spectrum of the protein fractions (Fig. S1A). This indicates that the new ^{13}C signal at 163 ppm should be assigned to the bicarbonate specifically

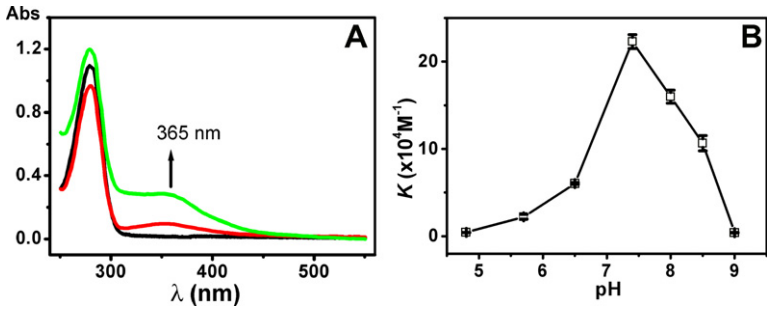

Fig. 2. Ferrous binding to MtsA in the presence of bicarbonate. (A) UV–vis absorption spectra of MtsA (26 μM, Black) in 50 mM Hepes, and upon addition of 10 mol equiv of Fe^{2+} in the absence (Red) and presence (Green) of 40 mol equiv. of HCO_3^-. (B) pH dependent ferrous binding to MtsA.

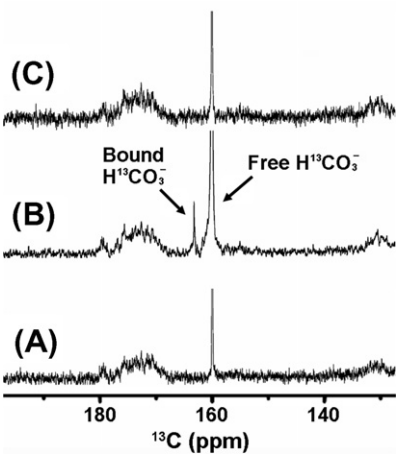

Fig. 3. 125 MHz ^{13}C NMR spectra of MtsA-Fe^{2+} interaction (90% H_2O, 10% D_2O, pH 7.4). (A) Apo-MtsA (1 mM) solution containing 0.1 M KCl and 40 mM $NaH^{13}CO_3$. (B) After adding 10 mM Fe^{2+} to the protein solution in (A), 0.1 M KCl was used to wash out the extra unbound Fe^{2+} with a gel filtration column and then $NaH^{13}CO_3$ solution was added to restore 40 mM bicarbonate concentration. (C) 20 mM EDTA was added to the protein solution in (B) to remove the protein-bound Fe^{2+}.

ligated to the protein-bound Fe^{2+}, as Fe^{2+} does not induce any additional peak besides the free bicarbonate signal in the absence of MtsA (Fig. S1B and S1C). To compare the two bicarbonate peaks in the free and the protein-Fe^{2+} bound forms, bicarbonate was added to restore the free $H^{13}CO_3^-$ signal at 160 ppm (Fig. 3). To further confirm that bicarbonate is directly bound to Fe^{2+}, EDTA, a well-known high-affinity chelator to Fe^{2+} ($\log K \sim 14.3$) [16], was titrated into the solution. Upon the introduction of EDTA, the peak at around 163 ppm disappeared, proving that bicarbonate was directly bound to iron (Fig. 3).

These NMR data suggested that the bound ferrous ion remains in their divalent form with low-spin state (diamagnetic form) rather than being oxidized to Fe^{3+} upon binding, as paramagnetic iron would have broadened the resonance signal beyond detection, as in Fe^{3+}-transferrin-(bi)carbonate complex [17]. The UV–vis data of Fe^{2+}-MtsA in the presence of bicarbonate is also characteristic for a low-spin ferrous in an octahedral geometry [23]. This phenomenon is indeed unusual (and thus interesting), our speculation is reasonably based on the experimental data. No low-spin iron with identical ligand sets was found in other proteins, however, an Fe^{2+} site with a similar ligand set has been observed in photosystem II in *Chlamydomonas reinhardtii* PSI-mutant where the low-spin iron was found to bind to four histidine residues and one bidentate glutamate or bicarbonate [18–20].

Our collective data firmly established that Fe^{2+} is the favorite binding ion of MtsA under physiologically relevant conditions. For the first time we demonstrated that an exogenous bicarbonate is required as a synergistic anion for the stable ferrous binding to MtsA, a surprisingly remarkable similarity to the iron binding modes of human transferrin [21,22]. MtsA shares 79% sequence identity with PsaA, a zinc-binding lipoprotein from *S. pneumoniae* [4]. Based on the crystal structure of PsaA, the ferrous binding site was modeled to involve His68, His140, Glu206 and Asp281. The involvement of bicarbonate in Fe^{2+} binding was supported complementarily by the above-mentioned NMR and UV results. Especially, ^{13}C NMR data confirmed that the exogenous bicarbonate directly takes part in the coordination to Fe^{2+} as the synergistic anion.

3.3. Metal bindings to MtsA

To further characterize the metal-binding properties of MtsA, stoichiometries and affinities for the interaction between MtsA and metal ions including Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, and Zn^{2+} were determined by equilibrium dialysis in combination with ICP-MS analysis (Fig. 4). These data showed that MtsA binds Fe^{2+}, Fe^{3+}, and Mn^{2+} in a 1:1 stoichiometry, while binding Zn^{2+} and Cu^{2+} in a 1:2 fashion. It is possible that, besides the specific metal-binding site, an additional binding site exists in MtsA for Zn^{2+} and Cu^{2+}, both requiring less binding ligands.

Fig. 4B presents the binding constants derived from the Hill plot fitting of the respective metal titration curves. The order of affinity for these metal ions follows Fe^{2+} ($(2.23 \pm 0.16) \times 10^5\ M^{-1}$) > Fe^{3+} ($(8.36 \pm 1.05) \times 10^4\ M^{-1}$) > Cu^{2+} ($(5.97 \pm 0.22) \times 10^4\ M^{-1}$) > Mn^{2+} ($(1.84 \pm 0.11) \times 10^4\ M^{-1}$) > Zn^{2+} ($(1.30 \pm 0.03) \times 10^4\ M^{-1}$), indicating that Fe^{2+} is the favorable binding ion for MtsA. This result clarifies the previously reported divergent data regarding the metal binding properties of MtsA, which may be derived from the differential experimental conditions (Luria-Bertani broth [6], PBS with NaCl [3], or Todd-Hewitt broth with yeast [4]). Moreover, recent studies have shown that increased level of Zn^{2+} in medium had no negative effects on MtsA expression [24], and that Zn^{2+} and Cu^{2+} had minor effects on the accumulations of ^{55}Fe and ^{54}Mn by bacteria [4]. All together, these observations suggest that Cu^{2+} and Zn^{2+} may not be transported via MtsA. Although a previous study suggested that MtsABC also plays an important role in Mn transport [4], our present data with others [6] suggested

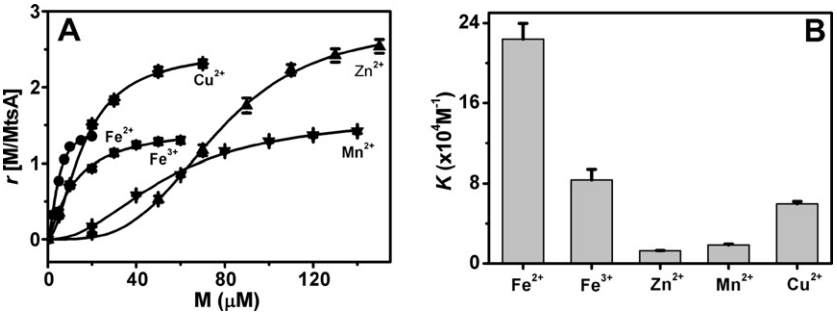

Fig. 4. Binding affinities of metal ions for MtsA were determined with equilibrium dialysis and ICP-MS. (A) Titration curves for Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}, respectively. (B) Binding constants (K) of metals to MtsA as analyzed with Hill plots shown in (A).

that MtsA exhibits too low affinity for Mn to have such a physiological function. It can be therefore speculated that MtsA works as a specific receptor targeting Fe^{2+} for iron acquisition through MtsABC system in *S. pyogenes*.

In summary, we have purified and characterized the lipoprotein MtsA, a critical component in MtsABC responsible for iron transport in *S. pyogenes*. Our results confidently established that Fe^{2+} is the principal binding ion for MtsA under physiologically relevant conditions. It is the first time that bicarbonate has been found to be required as a synergistic anion for stable iron binding in an iron transporter from Gram-positive bacteria. These findings will improve our understanding of iron trafficking in Gram-positive bacteria, and provide useful information for developing possible strategies to suppress the bacterial infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.03.020.

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