Copper Binding by Methanobactin and its Relevance for Copper Acquisition
by Methanotrophic Bacteria from Natural Organic Matter

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Summary

Methanotrophic bacteria play a key role in the regulation of methane release to the atmosphere due to their ability to oxidize a large fraction of the total methane from aquatic, marine, and terrestrial sources. Methanotrophs catalyze the oxidation of methane via a methane monooxygenase (MMO) that contains several copper atoms. Hence, the oxidation of methane depends on the availability of copper and the efficiency of copper acquisition by methanotrophic microorganisms. In natural environments such as soils, peat bogs, and surface waters, the presence of natural organic matter such as, e.g., humic acids, exhibiting a high affinity for copper, can control the bioavailability of copper. To mobilize copper, aerobic methanotrophs have developed a unique copper uptake system, similar to the siderophore-based iron uptake systems, involving the synthesis of a copper-binding ligand (so-called methanobactin). The aim of this thesis was to evaluate the potential role of methanobactin for copper acquisition in organic-rich natural systems.

The isolation of highly pure copper-free methanobactin is a prerequisite for the investigation of the biogeochemical functions of this chalcomphore molecule produced by methane-oxidizing bacteria. The analysis and characterizations of isolates obtained from Methylosinus trichosporium OB3b cultures with a previously published resin extraction procedure revealed that methanobactin contributed only 32% to the total organic carbon of the extracts. Spectroscopic results implied that beside methanobactin, substantial amounts of organic substances, exhibiting peptide or polysaccharide-like character, were present in the resin extracts. With an additional purification step using HPLC fractionation we succeeded in isolating pure methanobactin. Methanobactin released by Methylosinus trichosporium OB3b exhibited a high affinity for copper as demonstrated with metal titration experiments, whereby copper complexation increased.
with increasing pH. The pH-dependence was, however, only weak under the pH conditions investigated (pH 5 – 8). Depending on copper concentrations, the formation of three different types of copper-methanobactin complexes was observed. Dimer complexes, in which one copper ion is bound by two methanobactin molecules, prevailed at low copper-to-methanobactin ratios. With increasing copper concentrations, monomer copper-methanobactin complexes were formed, whereas at elevated copper concentrations methanobactin bound two copper ions. The occurrence of the different copper-methanobactin complexes was spectroscopically confirmed after separation of the complexes by size-exclusion chromatography. The determined stability constants for proton and copper binding can be used to predict the speciation of methanobactin in natural environments.

A well characterized humic acid extracted from a humified organic horizon of a Humic Gleysol was used as a model substance to study copper acquisition by methanobactin from natural organic matter. Kinetic experiments on ligand exchange between copper-humic acid and methanobactin studied with time-resolved UV–vis spectroscopy demonstrated a fast uptake of copper from humic acid. A substantial sorption of methanobactin into humic acid was not observed. Methanobactin was found to effectively mobilize copper from humic substances by forming strong monomer copper-methanobactin complexes even if soluble copper concentrations were low. Considering the fast complexation kinetics and the effective complexation of copper in equilibrium, methanobactin is very efficient for copper acquisition in organic-rich environments. The excellent agreement of the experimental results with model predictions using the conditional stability constants for copper binding by methanobactin and humic acid, confirms that the established speciation model for methanobactin can be applied for predictions in natural systems.

In this dissertation, our knowledge on copper binding, including temperature-dependence of copper complexation and pH-dependent stability of methanobactin, has been extended. The chemical properties of methanobactin provide valuable information to identify the ecosystems in which methanobactin may play an important role in copper uptake by methanotrophic bacteria.
Zusammenfassung


Zusammenfassung


1. Introduction

1.1. Research motivation

Methanotrophic microorganisms are gram-negative bacteria that have the unique ability to use methane as their sole source of carbon and energy [Hanson & Hanson, 1996]. In terrestrial environments and surface waters, aerobic methanotrophs were found to oxidize a large fraction of the total methane and, therefore, play a key role in the regulation of methane release to the atmosphere. Beside methane oxidation, some methanotrophs are able to degrade chlorinated hydrocarbons [Yoon & Semrau, 2008; Lontoh & Semrau, 1998]. Several factors, including pH, moisture content, temperature, methane, oxygen and nitrogen content, have been found to influence methane oxidation rates [Hanson & Hanson, 1996; Bender & Conrad, 1992]. Copper (Cu), which is central to the metabolism of methanotrophic bacteria, may be considered as one of the most important factors controlling methanotrophic activities [Semrau et al., 2010]. Methane oxidation depends on the availability of Cu and the efficiency of Cu acquisition by methanotrophs. Although the influence of Cu has been widely recognized, the effect of Cu availability on methanotrophic activities and the development of microbial communities is only sparsely studied in situ in natural systems. In natural environments such as soils, peat bogs, and surface waters, Cu is often bound to mineral surfaces and natural organic matter. First studies have investigated the capability of methanotrophic microorganisms for Cu mobilization from mineral Cu sources [Chi Fru et al., 2011; Kulczycki et al., 2011, 2007; Knapp et al., 2007], but little is known about Cu acquisition from natural organic matter.

In the following sections, an overview about Cu pools and speciation in the environments and its bioavailability in natural systems is presented. Furthermore, the role of Cu as an essential nutrient for methanotrophs and its function in methane oxidation are discussed.
1.2. Methanotrophic bacteria and methane oxidation

Methane is the most abundant organic trace gas in the atmosphere [Wuebbles & Hayhoe, 2002]. Total methane emissions are mostly biogenic and wetlands, including rice paddies, are one of the largest sources of methane produced by methanogenic microorganisms under anaerobic conditions [IPCC, 2007]. Methane production in lake sediments also contributes significantly to the global atmospheric methane budget [Bastviken et al., 2004]. A large fraction of methane is, however, oxidized by methanotrophic bacteria before it reaches the atmosphere [Hanson & Hanson, 1996] and these oxidative processes play an important role in the regulation of methane release [Reeburgh et al., 1993].

Methane-oxidizing bacteria are ubiquitous and are found in diverse environments, such as wetlands, rice paddies, peat bogs, sewage sludge, soils, freshwater and marine sediments, lakes, and groundwater [Bowman, 2006; Dedysh et al., 1998; Hanson & Hanson, 1996]. An overview about the diversity of methanotrophs and their habitats is given by Semrau et al. [2010]. According to the authors, most methanotrophs favor moderate pH (5–8) and temperature (20–35 °C) conditions. However, thermophilic and psychrophilic strains have been isolated growing best at temperature above 40 °C and below 15 °C, respectively. Furthermore, some methanotrophs are halophilic, alkalophilic or acidophilic. Methanotrophs have been classified into two major groups, Type I and Type II methanotrophs, based on physiological and biochemical characteristics. A major difference is the use of a ribulose monophosphate pathway for biomass assimilation by Type I methanotrophs, whereas Type II use a serine pathway. Further distinctive characteristics as, e.g., membrane arrangement and cell morphology are described in Hanson & Hanson [1996]. Type I and II methanotrophs belong to the phylum Proteobacteria including α-Proteobacteria (e.g., Methylosinus and Methylocystis strains) and γ-Proteobacteria (e.g., Methylococcus and Methylobacter strains). Recently, methanotrophic bacteria phylogenetically located under the phylum Verrucomicrobia have been isolated [Dunfield et al., 2007].
1.3. The role of copper in methane oxidation

All known methanotrophs oxidize methane to carbon dioxide with methanol, formaldehyde, and formate as intermediates. An imperative step of their metabolic pathway is the conversion of methane to methanol, a reaction catalyzed by methane monooxygenases (MMO). Whereas most aerobic methanotrophs produce a membrane bound or particulate MMO (pMMO), some strains also produce a cytoplasmic, or soluble MMO (sMMO) under Cu limiting conditions [Lieberman & Rosenzweig, 2004; Hanson & Hanson, 1996]. Both types of MMO contain metal atoms which are involved in the electron transfer to the substrate. sMMO is composed of three components: a hydroxylase which holds a di-iron active site, a reductase shuttling electrons to the active site, and a regulatory protein [Hakemian & Rosenzweig, 2007; Merkx et al., 2001]. pMMO, in contrast, contains several Cu atoms and possibly a di-iron center [Rosenzweig, 2008]. Copper has been found to play a key role in the regulation of pMMO and sMMO expression as well as in the regulation of other metabolic enzymes of methanotrophs [Semrav et al., 2010; Balasubramanian & Rosenzweig, 2008; Hakemian & Rosenzweig, 2007; Stanley et al., 1983]. Increasing methane oxidation rates were observed for the Type I methanotroph Methylomicrobium album BG8 with increasing Cu concentrations in the nutrient medium, indicating that methane oxidation by pMMO critically depends on Cu availability [Lontoh & Semrav, 1998]. Consequently, Cu is crucial to both, the regulation and the chemistry of pMMO and due to their high Cu requirement [Choi et al., 2003; Zahn & DiSpiro, 1996], methanotrophs necessitate an efficient Cu mobilization and uptake system.

1.4. Copper in the environment

An encompassing overview on the ecological cycling of Cu in the environment is given in Nriagu [1979]. In summary, the average Cu concentration in the lithosphere is about 24 mg/kg. Copper is found as a component of various minerals, whereby chalcopryite, containing 34 % Cu, is the most abundant in rocks. Copper is often associated with iron and manganese hydr(oxides), sulfides and carbonates [Benjamin & Honey, 1992] and is released from the minerals by weathering processes from rocks with Cu amounts ranging from 4 mg/kg in limestones up to 90 mg/kg in
basalts [Blume et al., 2010]. In oxidizing environments, Cu is mostly present as Cu$^{2+}$, whereas Cu$^+$ in only found under reducing conditions. Typically, total copper concentrations in soils range from 2 to 40 mg/kg, whereof less than 1% is present in its soluble form. In aerated soils, up to 75% of the total Cu can be bound to organic material and up to 70% may be associated with iron and manganese oxides [Blume et al., 2010]. The binding of Cu to both, mineral and organic sorbents is affected by a variety of factors of which the pH plays a key role. In general, Cu binding to minerals and organic matter strongly increases with increasing pH leading to a low availability of Cu under neutral and alkaline conditions [Heidmann et al., 2005a; Christl & Kretzschmar, 2001, 1999]. The formation of Cu-bearing solid phases is favored under neutral and alkaline conditions, which further limits Cu availability. Under acid conditions, natural organic ligands such as humic and fulvic acids usually exhibit a higher affinity for Cu than do many common mineral phases such as iron oxides or clay minerals [Heidmann et al., 2005b; Vermeer et al., 1999]. Thus, the presence of strong natural organic ligands is expected to control the availability of Cu in natural systems under acidic conditions. Likewise, biochelators exuded by algae were found to form strong Cu complexes in eutrophic lakes as reported by Xue & Sigg [1993]. They measured total Cu concentrations ranging from 6 to 75 nM in pH neutral lakes and rivers. Free Cu$^{2+}$ concentrations, however, were six to seven orders of magnitudes lower. Furthermore, the presence of sulfide may decrease free Cu concentrations under suboxic and anoxic conditions by forming sparingly soluble Cu-bearing sulfide phases as, e.g., in lake sediments and wetlands [Weber et al., 2009a,b]. A survey analyzing soils and pore water originating from wetlands comprising conditions differing in pH, Cu and organic matter content, revealed that total Cu concentrations in pore water and free Cu$^{2+}$ activities decrease with increasing organic carbon and increasing pH [Gerber, 2009]. Free Cu$^{2+}$ activities ranged from $10^{-8}$ to $10^{-11}$ at pH $< 4$ and from $10^{-13}$ to $10^{-17}$ at circumneutral pH. The measured activities were three orders of magnitudes lower than Cu$^{2+}$ activities observed in mineral soils [Sauve et al., 1997]. The extremely low Cu availabilities in circumneutral and/or organic-rich environments demands special mechanisms for Cu acquisition. Some organisms are known to produce chelating ligands to satisfy their nutrient requirements. Up to 45% of the total Cu were extracted from the above described wetland samples with solutions containing EDTA [Gerber,
1.5 Methanotrophs’ copper acquisition strategy

As described in the previous section, Cu availability is often low in many natural environments because it is bound in thermodynamically and kinetically stable soluble complexes or to solid phases. Thus, methanotrophic bacteria need to overcome this limitation in order to satisfy their high Cu requirement. Actually, studies conducted in the 1990s provided first indications for the existence of a specific Cu acquisition strategy. Methylosinus trichosporium OB3b and Methylococcus capsulatus Bath were found to release Cu binding compounds under Cu-depleted conditions [Morton et al., 2000; DiSpirito et al., 1998; Zahn & DiSpirito, 1996]. In analogy to the iron-chelating siderophores released by plants, bacteria, and fungi to overcome Fe deficiency [Winkelmann, 1991; Bossier et al., 1988; Neilands, 1981], such Cu binding ligands are termed chalkophores [Balasubramanian & Rosenzweig, 2008; Kim et al., 2004]. Kim et al. [2004] succeeded for the first time to isolate and elucidate the structure of a chalkophore produced by Methylosinus trichosporium OB3b, a small chromopeptide with a molecular mass of 1154 Da and showing high affinity for Cu that was named methanobactin. It is composed of seven amino acids and two chromophoric residues involved in Cu binding with the sequence 1-N-[mercapto-(5-oxo-(3-methylbutanoyl)oxazol-4-ylidene)methyl]-Gly-Ser-Cys-Tyr-[(pyrrolidin-2-yl)-(mercapto-(5-oxo-oxazol-4-ylidene)methyl)]-Ser-Cys-Met [Behling et al., 2008; Kim et al., 2004]. The synthesis of methanbactins appears not to be limited to one particular methanotrophic strain. Recently, methanobactin released from Methylocystis strain SB2, Methylococcus capsulatus Bath, and from Methylomicrobium album BG8 have been isolated [Choi et al., 2010; Krentz et al., 2010]. Likewise, a screening assay adapted from the chrome azurol S method used to detect siderophores [Schwyn & Neilands, 1987] proved that the ability to produce methanobactins is wide-spread in methanotrophs [Yoon et al., 2010]. Independent of their structural diversity, methanobactins are composed of amino acid residues and contain two heterocyclic rings (imidazole and/or oxazolone) involved in Cu binding [Krentz 2009]. Thus, the bioavailable Cu pool may be much larger than free Cu$^{2+}$ concentrations if the organisms needing Cu possess an appropriate Cu acquisition and uptake system.
et al., 2010; Behling et al., 2008]. Methanobactin has a high affinity for both, Cu(I) and Cu(II), whereby Cu(II) is immediately reduced to Cu(I) upon binding [El Ghazouani et al., 2011; Choi et al., 2006b; Hakemian et al., 2005]. Conditional stability constants for Cu(II) binding by methanobactin isolated from Methylosinus trichosporium OB3b have been determined by isothermal titration calorimetry [Choi et al., 2006b]. High initial binding with stability constants of $\log K = 8.4$ and $\log K = 6.4$ for Cu-to-methanobactin ratios above 0.25 and above 0.5, respectively, suggest the formation of tetramer, dimer, and monomer Cu-methanobactin complexes [Choi et al., 2006b]. A binding constant of $\log K = 20.8$ has been determined for Cu(I) at alkaline pH [El Ghazouani et al., 2011]. In the absence of Cu, methanobactin also binds a variety of other metals such as Ag, Au, Cd, Co, Fe, Hg, Mn, Ni, and Zn [Choi et al., 2006a].

In addition to its role in the external Cu mobilization system, methanobactin may also be involved in the regulation of pMMO expression, function as Cu chaperone for pMMO and be responsible for the electron flow to pMMO, or act as an oxygen radical scavenger [Balasubramanian et al., 2010; Choi et al., 2008, 2005, 2003; DiSpirito et al., 1998].

Recent studies revealed the influence of methanobactin on methanotrophic activity in the presence of Cu-bearing minerals [Chi Fru et al., 2011; Kulczycki et al., 2011; Knapp et al., 2007]. The authors found that methanobactin enhanced Cu release from the minerals and stimulates growth of methanotrophs when Cu is not readily available but added as a solid-phase Cu source to the nutrient medium. Furthermore, methanobactin was found to promote dissolution of Cu-doped silicate [Kulczycki et al., 2007].
1.6. **Research objectives and approach**

Although first studies investigated the influence of methanobactin on methanotrophic growth depending on the mineralogy of the Cu-bearing minerals, the biogeochemical aspects of Cu acquisition by methanobactin from environments with limited Cu availabilities are still poorly understood. Moreover, even though Cu availability in natural systems can be controlled to a large extent by the presence of natural organic matter and strong Cu complexation with a variety of organic ligands (*vide supra*), methanobactin-promoted Cu acquisition from natural organic matter has not yet been studied. The general goal of this project was, therefore, to determine the stability of methanobactin in natural environments and to evaluate the capability of methanobactin for Cu mobilization from natural organic matter. The results enhance our understanding of high affinity nutrient acquisition processes in general.

Sufficient amounts of Cu-free methanobactin are prerequisite to investigate the biogeochemical functions of chalkophores. Therefore, the aim addressed in the first part (Chapter 2) was to improve the previously reported one-step purification method to isolate Cu-free methanobactin [Choi et al., 2005] and to develop a procedure that can be used for semi-preparative work yielding highly pure Cu-free methanobactin. The one-step resin extraction method was extended with a subsequent purification step with HPLC using a reversed-phase C18 column. Different fractions were collected and analyzed to separate pure methanobactin and to characterize all component that were present in the product obtained from the resin extraction method. Characterization methods included various spectroscopic techniques, such as UV–vis, C-1s near-edge X-ray absorption fine structure (NEXAFS), and Fourier-transform infrared (FT–IR) spectroscopy, as well as electrospray ionization mass spectrometry (ESI–MS).

To better understand the potential role of methanobactin in Cu acquisition by methanotrophs, a chemical speciation model describing Cu binding to methanobactin and the formation of possible Cu-methanobactin complexes within the pH range of methanotrophs’ habitats is needed. In a second part (Chapter 3), a speciation model describing proton and copper binding by methanobactin isolated from *Methylosinus trichosporium* OB3b cultures was developed based on results obtained from potentiometric and metal titrations.
Chapter 1. Introduction

The third part (Chapter 4) assessed the competition between methanobactin and natural organic matter for Cu binding by size-exclusion chromatography, whereby humic acids were used as a model substance for natural organic matter. Furthermore, the kinetics of ligand exchange was studied by UV–vis spectroscopy. The experiments were conducted to answer the overall goal of the present study that is to evaluate the efficiency of methanobactin for Cu acquisition from natural organic matter.
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2. Isolation and purification of Cu-free methanobactin from Methylosinus trichosporium OB3b

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Abstract

The isolation of highly pure copper-free methanobactin is a prerequisite for the investigation of the biogeochemical functions of this chalkophore molecule produced by methane oxidizing bacteria. Here, we report a purification method for methanobactin from Methylosinus trichosporium OB3b cultures based on reversed-phase HPLC fractionation used in combination with a previously reported resin extraction. HPLC eluent fractions of the resin extracted product were collected and characterized with UV–vis, FT-IR, and C-1s NEXAFS spectroscopy, as well as with elemental analysis and ESI-MS. The results showed that numerous compounds other than methanobactin were present in the isolate obtained with resin extraction. Molar C/N ratios, mass spectrometry measurements, and UV–vis spectra indicated that methanobactin was only present in one of the HPLC fractions. On a mass basis, methanobactin carbon contributed only 32% to the total organic carbon isolated with resin extraction. Our spectroscopic results implied that besides methanobactin, the organic compounds in the resin extract comprised breakdown products of methanobactin as well as polysaccharide-like substances. Our
results demonstrate that a purification step is indispensable in addition to resin extraction in order to obtain pure methanobactin. The proposed HPLC purification procedure is suitable for semi-preparative work and provides copper-free methanobactin.

2.1. Introduction

Aerobic methanotrophic microorganisms are Gram-negative bacteria that have the unique ability to use methane as their only source of carbon and energy [Hanson & Hanson, 1996]. In a first step of their metabolic pathway, methanotrophs oxidize methane to methanol, a reaction catalyzed by methane monoxygenase (MMO). Whereas most aerobic methanotrophs produce a membrane-bound, or particulate MMO (pMMO), some strains also produce a cytoplasmic, or soluble MMO (sMMO) under copper-limiting conditions [Lieberman & Rosenzweig, 2004; Hanson & Hanson, 1996]. Both types of MMO contain metal atoms which are involved in the electron transfer to the substrate. pMMO contains several copper atoms [Semrau et al., 2010; Rosenzweig, 2008] and possibly a di-iron center [Rosenzweig, 2008], whereas sMMO holds a di-iron active site [Hakemian & Rosenzweig, 2007; Merkx et al., 2001]. Copper has been found to play a key role in the regulation of pMMO and sMMO expression as well as in the regulation of other metabolic enzymes of methanotrophs [Semrau et al., 2010; Balasubramanian & Rosenzweig, 2008; Hakemian & Rosenzweig, 2007; Stanley et al., 1983]. Furthermore, methane oxidation by pMMO critically depends on copper availability [Lontoh & Semrau, 1998]. Due to their high copper requirement, methanotrophs necessitate an efficient copper mobilization and uptake system.

Studies conducted in the 1990s provided the first evidence for the production of copper binding compounds (CBC) or copper binding ligands (CBL) released by methanotrophic bacteria under copper-depleted conditions [Morton et al., 2000; DiSpirito et al., 1998; Tellez et al., 1998; Zahn & DiSpirito, 1996]. Such copper binding ligands are termed chalkophores, in analogy to siderophores involved in the iron uptake in many organisms. Only recently, researchers have succeeded in isolating and elucidating the structure of a chalkophore from the methanotroph Methylosinus trichosporium OB3b, a small chromopeptide showing high affinity for copper that
was named methanobactin [Behling et al., 2008; Kim et al., 2005, 2004].

Copper-bound methanobactin isolated from *Methylosinus trichosporium* OB3b has a molecular mass of 1215 Da and is composed of seven amino acids and two chromophoric residues involved in copper binding [Kim et al., 2004]. Upon binding to methanobactin, Cu(II) is immediately reduced to Cu(I) [Choi et al., 2006b; Hakemian et al., 2005]. Initially, Kim et al. [2004] suggested that Cu(I) coordination is associated with two hydroxyimidazolates, each contributing with one N and one S atom to copper binding. Behling et al. [2008], however, proposed a revised structure of the oligopeptide methanobactin containing oxazolone rings instead of hydroxyimidazolate rings responsible for copper binding. The structure of the copper-bound methanobactin is shown in Fig. 2.1.

The copper binding peptide methanobactin is not only suspected to be involved in copper acquisition from the extracellular phase [Choi et al., 2006b; DiSpirito et al., 1998], but it may also
function as copper chaperone for pMMO [Balasubramanian & Rosenzweig, 2008; Choi et al., 2003; DiSpirito et al., 1998], be responsible for the electron flow to pMMO [Choi et al., 2008, 2005, 2003], and regulate pMMO expression [Choi et al., 2008; DiSpirito et al., 1998]. Furthermore, in vitro studies indicated that methanobactin can act as an oxygen radical scavenger [Choi et al., 2008, 2003]. Recent studies revealed the influence of methanobactin in natural systems by effectively mobilizing copper from mineral sources and soils [Knapp et al., 2007]. Additionally, methanobactin was found to promote dissolution of copper-doped silicate [Kulczycki et al., 2007].

Copper-bound methanobactin has been successfully isolated and purified for characterization and structural elucidation by a two-step purification method [Behling et al., 2008; Hakemian et al., 2005; Kim et al., 2005, 2004]. According to the protocols of the two-step purification method, methanobactin is stabilized with copper and isolated by a solid phase extraction followed by a reversed-phase high-performance liquid chromatography (HPLC) purification step. Methanobactin obtained by these procedures is suitable for characterization purposes. Sufficient amounts of copper-free methanobactin, devoid of buffers such as phosphate, are prerequisite to further investigations of the biogeochemical functions of chalkophores. Choi et al. [2005] described a one-step purification method to gain copper-free methanobactin. The one-step purification method consists of a resin extraction and has been applied in several studies [Knapp et al., 2007; Kulczycki et al., 2007; Choi et al., 2006a].

From the analysis of exudates released by Methylosinus trichosporium OB3b and isolated following the one-step purification protocol published by Choi et al. [2005], however, we found that the elemental composition (mainly C, N, and S) diverges largely from the theoretical composition of methanobactin according to the structure proposed by Behling et al. [2008].

Here, we report an improved method yielding highly pure, copper-free methanobactin extending the published one-step resin extraction method with a subsequent purification step with HPLC using a reversed-phase C18 column. Different fractions were collected from HPLC effluent and analyzed in order to separate pure methanobactin and to characterize other compounds that were present in the product of the one-step resin extraction method. Characterization methods included various spectroscopic techniques, such as UV–vis, C-1s near-edge X-ray absorption
fine structure (NEXAFS), and Fourier transform infrared (FT-IR) spectroscopy, as well as electrospray ionization mass spectrometry (ESI-MS).

2.2. Materials and Methods

2.2.1. Materials

All reagents used were at least analytical or liquid chromatography grade and purchased from Fluka, Merck, or Sigma-Aldrich. All solutions were prepared with high-purity deionized water (MilliQ, Millipore, ≥ 18.2 MΩ cm). Glassware was washed in 1 M NaOH and 1 M HCl for 24 hours and rinsed with deionized water. Either amber glassware was used or glassware was wrapped in aluminum foil to exclude light.

2.2.2. Methanobactin production and purification

*Methylosinus trichosporium* OB3b cells were cultured in nitrate minimal salts medium (NMS) amended with 0.2 µM CuCl₂ using a BIOSTAT® A plus bioreactor system (Sartorius) as previously described [Choi et al., 2005; Kim et al., 2005]. The cultures were grown in batch mode at 30 °C and continuously purged with a mixture of air and methane in a ratio of 2:1 (v/v) at a flow rate of 60 mL min⁻¹. When the cultures reached an optical density of 0.8–1 at a wavelength of 600 nm, 90% of the culture was harvested and replaced by fresh medium. The harvested medium was centrifuged twice at 9000 g for 30 min and immediately vacuum-filtered through a 0.2 µm PTFE filter (Millipore) combined with a glass microfiber pre-filter (Whatman®) to remove the cells. The filtrate was then loaded onto a 4 x 30 cm Diaion HP-20 column (Supelco). Subsequently, the column was washed with two column volumes of deionized water prior to elution with 60% methanol. The eluate was freeze-dried immediately.

After this resin extraction, the obtained isolates were dissolved in deionized water in amber glass vials and analyzed by reversed-phase chromatography on a HPLC system (Agilent 1100 Series) with a diode array UV–vis detector. To analyze the different components of the iso-
luates, chromatography was first performed on an analytical ProntoSil 120-5-C18aq column (4 x 250 mm, 5 µm, BISCHOFF Chromatography) with a K2 (4.0 x 20 mm, 5 µm) pre-column. Fractions were then separated using a semi-preparative ProntoSil 120-5-C18aq column (10 x 250 mm, 5 µm, BISCHOFF Chromatography) and a ProntoSil pre-column (8 x 33 mm, 5 µm) in series at a flow rate of 4.4 mL min$^{-1}$, with 10 mM NaCl (solvent A) and methanol (solvent B) as mobile phases. Prior to injection, the column was equilibrated with 40% solvent B and a linear gradient consisting of an initial solvent B concentration of 40% to 50% at 10 min and 100% at 15 min was used. UV–vis absorption of the eluate was monitored at wavelengths of 220, 254, 280, and 390 nm, which are characteristic for methanobactin. Additionally, the absorption spectra ranging from 220 to 600 nm were recorded for each peak. Eluate fractions were collected and freeze-dried for further characterization. Samples were stored at -20°C in the dark to avoid degradation [Kim et al., 2005].

2.2.3. Elemental analysis and molecular mass determination

To determine the molar C/N ratio of the collected fractions as well as of the resin extract isolated from the cultures, total organic carbon (TOC) and total nitrogen (TN) were measured with a TOC and TNb analyzer (DIMATOC®2000, DIMATEC Analysentechnik GmbH). Total Cu concentrations were determined by graphite furnace atomic absorption spectrometry (GF-AAS) with Zeeman background correction (GTA 120 AA240Z, Varian). Mass spectra were obtained with a Waters nanoACQUITY UPLC coupled to a Thermo Exactive Orbitrap mass spectrometer using electrospray ionization (ESI-MS) operated in negative ion mode. Freeze-dried material was diluted in deionized water, followed by addition of CuCl$_2$. Sample solutions were injected to a nanoACQUITY UPLC trap column (180 µm x 20 mm, 5 µm) for 30 s with 100% 5 mM formic acid at 15 µL min$^{-1}$ and an analytical Atlantis dC18 column (300 µm x 150 mm, 3 µm) with 30% 5 mM formic acid and 70% acetonitrile at 5 µL min$^{-1}$. 

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2.2.4. UV–vis spectroscopy

UV–vis absorption spectra of the resin extract and of the fractions collected during the follow-
ing HPLC purification step were recorded with a UV–vis spectrometer (Cary50 Bio, Varian). All samples were diluted with deionized water, which was also used as the blank and measured in a 1.0 cm quartz cell between 220 and 600 nm. TOC concentrations of all samples were measured and the absorption spectra were normalized to the relative carbon content of each fraction.

2.2.5. C-1s NEXAFS spectroscopy

C-1s near-edge X-ray absorption fine structure (NEXAFS) spectra were collected using the scanning transmission X-ray microscope (STXM) at beamline X-1A of the National Synchrotron Light Source (NSLS), Upton, NY. Sample solutions were freshly prepared by dissolving freeze-dried material in deionized water. Air-dried specimens were prepared by placing a 2 μL droplet of each solution onto an X-ray transparent Si₃N₄ window (Silson Ltd., Northampton, UK). For each dry film specimen, spectra were recorded at 15 different spots from 280 to 310 eV in steps of 0.1 eV. The recorded absorbance spectra were background corrected and normalized to the absorbance at 310 eV according to Christl & Kretzschmar [2007].

2.2.6. Fourier transform infrared (FT-IR) spectroscopy

FT-IR absorbance spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrophotometer equipped with a MIR TGS detector. The spectra were collected in transmission mode in the range of 4000–450 cm⁻¹ with a spectral resolution of 4 cm⁻¹. For the analysis, 0.2–0.5 mg of freeze-dried sample material were mixed with 300 mg dried KBr, homogenized with pestle and mortar, and pressed into a pellet under vacuum and under a pressure of 7.5·10⁵ kPa for 3 min. A background spectrum was recorded with a pellet containing 300 mg KBr. Sample pellets were freshly prepared prior to recording of the spectra. The recorded spectra were background corrected and a linear baseline was subtracted using MATLAB 7.8.0.
2.3. Results and Discussion

2.3.1. Reversed-phase HPLC analysis

The product of the previously published resin extraction method [Choi et al., 2005] was first analyzed with a reversed-phase C18 HPLC procedure. The resin extraction was ultimately developed for routine purification of preparative amounts of high-purity copper-free methanobactin. Here, we used the chromatographic separation to analyze the product obtained from the resin extraction for possible impurities. The chromatogram revealed several peaks at a detection wavelength of 390 nm, with one major peak eluted after 8 min (Fig. 2.2). Detection at a lower wavelength of 254 nm, however, displayed intense signals of fractions eluted with the void volume of the column. The detection of multiple peaks indicates the presence of a number of substances other than methanobactin in the isolate obtained from the cultures. Consequently, the one-step resin extraction procedure is insufficient to obtain pure methanobactin. Four fractions, marked as 1, 2, 3, and 4 in Fig. 2.2, were collected for further characterization using the semi-preparative column with a maximal load of 0.3 mg of the isolate per injection. Fraction 1 mainly consisted of substances with higher absorbance at 254 nm, but lower absorbance at higher wavelengths, whereas fractions 2 and 4 are composed of substances with higher absorbance at 390 nm. Fraction 3 comprises only the peak exhibiting the most intense absorbance at 390 nm.
Figure 2.2.: Reversed-phase chromatography of the resin extract isolated from *Methylosinus trichosporium* OB3b cultures was performed with a 10 mM NaCl/Methanol gradient. Absorbance was monitored at 390 nm (top) and 254 nm (bottom).
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### 2.3.2. C/N ratio

The molar carbon to nitrogen (C/N) ratios of the four HPLC fractions and of the resin extract are shown in Table 2.1. The theoretical C/N ratio of methanobactin is 4.5 according to the molecular structure proposed by Behling et al. [2008] (Fig. 2.1). The mean C/N ratio of the product isolated from 10 separate cultivation and extraction cycles was $7.9 \pm 2.4$. Total carbon measurements (not shown) revealed that the carbon content was approximately equal between the isolates from different harvests. Nitrogen content, however, was always much lower than the concentrations expected from the molecular structure of methanobactin and varied considerably between the isolates. Thus, a substantial amount of organic impurities are present in the isolates obtained with a single resin extraction.

The C/N ratios of the four fractions shown in Table 2.1 represent triplicate measurements. Fraction 1 showed the highest C concentrations with a C/N ratio of 8.7, followed by fraction 2 and 4 with C/N ratios of 6.3 and 6.1, respectively. All three fractions exhibited higher C carbon content as expected for methanobactin. Only the C/N ratio of fraction 3 approximately corresponded to the C/N ratio according to structure of methanobactin.

Table 2.1.: Molar C/N ratios of the product isolated from Methylosinus trichosporium OB3b cultures with resin extraction and of the four fractions collected during the following HPLC fractionation procedure as well as the molar C/N ratio of methanobactin according to the molecular structure proposed by Behling et al. [2008]. Additionally, the relative C and N contribution of fractions 1–4 to the total content of the resin extract is shown.

<table>
<thead>
<tr>
<th></th>
<th>C/N</th>
<th>Relative C content</th>
<th>Relative N content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resin extract</td>
<td>$7.9 \pm 2.4$</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>$8.7 \pm 1.5$</td>
<td>35%</td>
<td>23%</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>$6.3 \pm 0.2$</td>
<td>13%</td>
<td>14%</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>$4.7 \pm 0.1$</td>
<td>32%</td>
<td>42%</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>$6.1 \pm 0.3$</td>
<td>20%</td>
<td>21%</td>
</tr>
<tr>
<td>Molecular structure of methanobactin</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mass calculations showed that fraction 3 contributed only about one third to the total carbon content of the product isolated with resin extraction (Table 2.1). Thus, most of the organic carbon of the product of the one-step resin extraction procedure did not originate from methanobactin.

Since reversed-phase HPLC analysis and C/N ratios revealed the presence of numerous substances other than methanobactin, the composition of the four fractions collected during the second purification step was further characterized by means of spectroscopic techniques.

### 2.3.3. UV–vis

UV–vis absorption spectra of the four fractions, as well as of the isolate obtained from the cultures, are depicted in Fig. 2.3. The product obtained from the resin extract showed absorption maxima at 275, 342, and 392 nm and a shoulder at 298 nm. The spectra were similar to the UV–vis spectra of copper-free methanobactin as previously published [Choi et al., 2006b; Kim et al., 2005]. The absorption maxima at 342 and 392 nm may be assigned to the two chromophoric functional groups of methanobactin involved in Cu binding [Kim et al., 2005]. According to the revised structure of methanobactin postulated by Behling et al. [2008], Cu binding is associated with two alkylidene oxazolone rings, whereof one has a longer conjugated system leading to an absorption maximum at higher wavelengths [Choi et al., 2006b]. Fractions 1–4 showed clearly different spectroscopic properties. Similar to the isolate obtained after the first purification step, fraction 3 exhibited characteristic major peaks at 275, 342 and 392 nm, but the shoulder at 298 nm developed into a peak. Higher intensities of the two peaks at 342 and 392 nm compared to the absorption in the 245–315 nm region were observed for fraction 3. The absorption spectrum of fraction 4 was similar as shown for metal-free methanobactin after methanolysis due to the loss of one oxazolone group [Behling et al., 2008], leading to the conclusion that fraction 4 is composed of breakdown products of methanobactin and possibly precursor molecules from lysed cells. Analogously, fractions 1 and 2 may consist of breakdown products or by-product produced by *Methylosinus trichosporium* OB3b.

Therefore, UV–vis spectroscopy suggested that methanobactin was eluted with fraction 3.
Figure 2.3.: UV–vis spectra of the isolate obtained with resin extraction and of fractions 1–4 collected during HPLC fractionation (see Fig. 2.2). Absorbances were normalized to the relative carbon content of each fraction.
Due to their low absorbance in the range above 300 nm, fractions 1, 2 and 4 contributed only marginally to the absorption spectra of the isolate obtained with resin extraction, even though these fractions contain two third of its organic carbon. Thus, UV–vis spectroscopy is not a suitable tool to detect impurities in partially purified methanobactin.

### 2.3.4. C-1s NEXAFS

The C-1s NEXAFS spectra of the product isolated from *Methylosinus trichosporium* OB3b cultures and of the fractions collected with HPLC are shown in Fig. 2.4. The spectrum of fraction 1 differed clearly from the spectra of fractions 2–4. Fraction 1 showed an intense peak at 289.3 eV, but lower resonance at 288.2 eV. In contrast, fraction 3 revealed an intense absorbance at 288.2 eV. Furthermore, the spectrum of fraction 3 showed peaks at 285.1 eV, 285.4 eV, and 287.0 eV as well as at 286.6 eV and 287.7 eV. Fractions 2 and 4 showed similar spectroscopic properties as did fraction 3.

Resonances ranging from 288.6 to around 289 eV have been previously reported for the C-1s → \( \pi^*_{C=O} \) transition of carboxyl carbon of amino acids [Solomon et al., 2009; Zubavichus et al., 2005; Kaznacheyev et al., 2002; Boese et al., 1997]. The position of the carboxyl peak may be lowered by an energy shift of up to 0.4 eV towards 288.2 eV due to the formation of peptide bonds [Cooper et al., 2004; Boese et al., 1997]. Therefore, the intense signal at 288.2 eV indicates the presence of peptides in fraction 3. Peaks at 285.1 eV and 285.4 eV may be assigned to \( \pi^*_{C=C} \) states of aromatic carbon connected to C or H [Solomon et al., 2009; Kaznacheyev et al., 2002; Boese et al., 1997], whereas the peak at 287.0 eV may be attributed to C-1s → \( \pi^*_{C=C} \) transition of aromatic carbon bound to an O atom [Zubavichus et al., 2005]. Thus, all three peaks can be ascribed to the tyrosine group of methanobactin. The peak at 287.0 eV may also result from \( \pi^* \) transitions of C=N bonds [Boese et al., 1997] present in the two oxazolone rings of methanobactin. The absorption band at 286.6 eV may result from C-1s → \( \pi^*_{C=C} \) transition from ring structure substituted to N of the oxazolone binding group. Kaznacheyev et al. [2002] reported similar resonances for imidazol and histidine amino acids groups. The resonance at 287.7 eV may be related to the C-1s → \( \sigma^*_{C,H} \) transition or to the C-1s → \( \sigma^*_{C,S} \) transition, thus, in-
dicating the presence of pyrrolidine, cysteine and methionine units. All absorption bands of the C-1s NEXAFS spectrum of fraction 3 can be assigned to functional groups of methanobactin. Comparison of fractions 2 and 4 with fraction 3 showed that these fractions are also peptide-like material. Together with UV spectroscopic results discussed above, this suggests that fractions 2 and 4 contain breakdown products of methanobactin.

The major part of fraction 1, however, is clearly not peptide-like organic material because the C-1s NEXAFS spectrum showed only a weak peak at 288.2 eV. C−OH moieties, indicated by the strong resonance at 289.3 eV [Solomon et al., 2009], may be partly polysaccharide-like compounds, which are not retained by the C18 column used for fractionation of resin extracts. Similar C-1s NEXAFS spectra were reported for L-(1)-arabinose [Solomon et al., 2009] and xanthan [Mikutta et al., 2008]. Polysaccharides are known to contribute to a large extent to microbial extracellular polymeric substances [Flemming & Wingender, 2001]. Likewise, methanotrophic bacteria, including Methylosinus trichosporium OB3b, are known to produce extracellular polysaccharides [Fassel et al., 1992]. As a consequence, we conclude that fraction 1 contains mainly polysaccharides, e.g. exopolysaccharides, released by Methylosinus trichosporium OB3b during cultivation or dissolved decomposition products of lysed cell material. Principle component analysis of C-1s NEXAFS spectra of the product isolated with resin extraction from Methylosinus trichosporium OB3b cultures revealed the presence of two main components, namely a polysaccharide-like and a peptide-like component. Estimations with all recorded C-1s NEXAFS spectra indicated that fraction 1 contributed one third to the total carbon content of the product isolated with resin extraction. This finding is in accordance with the organic carbon mass balance as given in Table 2.1.

2.3.5. FT-IR spectroscopy

FT-IR spectra of all fractions collected during the purification of copper-free methanobactin are shown in Fig. 2.5. Peak assignments are based on values published by Bellamy [1980] and Parker [1971]. A broad intense band at about 3380 cm⁻¹ results from stretch vibrations of H-bonded hydroxyl (OH) groups and N−H stretch of secondary amides. Weaker bands at 2940
Figure 2.4.: C-1s NEXAFS spectra of the isolate obtained with resin extraction and of fractions 1–4 collected during HPLC fractionation (see Fig. 2.2). All spectra represent averages of 15 measurements collected on different spots on dry film specimens.
and 2840 cm\(^{-1}\) can be attributed to C–H stretching of aliphatic CH\(_3\) and CH\(_2\). A weak shoulder at 1760 cm\(^{-1}\) may result from C=O stretching of COOH and ketones. The intense band at 1660 cm\(^{-1}\) can be assigned to C=O stretching of amides (amide I band). Bands at 1540 and 1516 cm\(^{-1}\) can be ascribed to the amide II band of secondary amides found at 1570–1510 cm\(^{-1}\) in solids, which result from a mixed vibration involving N–H bending and C–N stretching [Parker, 1971]. Peaks around 1410 cm\(^{-1}\) can be attributed to O–H deformation, CH\(_3\) bending, C–O stretching of phenolic OH and COO\(^-\) antisymmetric stretching. The small band at around 1245 cm\(^{-1}\) can be assigned to C–O stretching and OH deformation of COOH. The intense peak at 1030 cm\(^{-1}\), however, results from C–O stretching as present, e.g., in aliphatic esters or polysaccharide-like substances.

The comparison of the different fractions demonstrated that the IR spectra were dominated by absorption bands at 1660, 1540, 1516, and 1030 cm\(^{-1}\) with varying intensities. The intensity ratio calculated for the bands at 1660 and 1030 cm\(^{-1}\) revealed a stronger absorption at 1660 cm\(^{-1}\) relative to the absorption at 1030 cm\(^{-1}\) for fraction 3 than for fractions 1, 2, and 4 (Table 2.2). Similar results were obtained for the intensities of absorption bands at 1540 and 1516 cm\(^{-1}\) relative to the absorption at 1030 cm\(^{-1}\). The sum of the absorption bands characteristic for amides (1660, 1540, and 1516 cm\(^{-1}\)) relative to the absorption at 1030 cm\(^{-1}\) was clearly higher for fraction 3 compared to fractions 1, 2, and 4, thus indicating a more pronounced peptide-like character for fraction 3. Fractions 2 and 4 showed similar absorption properties as did fraction 3 in the region from 1500–1200 cm\(^{-1}\), but lower absorption at 1660 cm\(^{-1}\) compared to the absorption at 1030 cm\(^{-1}\). Fractions 2 and 4 may therefore consist of degradation products of methanobactin. Likewise, fraction 1, containing mainly polysaccharide-like material according to C-1s NEXAFS spectroscopy (vide supra), might also include peptides, originating for example from degradation of lysed cells. Accordingly, FT-IR spectra confirmed the results shown with C-1s NEXAFS spectroscopy. NEXAFS spectra as well as the relative intensities of the FT-IR absorption bands can be used as a reference for future investigations.
Figure 2.5.: FT-IR spectra of fractions 1–4 collected during HPLC fractionation (see Fig. 2.2) of the resin extract. Spectra were recorded in transmission mode from KBr pellets containing 0.2–0.5 mg of freeze-dried sample material.
Table 2.2: Relative intensities of the FT-IR absorption bands of fractions 1–4 collected during the HPLC fractionation procedure.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1660 cm⁻¹</th>
<th>1540 cm⁻¹</th>
<th>1516 cm⁻¹</th>
<th>1660 + 1540 + 1516 cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1030 cm⁻¹</td>
<td>1030 cm⁻¹</td>
<td>1030 cm⁻¹</td>
<td>1030 cm⁻¹</td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>0.82</td>
<td>0.36</td>
<td>0.30</td>
<td>1.49</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.73</td>
<td>0.37</td>
<td>0.37</td>
<td>1.47</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>1.53</td>
<td>0.92</td>
<td>1.05</td>
<td>3.50</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>0.56</td>
<td>0.30</td>
<td>0.34</td>
<td>1.21</td>
</tr>
</tbody>
</table>

2.3.6. Purity and stability of isolated methanobactin

To further support the conclusion that methanobactin was eluted in fraction 3, this fraction was also analyzed with ESI-MS after copper addition for stabilization. ESI-MS analysis showed an intense signal at m/z 1215 [M–2 H + Cu⁺]⁻, thus confirming the presence of methanobactin in fraction 3 (Fig. 2.6). Furthermore, the spectra exhibited a molecular ion at m/z 1237, resulting from Cu-bound methanobactin with one Na ion adduct.

Copper analysis of fraction 3 (conducted with GF-AAS prior to copper addition) revealed a molar copper-to-methanobactin ratio of 0.0027. Methanobactin concentration was calculated from the carbon content of fraction 3. Consequently, methanobactin obtained with the combined resin extraction-HPLC procedure is virtually copper-free. To determine the stability of copper-free methanobactin after purification and storage, freeze-dried samples of fraction 3 were dissolved in deionized water and re-chromatographed using the HPLC procedure which was used for fractionation of resin extracts. Re-injection of copper-free methanobactin showed that a minor contribution of breakdown products was formed. An addition of CuCl₂ to the very same sample led to a chromatogram with a single peak (for chromatograms see Appendix A, Fig. A.1). These results indicate that the small amounts of breakdown products are formed during chromatography and that methanobactin can be stabilized by Cu addition.
Figure 2.6.: Negative ion ESI-MS spectrum of copper-bound methanobactin (m/z 1215.18).
2.4. Conclusions

The analysis of resin extracts of harvested *Methylosinus trichosporium* OB3b culture medium revealed that methanobactin was present in the extract, but it contributed only 32\% to the total isolated organic carbon of the extract. The extracted organic substances other than methanobactin were found to be peptide compounds as well as more polar compounds exhibiting primarily a polysaccharide-like character. We suggest based on our spectroscopic results that the peptide-like organic impurities represented mainly degradation products of methanobactin, whereas the polysaccharide-like material originated from exopolysaccharides or the decomposition of dead cells. The large variety of organic impurities detected with HPLC as well as the high quantity of these impurities present in the resin extract clearly demonstrated the need for a follow-up purification procedure in order to isolate pure methanobactin appropriate for experimental research on methanobactin. The presented HPLC fractionation using a C18 column was proven suitable for purification. We propose using a two-step procedure consisting of resin extraction followed by HPLC fractionation to isolate and purify methanobactin from *Methylosinus trichosporium* OB3b cultures. This procedure can be used for semi-preparative work. The isolated methanobactin obtained with this two-step procedure is virtually copper-free and thus well-suited for studies investigating copper binding properties of methanobactin as well as the role of methanobactin for copper acquisition of methanotrophs from organic and mineral sources.

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NSF under grant DBI-9605045. We are deeply grateful to Sue Wirick for her support at the beamline. This research was financially supported by the Swiss National Science Foundation (Grant 200021-113737).
References


kinetic, and thermodynamic properties of Cu(I) and Cu(II) binding by methanobactin from *Methylosinus trichosporium* OB3b. *Biochemistry, 45*(5), 1442–1453.


References


3. Copper complexation of methanobactin isolated from Methylosinus trichosporium OB3b: pH-dependent speciation and modeling

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**Abstract**

Methanobactins are copper-binding ligands produced by aerobic methanotrophic microorganisms. A quantitative understanding of their potential role in methanotrophic copper acquisition requires the investigation of their copper complexes under relevant pH conditions. In this study, a chemical speciation model describing the pH-dependence of copper binding and the formation of the different complexes by methanobactin released by *Methylosinus trichosporium* OB3b was developed. Potentiometric and spectrophotometric titrations of the free ligand indicated the presence of four protonation sites consistent with the molecular structure of methanobactin. Metal titrations revealed a distinct pH-dependence of copper binding to methanobactin between pH 5 and 8. Based on evidence from size-exclusion chromatography coupled to ICP-MS, the copper binding was quantitatively described with three different types of copper-methanobactin complexes which can additionally undergo protonation reactions. The
high affinity observed upon initial copper additions resulted from the predominant occurrence of copper-methanobactin dimer complexes, \( \text{mb}_2\text{H}_4\text{Cu} \) and \( \text{mb}_2\text{H}_3\text{Cu} \) with \( \log K \) values of 58 and 52, respectively. With increasing copper-to-methanobactin ratios, methanobactin bound copper as monomers, \( \text{mbHCu} \) (\( \log K = 25 \)) and \( \text{mbCu} \) (\( \log K = 18 \)), whereas at elevated copper activities methanobactin was able to bind two copper ions (\( \text{mbHCu}_2 \) and \( \text{mbCu}_2 \)). Model calculations based on the fitted complexation constants suggest that in natural systems, copper-methanobactin complexes are mostly present as monomers.

### 3.1. Introduction

Methanobactins are copper-binding molecules produced by many aerobic methane oxidizing bacteria [Yoon et al., 2010; Semrau et al., 2010]. In analogy to siderophores exuded by numerous organisms to overcome iron deficiency [Winkelmann, 1991; Bossier et al., 1988; Neilands, 1981], methanobactins are termed chalkophores due to their function as extracellular component of a copper acquisition mechanism to cover methanotrophs’ high demand for copper [Semrau et al., 2010; Kim et al., 2004]. Moreover, methanobactins share structural similarities with the amino acid containing pyoverdin class of siderophores [Budzikiewicz, 2010]. Besides playing an important role in the copper uptake system, methanobactins regulate methane mono-oxygenase expression [Choi et al., 2008; DiSpirito et al., 1998] and are believed to act as a copper chaperone [Balasubramanian & Rosenzweig, 2008; Choi et al., 2003; DiSpirito et al., 1998] and as oxygen radical scavenger [Choi et al., 2008, 2003].

Several methanotrophs were found to exude copper binding compounds [El Ghazouani et al., 2011; Choi et al., 2010; Krentz et al., 2010; Kim et al., 2004; DiSpirito et al., 1998]. The best characterized methanobactin is produced by *Methylosinus trichosporium* OB3b. It has a molecular mass of 1154 Da and is composed of seven amino acids (Fig. 3.1) [Behling et al., 2008]. The two chromophoric groups constituted of modified amino acids, i.e., oxazolone rings which are associated with an enethiol, are involved in copper binding [El Ghazouani et al., 2011; Behling et al., 2008]. Methanobactin binds Cu(I) and Cu(II), whereby Cu(II) is immediately reduced to Cu(I) upon binding [Choi et al., 2006b; Hakemian et al., 2005]. The analysis of the crystal
structure revealed that Cu is bound by methanobactin in a distorted tetrahedral geometry by two N and two S provided by the modified residues [El Ghazouani et al., 2011; Kim et al., 2004]. Regardless of their structural diversity, methanobactins, exuded from different methanotrophs, are all composed of amino acid and non-amino acid residues and contain two heterocyclic rings (imidazoles and/or oxazolones) responsible for their high copper affinity [Bandow et al., 2012; Krentz et al., 2010; Behling et al., 2008]. Methanobactin also binds a variety of other metals such as Ag, Au, Cd, Co, Fe, Hg, Mn, Ni, and Zn [Choi et al., 2006a]. For methanobactin produced by Methylosinus trichosporium OB3b, a high Cu affinity has been determined using isothermal titration calorimetry [Choi et al., 2006b]. After an extremely high initial binding, conditional stability constants of log $K = 8.4$ and log $K = 6.4$ were found at pH 6.8 for molar Cu-to-methanobactin ratios of $0.5 > \text{Cu/m}b > 0.25$ and $\text{Cu/m}b > 0.5$, respectively [Choi et al., 2006b]. Based on these results, the authors suggested the formation of tetramer, dimer and monomer Cu-methanobactin complexes [Choi et al., 2006b]. In a more systematic study, the Cu(I) affinity for methanobactin exuded by Methylosinus trichosporium OB3b was studied at different pH with experiments using bathocuproine as a competitive ligand [El Ghazouani et al., 2011]. A binding constant of log $K = 20.8$ was derived from data at alkaline pH, with decreasing Cu affinities below pH 8.

Even though conditional affinity constants were determined for both, Cu(II) and Cu(I) to methanobactin, it is still difficult to predict the speciation of methanobactin under variable pH conditions as detailed information on the pH-dependence of Cu binding is scarce. In common habitats of aerobic methane oxidizing bacteria, copper is mostly present as Cu(II) bound to organic matter and mineral phases and thus not readily available. To better understand the role of methanobactin in the Cu acquisition by bacteria, a chemical speciation model describing the pH-dependent Cu(II) binding to methanobactin and the formation of possible Cu-methanobactin complexes, is required. Since Cu binding is linked to proton binding due to concurrent protonation reactions, it is indispensable to study both processes together. Moreover, knowledge about proton binding is essential to assess which Cu binding mechanisms dominate at a given pH value. The aim of this study was, therefore, to develop a chemical speciation model describing proton and copper binding by methanobactin isolated from Methylosinus trichosporium OB3b.
Chapter 3. Copper complexation of methanobactin

Figure 3.1.: Schematic drawing of copper-bound methanobactin modified from Behling et al. [2008]. The four sites of methanobactin identified to undergo protonation–deprotonation reactions are marked and the respective protonation constants ($\log K_1-4$) are indicated. Log $K_2$ and log $K_3$ resulting from the enethiol-oxazolone moieties cannot be ascribed to a specific group.
cultures (Fig. 3.1) based on results obtained from potentiometric and metal titrations. The formation of different Cu-methanobactin complexes was further investigated by size-exclusion chromatography coupled to ICP-MS to validate the proposed binding mechanisms.

3.2. Experimental section

3.2.1. Materials

Solutions were prepared with high-purity deionized water (18.2 MΩ cm, MilliQ, Millipore). Prior to use, the water was boiled for 30 min while purging with N$_2$. All reagents were at least analytical or liquid chromatography grade and purchased from Fluka, Merck, or Sigma-Aldrich. All vessels were washed in 1 M NaOH and 1 M HCl for 24 hours and rinsed with deionized water. Amber glassware or vessels wrapped in aluminum foil were used to avoid potential photodegradation [Kim et al., 2005].

3.2.2. Methanobactin purification and stock solution preparation

Methanobactin was isolated from Methylosinus trichosporium OB3b cells cultured in nitrate minimal salts medium (NMS) amended with 0.2 µM CuCl$_2$ in a BIOSTAT® A plus bioreactor system (Sartorius) as previously reported [Choi et al., 2005; Kim et al., 2005]. Methanobactin, abbreviated mb in the following, was isolated from the spent medium by resin extraction (Diaion HP-20, Supelco) followed by a second purification step using reversed-phase chromatography on a C18 column with methanol and a 0.01 M NaCl solution as the mobile phases. The collected mb, shown to be virtually Cu-free (Cu-to-mb ratio of 0.003) after isolation and purification [Pesch et al., 2011], was freeze-dried and stored in the dark at -20°C before use. Details about isolation and purification of mb were described earlier [Pesch et al., 2011]. Methanobactin stock solutions were prepared with deionized CO$_2$-free water and the concentration of mb in the solutions was determined by measuring the carbon content with a TOC analyzer (DIMATOC®-2000, DIMATEC Analysentechnik GmbH) or by Cu(I) titration of mb solutions in the presence of
bicinchoninic acid [El Ghazouani et al., 2011] (see Appendix D Fig. B.1 for details). The Cu concentrations of Cu stock solutions were checked by atomic absorption spectrometry (Varian SpectrAA 220FS, Varian Inc.).

3.2.3. Potentiometric titrations

The pH-dependent protonation behavior of mb was investigated with potentiometric acid–base titrations at 25 ±1°C. A 90 µM Cu-free mb solution with an ionic strength of 0.01 M NaCl was titrated in a Teflon titration vessel using diluted Titrisol® (Merck) solutions of NaOH and HCl (0.005 – 0.05 M). During the titrations, the titration vessel was sealed and the headspace was continuously flushed with water-saturated CO₂-free nitrogen gas. Proton activity in solution was monitored with a pH electrode (6.0234.100, Metrohm). The solution was stirred for 5 min after each NaOH or HCl addition and then equilibrated for 2 min without stirring. The electrode readings were recorded when the potential drift dropped below 1.6 mV min⁻¹. Blank titrations of a 0.01 M NaCl solution were performed with the same setup to verify CO₂-free conditions during the experiment and to be able to correct for proton consumption due to hydrolysis reactions of the background solution. Methanobactin solution was first titrated with base (forward titration) and then titrated with acid (backward titration). Forward and backward titrations gave identical results. Here, only forward titrations are reported.

An additional acid–base titration was conducted under laboratory atmosphere in a 1.0 cm quartz cell to determine the pH-dependent absorbance of mb with an UV–vis spectrometer (Cary50 Bio, Varian). Copper-free mb was dissolved in deionized water to obtain a 45 µM mb solution. After each addition of NaOH or HCl, the solution was thoroughly shaken and equilibrated for 5 min before the pH of the solution was measured. Absorption spectra were subsequently recorded between 220 and 600 nm. During the titration and pH measurement step, the quartz cuvette was wrapped in aluminum foil which was removed only shortly before recording the UV–vis spectra. Absorption spectra were corrected for the changes of mb concentration due to additions of small amounts of NaOH and HCl and evaporation of the solution during the time of the experiment. Evaporation of solution was determined gravimetrically.
3.2.4. Metal titrations

The binding of Cu(II) to mb was investigated with copper titration experiments at constant ionic strength (0.01 M NaCl) and constant pH values (pH 5 – 8) at 25 ± 1 °C. The titrations were performed with the same setup as the potentiometric titrations. Proton and free metal-ion activities in solution were monitored using a pH electrode and a Cu$^{2+}$ ion-selective electrode (ISE) (Orion 9429SC) coupled to a Ag/AgCl reference electrode (6.0733.10, Metrohm). The ISE was calibrated by titrating 20 mL of a 1 mM CuCl$_2$ solution with a 1.787 mM ethylenediamine solution as described by Christl et al. [2001]. Solutions of 45 and 100 µM Cu-free mb were titrated with 1 mM CuCl$_2$ containing 0.01 M NaCl to keep the ionic strength constant during the titration experiment. The pH value was adjusted after each titration step with NaOH and HCl. After each copper addition, the solution was stirred for 5 min, equilibrated for 10 min and electrode readings were recorded when both potential drifts dropped below 1 mV min$^{-1}$ or after a maximum time of 20 min. Each experiment was stopped at a Cu$^{2+}$ activity close to the level where copper(hydr-)oxide precipitation is expected according to chemical speciation calculations.

3.2.5. Size-exclusion chromatography

To investigate the potential formation of different Cu-mb complexes, mb solutions were spiked with varying Cu concentrations and the complexes were separated by size-exclusion chromatography (SEC) coupled to ICP-MS (see below). Different Cu-to-mb ratios were obtained by mixing a Cu standard solution with aliquots of a mb stock solution in 0.01 M NaCl and 0.005 M MES (2-(N-morpholino)ethanesulfonic acid) for pH 6.0 ± 0.05 or MOPS (3-(N-morpholino)-propanesulfonic acid) for 8.0 ± 0.05. The experiments were conducted in duplicates to show the reproducibility of the method. Before measurement, the samples were filtered with a 0.2 µm nylon filter (WICOM International AG) and loaded onto a Superdex$^\text{TM}$ Peptide 10/300 GL column (GE Healthcare) using a double piston pump with a flow rate of 0.7 mL min$^{-1}$ and an injection valve with a 100 µL sample loop of an ion chromatographic system (Metrohm 761 Compact IC, Metrohm AG). Buffers containing 0.01 M KCl and 0.005 M MES or MOPS were used as mobile phases for pH 6 and 8, respectively. The different mb complexes were
separated with the SEC column and the absorbance of the eluate was monitored with a diode array UV–vis detector (Agilent 1100 Series HPLC, Agilent Technologies) at wavelengths of 254, 280, 392, and 422 nm. Additionally, the absorption spectra ranging from 220 to 600 nm were recorded for each observed peak. Thereafter, the eluate was acidified with a 1 M HCl solution to a concentration of 10 %v/v and transported to the ICP-MS (Agilent 7500 ce Series, Agilent Technologies) for Cu measurements. The piston pump, injection valve, sample loop, and the tubings leading to the SEC column consisted of polyether ether ketone (PEEK) material. Polyfluoralkoxy (PFA) tubings were used between the outlet of the UV–vis detector and the nebulizer of the ICP-MS system to avoid metal contamination of the samples. After 8–10 separation cycles, the column was consecutively rinsed with 3 column volumes of 0.1 M KOH, 0.01 M HCl, 0.01 M K$_2$-EDTA, and then re-equilibrated with MES or MOPS eluent before the next series of experiments.

For calibration, solutions containing Cu-free mb and Cu-bound mb at pH 6 and 8 were injected into the SEC-ICP-MS system. Chromatograms of eluates were recorded at 422 nm. At this wavelength, the absorbance of mb is not affected by the presence of Cu (isosbestic point) El Ghazouani et al. [2011]. The recorded peaks were integrated using OriginPro 8.5 (OriginLab Corporation) to establish a calibration for the detected mb species. The slopes of the calibration for Cu-free mb and Cu-bound mb were identical showing that evaluation of spectra at the isosbestic point is suitable to determine the mb concentration in each fraction. Mass balance of mb was verified by calculating the sum of eluted mb. The different mb complexes eluted at different times were identified with full-range UV–vis spectra. Additionally, the subsequent Cu measurement (see below) allowed to quantitatively determine the Cu-to-mb stoichiometry of each fraction. Filtered and unfiltered mb samples injected directly to the UV–vis detector showed that the recovery of mb was about 85 % after filtration.

Copper was quantified using Cu-EDTA standards. Standards were prepared from K$_2$-EDTA and CuCl$_2$ stock solutions and diluted on a daily basis to concentrations ranging from 5 to 4000 µg L$^{-1}$ with the same background concentrations of KCl, MES and MOPS as contained in the samples and the eluents. Cu standards were injected directly into the analytical system with a 100 µL sample loop bypassing the SEC column and the UV–vis detector.
3.2 Experimental section

3.2.6. Data analysis and modeling of conditional stability constants

The amount of protons absorbed to mb was calculated by subtracting the potentiometric blank tiritations from the mb titrations. For each metal titration step, the sum of inorganic copper species was calculated with the speciation program ECOSAT [Keizer & van Riemsdijk, 1998] using the metal hydrolysis constants and metal (hydr-)oxide solubility constants from Smith & Martell [1989]. The amount of Cu bound by mb was deduced from the total amount of Cu added to the solution and the sum of inorganic dissolved copper species. Additionally, $\text{H}^+$/Cu$^{2+}$ exchange ratio were calculated from the amounts of NaOH and HCl needed to adjust pH after each Cu titration step.

The optimization of the stability constants were carried out using ECOSAT [Keizer & van Riemsdijk, 1998] coupled to the parameter fitting program FIT [Kinniburgh & Tang, 1998]. According to the molecular structure of mb, a four-site protonation model was used to determine the proton binding constants ($\log K_{1-4}$) for mb in a first step. The value of $\log K_1$ representing the protonation of the tyrosine group was derived from the pH-dependent UV–vis absorption spectra according to Hirt et al. [1961]. The curve was fitted with a sigmoidal equation (Eq. 3.1) using OriginPro 8.5 (OriginLab Corporation):

$$Abs_{245} = y_0 + \frac{a}{1 + e^{x_0 - pH}}$$  \hspace{1cm} (3.1)

where $Abs_{245}$ is the absorption at 245 nm. The fit parameters $a$, $b$, and $y_0$ are required to describe the shape and position of the sigmoidal curve. The inflection point corresponds to the $\log K_\alpha$ value ($x_0 = \log K_\alpha$). The $\log K_\alpha$ derived for the tyrosine group was used as a fixed input parameter for the determination of the protonation constants $\log K_{2-4}$ from the potentiometric titration data. In a second step, the conditional stability constants of mb for copper were optimized using the previously calculated protonation constants and the results obtained from metal titrations and size-exclusion chromatography.
3.3. Results and Discussion

3.3.1. Proton binding by methanobactin

Protonation constants for mb were determined based on the experimental results obtained from potentiometric and spectrophotometric titrations. According to the molecular structure of mb depicted in Figure 3.1, four functional groups are expected to be susceptible to protonation and deprotonation reactions. These include two sulfhydryl protons originating from the enethiols associated with the oxazolone rings and two hydroxyl protons from the phenolic moiety of the tyrosine residue and from the carboxylic moiety of the terminal methionine residue, respectively. The pH-dependent absorption behavior of Cu-free mb was monitored by UV–vis spectroscopy. UV–vis absorption scans can be found in Appendix D (Fig. B.2). Almost no absorption changes were observed between pH 6 and 9. Above pH 9 and below pH 6, major spectral shifts were observed at 245, 342, and 392 nm, respectively (Fig. 3.2A and 3.2B). Similar spectral features have been recorded for Cu-bound mb [Kim et al., 2005]. The phenolic group of tyrosine exhibits an absorption maximum at a wavelength of 275 nm and an absorption minimum at 245 nm [Greenstein & Winitz, 1961]. The phenoxide form of tyrosine, in contrast, shows an absorption maximum at 294 nm and a second maximum with a much higher absorption coefficient at 240 nm [Greenstein & Winitz, 1961]. Figure 3.2A reveals an absorption increase at 245 nm with increasing pH. The absorption spectrum of tyrosine is not very sensitive to pH variations until it reaches the alkaline pH range [Greenstein & Winitz, 1961] and the spectral change observed for mb can be ascribed to the dissociation reaction of the phenolic group of the tyrosine amino acid residue. A log $K$ of 10.6 ±0.1 was deduced for the protonation of the tyrosine group of mb from the curve describing the spectral changes at 245 nm using equation 3.1. This log $K$ value is in accordance with protonation constants reported for phenolic groups of peptides elsewhere [Martell & Smith, 1982]. The pH-dependent spectral features of mb displayed smaller differences at wavelengths of 342 and 392 nm compared to the absorption changes observed at 245 nm (Fig. 3.2). The absorption bands at 342 and 392 nm can be assigned to the two chromophoric moieties of mb involved in Cu binding [Choi et al., 2006b; Kim et al., 2005]. The observed spectral shifts implied that both enethiol-oxazolone groups follow similar protonation
3.3 Results and Discussion

reactions. According to the obtained results, both groups may be involved in the protonation of mb below pH 6.

Potentiometric titration data are shown in Figure 3.3. Deprotonation of mb was observed over the total pH range investigated and increased with increasing pH. After a first sharp increase of the negative charge up to pH 5.5, a plateau region was observed followed by a second rise above pH 9. The results revealed that at least two protons per mb molecule were released between pH 4 and 10. The exponential increase towards pH 10 suggested the release of a third proton. The experimental data and log $K_1$ were used as input parameters to optimize the protonation constants with FIT. The fits indicated that proton binding by mb can quantitatively be described with a four-site protonation model. All obtained log $K$ values and their corresponding reactions are described in Table 3.1. A log $K$ of 5.0 ± 0.1 (log $K_3$) was calculated for the first increase of the pH-dependent proton release per mb (Fig. 3.3) which corresponds to a loss of proton from one of the enethiol-oxazolone moieties. These results are consistent with the observations of the spectrophotometric titrations (Fig. 3.2B). Deprotonation of one enethiol-oxazolone group stabilizes the proton binding to the second enethiol-oxazolone group and therefore leads to a higher protonation constant (log $K_2 = 9.7 ± 0.1$) of the latter moiety. Spectral shifts at 342 and 392 nm, however, showed that the UV–vis absorption of each moiety may be affected by protonation of mb below pH 6. Thus, it is difficult to ascribe log $K_2$ and log $K_3$ to a specific group. For the fourth protonation of mb, only an upper limit of log $K_4 < 1.7$ can be estimated from the experimental data. This limit can be ascribed to the carboxylic group of the terminal methionine residue (Fig. 3.1). The determined dissociation constant is consistent with literature values reported for carboxylic moieties of methionine residues [Smith & Martell, 1989; Martell & Smith, 1982]. Overall, the titration data and the determined log $K$ values explained proton binding by mb and confirmed the presence of proton binding sites according to its chemical structure (Fig. 3.1). Based on the protonation of mb consistent with the molecular structure, the pH-dependent binding of Cu by mb is presented and discussed in the following.
Figure 3.2.: Absorbance of Cu-free methanobactin in 0.01 M NaCl as a function of pH (A) at 245 nm corresponding to the deprotonation of the tyrosine moiety of methanobactin and (B) at 342 nm (filled circles) and 392 nm (open circles) corresponding to the deprotonation of the two chromophoric moieties of methanobactin. The absorbance was corrected for changes of methanobactin concentration due to acid and base additions. The line in plot (A) represents a sigmoidal curve fit (see section 3.2.6) to derive the inflexion point corresponding to the protonation constant of the tyrosine moiety.
Figure 3.3.: Deprotonation of 90 µM Cu-free methanobactin (mb) as a function of pH in 0.01 M NaCl at 25 ±1°C. Symbols represent data obtained from potentiometric acid–base titrations. The line represents the fit derived from a four-site protonation model (see Table 3.1). The origin of the y-axis refers to the fully protonated form of mb.
Table 3.1.: Conditional stability constants for proton and copper binding by methanobactin optimized for data recorded in 0.01 M NaCl at 25 °C. The Davies equation implemented in the speciation program ECOSAT [Keizer & van Riemsdijk, 1998] was used to convert constants to $I = 0$ M as listed. The reactions describing Cu binding by methanobactin are formulated starting from $\text{mbH}_2^{2-}$ as this complex dominates between pH 6 and 8.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Conditional stability constant</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{mb}^{4+} + H^+ \rightleftharpoons \text{mbH}^{3-}$</td>
<td>$\log K_1$</td>
<td>$10.6 \pm 0.1$</td>
</tr>
<tr>
<td>$\text{mbH}^{3+} + H^+ \rightleftharpoons \text{mbH}_2^{2-}$</td>
<td>$\log K_2$</td>
<td>$9.7 \pm 0.1$</td>
</tr>
<tr>
<td>$\text{mbH}_2^{2-} + H^+ \rightleftharpoons \text{mbH}_3^{-}$</td>
<td>$\log K_3$</td>
<td>$5.0 \pm 0.1$</td>
</tr>
<tr>
<td>$\text{mbH}_3^{-} + H^+ \rightleftharpoons \text{mbH}_4$</td>
<td>$\log K_4$</td>
<td>$\leq 1.7$</td>
</tr>
<tr>
<td>$\text{mbH}_2^{2-} + Cu^{2+} \rightleftharpoons \text{mbHCu}^{-} + H^+$</td>
<td>$\log K_{\text{mbHCu}}$</td>
<td>$24.6 \pm 0.1$</td>
</tr>
<tr>
<td>$\text{mbH}_2^{2-} + Cu^{2+} \rightleftharpoons \text{mbCu}^2- + 2 H^+$</td>
<td>$\log K_{\text{mbCu}}$</td>
<td>$17.7 \pm 0.1$</td>
</tr>
<tr>
<td>$\text{mbH}_2^{2-} + 2 Cu^{2+} \rightleftharpoons \text{mbHCU}^2+ + H^+$</td>
<td>$\log K_{\text{mbHCU}2}$</td>
<td>$27.9 \pm 0.2$</td>
</tr>
<tr>
<td>$\text{mbH}_2^{2-} + 2 Cu^{2+} \rightleftharpoons \text{mbCu}_2 + 2 H^+$</td>
<td>$\log K_{\text{mbCu}_2}$</td>
<td>$23.9 \pm 0.2$</td>
</tr>
<tr>
<td>$2 \text{mbH}_2^{2-} + Cu^{2+} \rightleftharpoons \text{mb}_2 H_3 Cu^{3-} + H^+$</td>
<td>$\log K_{\text{mb}_2 H_3 Cu}$</td>
<td>$52.3 \pm 0.1$</td>
</tr>
<tr>
<td>$2 \text{mbH}_2^{2-} + Cu^{2+} \rightleftharpoons \text{mb}_2 H_4 Cu^{2-}$</td>
<td>$\log K_{\text{mb}_2 H_4 Cu}$</td>
<td>$57.9 \pm 0.1$</td>
</tr>
</tbody>
</table>

3.3.2. Copper binding by methanobactin

Isotherms of copper binding by mb derived from Cu(II) titration experiments are shown in Figure 3.4. The observed binding properties were similar between pH 5 and 8. The results showed that the Cu affinity increased with increasing pH but the pH-dependence for Cu-binding by mb was weak within the studied pH range. A first increase in bound Cu was found up to a molar Cu-to-mb ratio of 0.5 ($\log Cu^{2+}_{\text{bound}}$/mb = -0.3), suggesting the formation of 1:2 Cu-mb complexes. A second increase was observed at higher Cu activities. At pH 5 and 6, the shape of the recorded isotherms as well as bound Cu-to-mb ratios ($\log Cu^{2+}_{\text{bound}}$/mb > 0) indicated that mb bound a second Cu ion. Thus, 2:1 Cu-mb complexes are formed at elevated Cu activities. According to literature, in a 1:1 Cu-mb complex, copper is bound by the two oxazolone groups of mb in a distorted tetrahedral geometry [El Ghazouani et al., 2011; Behling et al., 2008; Kim et al., 2004]. The presence of a second binding site has previously been observed [El Ghazouani et al., 2011; Choi et al., 2006b] and different potential binding sites can be deduced from the
3.3 Results and Discussion

Figure 3.4: Cu binding by methanobactin (mb) for pH 5–8 in 0.01 M NaCl at 25 ±1 °C as a function of Cu$^{2+}$ activity. Closed symbols and solid lines correspond to the metal titration data and fits obtained from the experiments conducted with 45 µM mb. Open symbols and the dashed line correspond to the metal titration data and fit obtained from the experiment conducted at pH 6 with 100 µM mb. The fits were obtained from the optimization of the stability constants (see Table 3.1).

structure of mb (Fig. 3.1). The second Cu atom may be bound to the carboxylic group of mb, deprotonated at the pH range considered or to the phenolic group of the tyrosine residue. El Ghazouani et al. [2011] suggested a second, weaker binding site that involves the ring oxygen of oxazolone A and the adjacent carbonyl oxygen.

It has been reported that Cu(II) bound to mb by the two chromophoric residues is immediately reduced to Cu(I) [Hakemian et al., 2005]. If a second Cu ion is bound to mb, it remains in its oxidized form [El Ghazouani et al., 2011]. The mechanisms and the source of electrons for the reduction of Cu are still unknown. Different possibilities have been discussed and are summarized in the following (for details see Krentz et al. [2010]). It has been suggested that the electrons may come from the sulfhydryl groups of the cysteine residues. This would imply that the disulfide bridge is formed upon Cu binding. Recent investigations, however, showed the presence of the disulfide bonding in Cu-free mb [El Ghazouani et al., 2011]. Alternatively,
the formation of Cu-mb complexes may lead to a preferential localization of an electron from the two chromophoric systems of mb towards the Cu atom. Consequently, the source of the electron can hardly be attributed to one specific atom of mb and experimental evidence on an electron donating atom may be difficult to obtain.

3.3.3. Formation of different methanobactin complexes

To verify the formation of the different Cu-mb complexes, mb solutions were spiked with varying Cu concentrations and the complexes were separated on a SEC column. Figure 3.5A shows an absorption chromatogram at 280 nm for Cu-free mb and Cu-bound mb injected to the SEC column at pH 8. For Cu-free mb, a major peak was eluted after 22.2 min, followed by two minor peaks at 24.2 and 27 min. When mb was equilibrated with Cu before injection, the peak eluted at 22.2 min disappeared and an intense peak at 27 min evolved. The chromatogram of mb equilibrated with an excess of Cu revealed an additional peak after 29.0 min. The peak at 22.2 min can be clearly identified as Cu-free mb based on the full-range UV-vis spectra (Fig. 3.5A, Inset 1), whereas the peak at 27 min resulted from Cu-bound mb (Inset 2). A Cu-to-mb ratio of 2 of the peak at 29 min (vide infra) indicated the presence of a mb-Cu₂ complex. Spectroscopic properties of the small peak eluted after 24.2 min indicated that this fraction may be composed of mb fragments degraded during the chromatography process.

To further specify the formation of the different Cu-mb species, Cu and mb concentrations were quantified and Cu-to-mb ratios were calculated. Three different fractions were considered based on the peaks identified from the UV–vis elution profile. Fraction I included Cu-free mb and ranged from 19.2 to 23 min, fraction II (mb-Cu) ranged from 25 to 28.4 min, and fraction III (mb-Cu₂) from 28.4 to 30.8 min. The average recovery of methanobactin in these samples was 88% for both Cu-free mb and Cu-bound mb, with the exception of the condition where the Cu concentration was twice the concentration of mb. The amount of Cu bound to mb for the experiments containing 31 µm mb but varying total Cu concentrations are depicted in Figure 3.5B. For the experiments without Cu addition, all eluted fractions remained virtually Cu-free. Trace Cu concentrations which were detected corresponded to the amount of Cu con-
3.3 Results and Discussion

Figure 3.5.: (A): Separation of Cu-free and Cu-bound methanobactin (mb) complexes by size-exclusion chromatography. The absorbance of eluates at 280 nm is plotted as a function of elution time. Inset 1: full-range UV–vis spectra of Cu-free mb, Inset 2: full-range UV–vis spectra of Cu-mb. (B): Formation of Cu-free and Cu-bound mb complexes with 31 µM mb and increasing total Cu concentrations at pH 8 and separated by size-exclusion chromatography.
Chapter 3. Copper complexation of methanobactin

tained in mb after purification (Cu/mb = 0.003) [Pesch et al., 2011]. When Cu concentrations were lower than mb concentrations, Cu-free mb was still observed, but most of the Cu and mb was eluted as a 1:1 mb-Cu complex. The contribution of Cu-free mb species to the overall elution profile decreased with increasing Cu concentrations. Dimer complexes (mb₂-Cu), expected to be formed at Cu/mb ratios < 1, could not be separated by SEC. UV–vis chromatograms and Cu measurements of standards containing Cu-free or 1:1 mb-Cu complexes proved that these complexes were not labile during size-exclusion chromatography. Dimer complexes (mb₂-Cu), however, were labile and dissociated into 1:1 mb-Cu and Cu-free mb complexes as shown by Cu and mb mass balance calculations. For the experiment with an initial Cu/mb ratio of 2, 1:1 complexes were eluted with fraction II, whereas fraction III contained mb-Cu₂ complexes. However, for this experiment the recovery of mb was low which may result from precipitation of mb-Cu₂ complexes as also indicated by the loss of Cu during the experiment.

3.3.4. Conditional stability constants for copper binding

Acid–base titration data and protonation constants determined for mb were used to optimize conditional stability constants for Cu-mb complexes. Based on Cu isotherms, the separation of different Cu-mb complexes performed in this study, and information on Cu binding by mb from previous studies (*vide supra*), it is assumed that 1:2, 1:1, and 2:1 Cu-mb complexes form. Fits obtained from proton binding to mb indicated the existence of different protonation states of Cu-mb complexes. Model calculations to derive the conditional stability constants for Cu binding were, therefore, based on the assumption that three different groups of Cu-mb complexes are formed which can undergo protonation reactions. All constants and equations describing the binding reactions are summarized in Table 3.1. The model fits (Fig. 3.4) described Cu binding by mb fairly well (R² = 0.96). The calculated Cu activity-dependent formation of the different Cu-mb complexes is shown in Figures 3.6A and 3.6B for pH 5 and 8, respectively. Speciation plots for pH 6 and 7 are displayed in Appendix D (Fig. B.3). The high initial affinity is attributed to the formation of Cu-mb dimers, mainly mb₂H₄Cu at pH 5 and mb₂H₃Cu at pH 8. With increasing Cu activities, mb bound Cu as monomers (mbHCu and mbCu), whereas at
3.3 Results and Discussion

Elevated Cu activities (log Cu$^{2+}$ bound/mb > 0) mb complexes containing two Cu ions (mbHCu$_2$ and mbCu$_2$) were calculated to form. The formation of oligomers has previously been proposed by Choi et al. [2006b]. They suggested that the high Cu affinity (log $K > 18.8$) at a molar Cu-to-mb ratio of 0.2 can only be explained if mb binds Cu as an oligomer. Binding constants were found to decrease with increasing Cu additions and consequently different Cu-mb complexes must be formed. In fact, UV–vis and X-ray photoelectron spectra indicated the presence of dimers at a Cu-to-mb ratio below 0.5 and the formation of Cu-mb monomer above 0.5 [Choi et al., 2006b]. Binding constants of log $K = 8.4$ and log $K = 6.1$ were derived from results obtained from isothermal titration calorimetry for Cu-mb dimer and monomer, respectively. It is important to note that these constants are only valid under the studied experimental pH conditions. A Cu(I) binding constant of log $K = 20.8$ was determined above pH 8 by assuming the formation of 1:1 Cu-mb complexes and almost identical affinities were observed for Cu(II) [El Ghazouani et al., 2011]. Similarly to the results of the present study, a decrease in Cu affinity was observed if the pH dropped below 8.

The binding of Cu to mb alters the properties of the chelator by changing the protonation behavior of mb. Considering the binding constants of Cu(II) determined in this study (Table 3.1), log $K$ values for both monomer Cu-mb complexes (mbHCu and mbCu) differed by 7 log units. The only differentiation between mbHCu and mbCu is a proton released from the tyrosine moiety of the latter complex. The log $K_4$ value of the phenol moiety, which helps to stabilize the Cu binding site by $\pi$ interactions with the oxazolone A ring, was lowered from 10.6 to 7 if the conformation of mb is altered due to Cu binding. Similarly, El Ghazouani et al. [2011] deduced a log $K$ of 7 responsible for the protonation event triggered by Cu(I) binding from metal titrations at varying pH. A similar difference was found between log $K$ values for mbHCu$_2$ and mbCu$_2$. A lower difference was observed for both dimer complexes (mb$_2$H$_4$Cu and mb$_2$H$_3$Cu). But here, the released proton originates from one of enethiol groups and not from the phenolic moiety.
Figure 3.6.: Cu-methanobactin speciation in 0.01 M NaCl at 25 ±1 °C as a function of Cu$^{2+}$ activity (A) at pH 5 and (B) at pH 8 as calculated with the optimized stability constants given in Table 3.1.
3.3.5. Proton to copper exchange ratio

Proton to copper exchange ratios calculated from the titration data as well as the fitted exchange ratios calculated from the deduced stability constants are shown in Figure 3.7. The experimental data showed that within the studied pH range one proton was released per Cu bound by mb. Relevant information regarding the Cu binding sites may be derived from the H$^+$ to Cu$^{2+}$ exchange ratios. If dimer mb$_2$-Cu complexes are formed by the complexation of Cu with two tyrosine moieties, two protons are expected to be exchanged per Cu bound. The carboxylic moieties, in contrast, are deprotonated within the studied pH range. Thus, the H$^+$ to Cu$^{2+}$ exchange ratio is expected to be zero if the dimer formation results from complexation by the carboxylic moieties. The observed exchange ratios of about one support the Cu binding model established in this study, where Cu is bound by the two oxazolone moieties of mb. At elevated Cu activities, H$^+$ to Cu$^{2+}$ exchange ratios decreased to 0.5 at pH 5, indicating that the second Cu ion bound by mb may be complexes by the deprotonated carboxylic moiety. The ratios calculated based on the fitted stability constants (Table 3.1) are in accordance with the ratios calculated from the titration data. At pH 8, the calculated ratios increased with increasing Cu activity and were higher than the ratios calculated from the experimental data. At higher pH, the proton consumption due to the formation of inorganic Cu species in solution quantitatively exceeded proton consumption of mb. Consequently, a minor variation of the stability constants can lead to a pronounced effect in the calculation of H$^+$ to Cu$^{2+}$ exchange ratios.

3.4. Consequences for copper mobilization

Based on potentiometric and metal titration experiments, a chemical speciation model describing Cu binding to mb and the formation of the different complexes has been successfully established. The optimized stability constants for proton and Cu binding can be used to predict mb behavior and to estimate mb-promoted Cu mobilization in natural environments.

Although many methanotrophs were found to produce methanobactins [Yoon et al., 2010; Semrau et al., 2010], mb has never been detected in natural samples so far. We expect, however,
that concentrations found in habitats of methanotrophs are much lower than those in laboratory cultures. Predictions calculated with the stability constants for proton and Cu binding (Table 3.1) revealed that both, total Cu binding of methanobactin and Cu-methanobactin speciation strongly depend on pH and Cu\(^{2+}\) activity (Fig. 3.6) but also on the total methanobactin concentration (Fig. 3.8). Based on the model calculations, we expect that Cu-mb complexes are mostly found as monomers in natural systems. In soils, sediments, and surface waters with neutral and alkaline pH, the low bioavailability of Cu results from the high sorption of Cu to organic and inorganic matter and from the low solubilities of many Cu minerals. Due to methanobactin’s high Cu affinity, especially under alkaline conditions, it seems that methanotrophic microorganisms can effectively enhance Cu acquisition by producing and releasing chalkophores. Organisms producing high-affinity chelating compounds, however, necessitate a mechanism to liberate the chelated nutrient. The slight weakening of the Cu bond by protonation observed in this study potentially represents a mechanism to facilitate Cu release from methanobactin. At lower pH, metal-free methanobactin was found to be unstable [Krentz et al.,
3.4 Consequences for copper mobilization

Figure 3.8.: Total Cu binding by methanobactin at pH 6 as a function of Cu\(^{2+}\) activity and total methanobactin concentrations. The calculations are based on the stability constants derived for proton and copper binding by methanobactin (Table 3.1).

2010; Behling et al., 2008], thus lowering its capability to act as a Cu acquisition member. Free Cu and bioavailable Cu concentrations increase with decreasing pH. Consequently, a specific strategy to promote Cu mobilization may not be necessary. In fact, methanotrophs growth experiments with different Cu minerals as Cu sources indicated that methanobactin enhanced Cu uptake in the presence of low soluble Cu minerals such as CuO but had no effect on methanotrophic growth if more soluble minerals (e.g., CuCO\(_3\)-Cu(OH)\(_2\)) were used [Chi Fru et al., 2011; Knapp et al., 2007]. Thus, methanobactin may mainly play a role in the Cu acquisition system in slightly acidic to alkaline environments.

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References


References


4. Competitive ligand exchange between 

Cu-humic acid complexes and 

methanobactin

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Abstract

Copper has been found to play a key role in the physiology of methanotrophic microorganisms and methane oxidation may critically depend on the availability of Cu. In natural environments such as soils, sediments, peat bogs, and surface waters, the presence of natural organic matter can control the bioavailability of Cu by forming strong metal complexes. In order to promote Cu acquisition, methanotrophs exude methanobactin, a ligand known to have a high affinity for Cu. In this study, the capability of methanobactin for Cu acquisition from natural organic matter was investigated using humic acid as a model substance. The kinetics of ligand exchange between Cu-humic acid and methanobactin was observed by UV–vis spectroscopy and the speciation of Cu bound to methanobactin was determined by size-exclusion chromatography coupled to an ICP-MS. The results showed that Cu was mobilized from humic acid by a fast ligand exchange reaction following a second-order rate law with first-order kinetics for both, methanobactin and Cu-humic acid complexes. The reactions rates decreased with decreasing temperature. Equilibrium experiments indicated that methanobactin was not sorbed to humic
acid and proved that methanobactin is competitive with humic acid for Cu binding by forming strong 1:1 Cu:methanobactin complexes. Consequently, our results demonstrate that methanobactin can efficiently acquire Cu in organic-rich environments.

4.1. Introduction

The uptake of micronutrients from their natural environment poses a challenge for many microorganisms and plants. Although ubiquitous in terrestrial and aquatic systems, some essential nutrients such as iron (Fe) and copper (Cu) can be growth limiting for numerous organisms due to their low bioavailability [Marschner, 2012; Semrau et al., 2010; Puig et al., 2007]. Under aerobic conditions, Fe deficiency often results from low solubility of iron oxides and slow dissolution kinetics of Fe-bearing minerals at neutral and alkaline pH, whereas Cu is mainly bound to natural organic matter (NOM) and manganese and iron oxides [Kraemer et al., 2006; Adriano, 2001; Nriagu, 1979].

In order to overcome nutrient deficiency, various microorganisms have developed a variety of acquisition strategies to enhance mineral dissolution and mobilize nutrients by changing the extracellular environment [Blume et al., 2010]. Some organisms were shown to decrease the solution pH by exuding protons and organic acids or to mobilize nutrients by producing chelating ligands [Telford & Raymond, 1996; Winkelmann, 1991; Bossier et al., 1988; Neilands, 1981]. Siderophores are examples for such ligands known to have a high affinity for Fe and to promote iron mineral dissolution [Kraemer, 2004; Boukhalfa & Crumbliss, 2002; Albrecht-Gary & Crumbliss, 1998]. Analogously, aerobic methanotrophic microorganisms, which have a high demand for Cu, secrete Cu chelating compounds, so-called chalkophores, to overcome Cu deficiency [Balasubramanian & Rosenzweig, 2008; Kim et al., 2004]. Almost all aerobic methanotrophs oxidize methane to methanol, a reaction catalyzed by Cu-containing particulate methane monooxygenase (pMMO) [Lieberman & Rosenzweig, 2004; Hanson & Hanson, 1996]. Some strains also produce a cytoplasmic or soluble MMO (sMMO) under Cu-limiting conditions. Copper has been found to play a key role in the regulation of pMMO and sMMO expression as well as in...
the regulation of other metabolic enzymes of methanotrophs [Semrau et al., 2010; Balasubramanian & Rosenzweig, 2008; Hakemian & Rosenzweig, 2007; Stanley et al., 1983]. Furthermore, methane oxidation by pMMO critically depends on Cu availability [Lontoh & Semrau, 1998]. It is assumed that many methanotrophs possess an efficient Cu mobilization and uptake system [Semrau et al., 2010], involving the synthesis of methanobactins (chalkophores) which have only been isolated and characterized from a few strains [Bandow et al., 2012; Choi et al., 2010; Krentz et al., 2010; Behling et al., 2008; Kim et al., 2005]. The best characterized methanobactin is a modified peptide with a molecular mass of 1154 Da produced by *Methylosinus trichosporium* OB3b [Behling et al., 2008]. A common characteristic of all methanobactins isolated so far is the presence of two heterocyclic rings (imidazole and/or oxazolone associated with an enethiol) involved in Cu binding [Bandow et al., 2012; El Ghazouani et al., 2011; Krentz et al., 2010; Behling et al., 2008]. Methanobactin has a high affinity for Cu(I) and Cu(II), the latter being reduced upon binding [El Ghazouani et al., 2011; Choi et al., 2006; Hakemian et al., 2005]. In a previous study, a very strong and pH-dependent Cu complexation by methanobactin was demonstrated using potentiometric and metal titrations [Pesch et al., 2012]. A conditional stability constant of log $K \approx 25$ has been determined for 1:1 Cu:methanobactin complexes. Additionally, the data indicated that with increasing Cu-to-methanobactin ratios either dimer (mb$_2$-Cu), monomer (mb-Cu) or methanobactin complexes which bind two Cu atoms (mb-Cu$_2$) are formed.

Most previous studies investigated methanotrophic activity and the influence of methanobactin as a response of soluble Cu concentrations [DiSpirito et al., 1998; Lidstrom & Semrau, 1995]. Only recently, methanotrophic behavior has been analyzed in the presence of Cu-bearing minerals [Chi Fru et al., 2011; Kulczycki et al., 2011; Knapp et al., 2007]. In oxic environments, however, dissolved Cu concentrations are often low and Cu(II) is mainly bound to organic matter or manganese and iron oxides at circumneutral pH [Blume et al., 2010]. At acidic pH, Cu bound to NOM is the prevalent form. Humic and fulvic acids were shown to have high affinity for Cu [Christl et al., 2001] and exhibit a higher affinity for Cu than do many mineral phases under acidic conditions [Heidmann et al., 2005]. Thus, NOM is an important sorbent for Cu potentially controlling Cu availability under acidic conditions and in organic-rich systems.
such as top soils and peat bogs. Even though, the speciation of Cu(II) in natural systems is often dominated by stable organic complexes, methanotrophic activity in the presence of Cu originating from NOM has not been studied so far. Based on the strong binding of Cu by methanobactin [Pesch et al., 2012], methanobactin may readily acquire weakly bound Cu from NOM, but Cu acquisition may be restricted by high affinity sites of NOM. Furthermore, kinetic limitations and interactions between NOM and methanobactin may affect Cu acquisition from NOM.

The aim of this study was to investigate methanobactin-induced Cu acquisition from NOM. A purified humic acid was used as model substance to investigate the competition between methanobactin and NOM for Cu. The speciation of Cu bound to methanobactin in the presence of humic acid was determined by size-exclusion chromatography coupled to ICP-MS and compared to speciation calculations based on Cu binding constants published for methanobactin and humic acid, respectively. Furthermore, the kinetics of ligand exchange was studied by time-resolved UV–vis spectroscopy at different temperatures in order to evaluate the efficiency of methanobactin for Cu acquisition in natural systems.

4.2. Materials and methods

4.2.1. Materials

Solutions were prepared with high-purity deionized water (18.2 MΩ cm, Milli-Q, Millipore). All reagents used were at least analytical grade and purchased from Fluka, Merck, or Sigma-Aldrich. All vessels were washed in 1 M NaOH and 1 M HCl for 24 hours and rinsed with deionized water. Amber glassware or vessels wrapped in aluminum foil were used to avoid potential photodegradation [Kim et al., 2005].

Methanobactin was isolated from Methylosinus trichosporium OB3b cells cultured in nitrate minimal salts medium (NMS) amended with 0.2 µM CuCl₂ in a BIOSTAT® A plus bioreactor system (Sartorius) as previously reported [Choi et al., 2005; Kim et al., 2005]. Methanobactin produced by Methylosinus trichosporium OB3b, abbreviated mb in the following, was isolated
from the spent medium by resin extraction (Diaion HP-20, Supelco) followed by a second purification step using reversed-phase chromatography on a C18 column with methanol and a 0.01 M NaCl solution as the mobile phases. The collected mb, shown to be virtually Cu-free (Cu-to-mb ratio of 0.003) after isolation and purification [Pesch et al., 2011], was freeze-dried and stored in the dark at -20 °C before use. Details about the isolation and purification of mb were described earlier [Pesch et al., 2011]. The concentration of mb stock solutions was determined by Cu(I) titration of mb solutions in the presence of bicinechoninic acid [El Ghazouani et al., 2011].

As a model substance for NOM, a humic acid (HA) was chosen which was extracted from a humified organic horizon of a humic Gleysol in northern Switzerland and purified by dialysis at a molecular weight cut-off of 12000 Da [Christl et al., 2000]. This HA was used because it has been well characterized [Christl et al., 2000] and its proton and Cu binding properties have been studied in detail [Christl & Kretzschmar, 2001; Christl et al., 2001]. Humic acid stock solutions used for the experiments were spiked with different CuCl₂ concentrations at pH 6 and 8 and equilibrated for 24 h at 25 °C. The stock solutions were then stored at 4 °C in the dark for 1 – 2 months before use to assure equilibrium Cu binding to HA and thereby mimicking natural conditions.

All experiments were performed in 0.01 M NaCl buffered to pH 6.0 ±0.05 with 0.005 M MES (2-(N-morpholino)ethanesulfonic acid) and pH 8.0 ±0.05 with 0.005 M MOPS (3-(N-morpholino)-propanesulfonic acid), respectively. These buffers were chosen because they do not bind Cu and are therefore suitable for metal speciation studies [Mash et al., 2003]. The Cu concentrations of stock solutions were checked with atomic absorption spectrometry (Varian SpectrAA 220FS, Varian Inc.).

### 4.2.2. Kinetics of ligand exchange

The kinetics of ligand exchange between Cu-humic acid and mb was studied by UV–vis spectroscopy. The experiments were directly conducted in a 1.0 cm quartz cuvette and the complexation of Cu by mb from Cu-spiked HA was determined by monitoring the decrease of absorption at 392 nm over time with a time resolution of 3 seconds using a UV–vis spectrometer (Cary50
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Bio, Varian). The kinetics experiments were conducted at 23 ±1 °C in a thermostat-controlled room in a total volume of 2.5 mL which was continuously stirred with a magnetic stirrer. At the start of the experiment, aliquots of a mb stock solution were added to the Cu-spiked HA stock solutions to obtain final mb concentrations ranging from 23 to 62 µM. HA and Cu concentrations were varied to investigate the influence of reagent ratios on the kinetics of the ligand exchange reaction. All experimental concentrations and Cu-to-mb ratios are summarized in Table 4.1. All experiments were performed in duplicates. Additional kinetics experiments were performed with 0.07 g L⁻¹ HA, total Cu and mb concentrations of 30 and 23 µM, respectively at pH 6.0 ±0.05 in a thermostat-controlled room at 4 °C and 14 °C to investigate the temperature-dependence of the ligand exchange reaction. The respective temperatures were controlled inside the spectrophotometer.

UV–vis absorption scans between 220 and 600 nm were recorded for Cu-free mb and HA separately for each concentration and pH-value used in the experiments. Absorption spectra of Cu-free HA and Cu-HA were identical for the experimental conditions of the kinetic experiments. Because the reaction was fast, the starting point of the experiments (absorption at t=0) was calculated by addition of the single spectra of mb and HA. Absorption spectra of a 20 µM mb solution titrated with a CuCl₂ solution at pH 6 were taken to calculate the decrease in absorption at 392 nm per mol Cu-mb (ΔAbs/mb-Cu = -0.011) formed (Appendix C Fig. C.1). A linear decrease was observed up to a molar Cu-to-mb ratio of 1.1. The absorption at 392 nm relative to the absorption at the isosbestic point at 422 nm was used to calculate the decline of Cu-free mb and the formation of Cu-bound mb over time. The initial amount of Cu bound to HA was calculated with the NICA-Donnan model [Koopal et al., 2005] using the speciation program ECOSAT [Keizer & van Riemsdijk, 1998] and the model parameters published for the HA used here [Christl & Kretzschmar, 2001; Christl et al., 2001].

4.2.3. Competitive ligand exchange in equilibrium

Competitive ligand exchange between HA and mb was studied in batch experiments and analyzed using size-exclusion chromatography (SEC) coupled to an ICP-MS. Pre-equilibrated
Cu-spiked HA stock solutions were mixed with aliquots of an mb stock solution in 0.01 M NaCl and 0.005 M MES or MOPS for pH 6.0 ±0.05 and 8.0 ±0.05, respectively. HA, mb and Cu concentrations were varied to study the influence of reagents ratios on the competitive ligand exchange. All experimental concentrations and Cu-to-mb ratios are listed in Table 4.2. Selected experiments were conducted in duplicates to verify the reproducibility of the applied method. The samples were equilibrated for 4 h at 25 °C to assure a completed ligand exchange reaction. Before injection to the SEC-ICP-MS system, the samples were passed through a 0.2 µm nylon filter (WICOM International AG). Methanobactin and HA were separated on a Superdex™ Peptide 10/300 GL column (GE Healthcare). The formation of the different mb complexes were quantified with a diode array UV–vis detector and Cu concentrations were measured by ICP-MS as described previously Pesch et al. [2012].

4.3. Results and Discussion

4.3.1. Additivity of UV–vis spectra

Figure 4.1A shows the absorption spectra recorded between 220 and 600 nm for 0.07 g L⁻¹ HA as well as for 40 µM Cu-free and Cu-bound mb solutions. The absorption maxima at 342 and 392 nm can be attributed to the two enethiol-oxazolone moieties of mb [Behling et al., 2008; Kim et al., 2005]. A striking decrease of the absorption, especially at 392 nm, is observed upon Cu binding by mb [El Ghazouani et al., 2011; Choi et al., 2006] (see also Appendix C Fig. C.1). The absorption scans of mixtures containing Cu-free mb and HA or Cu-bound mb and HA in equal concentrations as for the single solutions (Fig. 4.1A) are depicted in Figure 4.1B. The spectra calculated for mixed solutions (dotted lines) based on the spectra of the single systems agree very well with the experimental spectra recorded for the mixed systems. Thus, the absorption spectra are additive and the kinetics of Cu complexation by mb can be traced in situ over time by continuously measuring the decrease of absorption at 392 nm relative to the isosbestic point at 422 nm upon mb addition to Cu-spiked HA solutions (Fig. 4.1B).
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Figure 4.1.: Additivity of UV–vis spectra. (A) UV–vis absorption spectra of 40 µM Cu-free mb (mb) (grey line), 40 µM Cu-bound mb (mb-Cu) (black line), and 0.07 g L\(^{-1}\) humic acids (HA) (dashed line). (B) Absorption spectra of a mixture of 40 µM mb and 0.07 g L\(^{-1}\) HA (grey line), 40 µM mb-Cu and 0.07 g L\(^{-1}\) HA (black line), the sum of the single mb and HA spectra as plotted in A (grey dotted line), and the sum of the single mb-Cu and HA spectra as plotted in A (black dotted line).

Figure 4.1.
4.3.2. Kinetics of ligand exchange

Selected results of the experiments on the kinetics of ligand exchange between Cu-HA complexes and mb at pH 6 and 8 are shown in Figure 4.2. For all experiments, the kinetics of ligand exchange can be described by a second-order rate law that is first-order in mb and Cu-HA complexes, respectively, as described by Equation 4.1,

\[ k \cdot t = \frac{1}{CuHA_0 - mb_0} \cdot \ln \left( \frac{CuHA_t \cdot mb_0}{CuHA_0 \cdot mb_t} \right) \]  

(4.1)

where \( k \) is the complexation rate, \( t \) the elapsed time of the experiment, \( mb_0 \) and \( CuHA_0 \) the initial concentrations of Cu-free mb and Cu-HA complexes, respectively, and \( mb_t \) and \( CuHA_t \) the concentrations of the reagents at time \( t \).

The overall exchange reaction can be divided into two different phases. Independent of the initial Cu, mb, and HA concentrations and pH, a faster initial ligand exchange was observed, followed by a slower reaction until equilibrium was reached. Reaction rates were calculated separately for both phases as exemplarily demonstrated in Figure 4.3. The calculated exchange rates \( k_1 \) and \( k_2 \) as well as the corresponding half-lives are summarized in Table 4.1. The reaction rates of the first reaction phase \( (k_1) \) ranged from 0.02 to 0.16 \( \mu \text{M}^{-1}\text{min}^{-1} \), whereas \( k_2 \) ranged from 0.003 to 0.013 \( \mu \text{M}^{-1}\text{min}^{-1} \). The corresponding half-lives of the initial exchange reaction \( (t_{1/2}(k_1)) \) were below 1.3 min for all experiments. During the initial, fast ligand exchange phase, 60 to 80% of the added Cu-free mb reacted to form Cu-mb complexes (Table 4.1). Cu-mb formation was lowest (60%) for the experiment conducted at pH 8 with total Cu and mb concentrations of 30 and 23 \( \mu \text{M} \), respectively. For each pair of experiments with equal Cu and mb concentrations, Cu-mb formation was lower at pH 8 as compared to pH 6. Likewise, Cu-mb formation was reduced with increasing concentration of HA. Mixing of the reagents after mb addition may have had some influence on reaction rates during the first seconds of the experiment and on the rate coefficient \( k_1 \). However, as discussed below, the slower reaction phase is more relevant for natural systems that are characterized by low Cu loadings of HA.

The half-lives of the second ligand exchange phase \( (t_{1/2}(k_2)) \) ranged from 1 to 6 min. For the experiments with 23 \( \mu \text{M} \) mb and 30 \( \mu \text{M} \) Cu equilibrated with 0.07 g L\(^{-1}\) HA and 40 \( \mu \text{M} \) mb
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Figure 4.2.: Kinetics of Cu binding by methanobactin (mb) using Cu bound humic acid as the Cu source. Cu bound to mb (mb-Cu) over time with varying mb, Cu and HA concentrations at 23 °C in 0.01 M KCl and (a) 0.005 M MOPS at pH 8 and (b) 0.005 M MES at pH 6. (c) Cu bound to mb (mb-Cu) over time with 23 µM mb, 31 µM Cu and 0.07 g L⁻¹ HA in 0.01 M KCl and 0.005 M MES at pH 6 at 4 °C, 14 °C, and 23 °C.
4.3 Results and Discussion

Figure 4.3.: Determination of the ligand exchange reaction rate. Kinetics of ligand exchange between Cu-humic acid (HA) and mb (mb) with total concentrations of 23 µM mb, 31 µM Cu and 0.07 g L⁻¹ HA at pH 8. Two phases are apparent and each can be described by second-order rate law that is first-order in mb and Cu-HA. Reaction rates $k_1$ and $k_2$ were calculated by linear regression for the first and the second ligand exchange phase, respectively.
and 52 µM Cu equilibrated with 0.1 g L$^{-1}$, $t_{1/2}(k_2)$ increased and the ligand exchange rates $k_2$ decreased with increasing pH. The reactions rates of the other experiments were all within the same order of magnitude. In general, the reaction rates were almost as fast as complexation of Cu by mb if Cu is added in a soluble inorganic form. Cu(II) binding by mb was found to be completed within 10 min [El Ghazouani et al., 2011; Choi et al., 2006]. Considering the total concentrations of Cu, mb and HA in the different experiments (Table 4.1), no obvious trend can be deduced between the reaction rates and Cu, mb, or HA concentrations. A slight trend was however noticeable when $t_{1/2}$ is compared to Cu activity in solution (Appendix C Fig. C.2). The half-lives were found to increase with decreasing Cu activity. Thus, the reaction rates decreased if Cu dissociation from HA was lower.
Table 4.1.: Results obtained from kinetics of ligand exchange experiments between Cu-humic acid (HA) and methanobactin (mb) at 4, 14, and 23 °C with varying mb, Cu, and HA concentrations in 0.01 M NaCl and 0.005 M MES and MOPS for pH 6 and 8, respectively.

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature</th>
<th>Cu/mb</th>
<th>(k_1)</th>
<th>(k_2)</th>
<th>(t_{1/2}(k_1))</th>
<th>(t_{1/2}(k_2))</th>
<th>Cu-mb formation (during phase (k_i))</th>
<th>low affinity sites (Cu_{la}) (^1)</th>
<th>Cu_{la}/mb</th>
<th>Cu_{la}/mb_{tot}</th>
<th>Cu_{la}</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>23(^\circ)C</td>
<td>62 (\mu)M mb, 78 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>2.6</td>
<td>0.163</td>
<td>0.013</td>
<td>0.1</td>
<td>0.9</td>
<td>75.5</td>
<td>24.2</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>23(^\circ)C</td>
<td>23 (\mu)M mb, 31 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>0.022</td>
<td>0.005</td>
<td>1.3</td>
<td>6.1</td>
<td>59.5</td>
<td>12.3</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>23(^\circ)C</td>
<td>40 (\mu)M mb, 52 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>0.022</td>
<td>0.005</td>
<td>1.3</td>
<td>6.1</td>
<td>59.5</td>
<td>12.3</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>23(^\circ)C</td>
<td>40 (\mu)M mb, 52 (\mu)M Cu, 0.10 g L(^{-1}) HA</td>
<td>1.3</td>
<td>0.024</td>
<td>0.003</td>
<td>0.7</td>
<td>6.2</td>
<td>67.3</td>
<td>20.3</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>23(^\circ)C</td>
<td>23 (\mu)M mb, 31 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.9</td>
<td>67.7</td>
<td>43.2</td>
<td>1.6</td>
<td>1.1</td>
<td>83.6</td>
</tr>
<tr>
<td>8</td>
<td>23(^\circ)C</td>
<td>40 (\mu)M mb, 52 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.9</td>
<td>67.7</td>
<td>43.2</td>
<td>1.6</td>
<td>1.1</td>
<td>83.6</td>
</tr>
<tr>
<td>6</td>
<td>23(^\circ)C</td>
<td>40 (\mu)M mb, 52 (\mu)M Cu, 0.10 g L(^{-1}) HA</td>
<td>1.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.9</td>
<td>67.7</td>
<td>43.2</td>
<td>1.6</td>
<td>1.1</td>
<td>83.6</td>
</tr>
<tr>
<td>8</td>
<td>23(^\circ)C</td>
<td>23 (\mu)M mb, 31 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>0.024</td>
<td>0.003</td>
<td>0.7</td>
<td>6.2</td>
<td>67.3</td>
<td>20.3</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>23(^\circ)C</td>
<td>62 (\mu)M mb, 78 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>0.107</td>
<td>0.007</td>
<td>0.1</td>
<td>2.0</td>
<td>73.6</td>
<td>59.3</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>23(^\circ)C</td>
<td>23 (\mu)M mb, 31 (\mu)M Cu, 0.07 g L(^{-1}) HA</td>
<td>1.3</td>
<td>0.022</td>
<td>0.005</td>
<td>1.3</td>
<td>6.1</td>
<td>59.5</td>
<td>12.3</td>
<td>0.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^1\) The amounts of Cu supplied as bound to low affinity sites of humic acid (Cu_{la}) were calculated using the NICA-Donnan model parameters published by Christl et al. [2001].

\(^2\) n.d. denotes not determined because very fast reaction kinetics disabled quantification.
Chapter 4. Competitive ligand exchange between Cu-humic acid complexes and methanobactin

Two different mechanistic pathways can be distinguished to describe the overall ligand exchange reaction. In the adjunctive pathway, a competitive ligand directly attacks the initial complex, whereas the disjunctive pathway involves the dissociation of the initial complex before complexation by the competitive ligand [Hering & Morel, 1988]. For an unambiguous discrimination between adjunctive and disjunctive pathways, experimental molar ratios of reactants have to span a wide range [Hering & Morel, 1990]. However, due to the detection limits of the UV–vis method used in this study, experimental variations in Cu, mb, and HA concentrations were fairly limited. Alternatively, the Cu loadings of humic acids may be used to gain information on the type of pathway. Hering & Morel [1990] demonstrated that at high Cu loading of humic acids (as used in this study), the disjunctive pathway is expected to be the dominating mechanism for ligand exchange reactions. Also, our observation of a fast and a slow ligand exchange phase is consistent with their study [Hering & Morel, 1990]. They observed that at a Cu-to-HA ratio of 45 µmol Cu g⁻¹ HA and above the ligand exchange reaction cannot be explained with a single overall rate, but with two rate constants: one for reaction with fast-reacting sites and one for the reaction with slow-reacting sites [Hering & Morel, 1990]. In this study, Cu loading of HA is almost ten times higher (> 400 µmol Cu g⁻¹ HA) for all experiments and we also find two phases with different rate constants, which we interpret as an exchange reaction involving two different sites on the HA. Previous studies investigating Cu binding by the HA used for the present experiments have shown that Cu binding can be quantitatively described with the NICA-Donnan model based on the presence of low and high affinity sites [Christl et al., 2001]. The amount of Cu bound to the low affinity sites for each experimental condition is specified in Table 4.1. If we assume that first Cu originating from low affinity sites (Cu₁₉₈) is primarily complexed by mb via a fast ligand exchange, the overall ligand exchange reaction rate is expected to depend on the extent to which the low affinity pool is exploited. Molar Cu₁₉₈-to-mb ratios greater than 1 obtained for the majority of the experiments indicate that mb can easily mobilize Cu from HA, thus, leading to the fast complexation rates observed in this study. For the experiments at pH 8 with initial mb concentrations of 23 or 39 µM and HA concentrations of 0.07 or 0.1 g L⁻¹, molar Cu₁₉₈-to-mb ratios < 1 were found, indicating that the low affinity pools are depleted. Hence, mb has to mobilize Cu from high affinity sites,
which may explain the higher half-lives observed for these experiments. In natural environments, lower Cu loadings of the humic material are expected and average Cu concentrations of 5.5 \( \mu \text{mol g}^{-1} \) humic acids have been reported [Nriagu, 1979]. Consequently, Cu mobilization from high affinity binding sites, reflected in the second ligand exchange rate \( (k_2) \), may be the rate-determining reaction under natural conditions.

### 4.3.3. Influence of temperature on ligand exchange reactions

Figure 4.2C describes the temperature-dependence of the ligand exchange between mb and HA with a total mb concentration of 23 \( \mu \text{M} \) and 31 \( \mu \text{M} \) of Cu equilibrated with 0.07 g L\(^{-1}\) HA at pH 6. Similar to the experiments conducted at 23 \(^\circ\)C, two exchange reaction phases were observed. For both phases, the reaction rates were lowered with decreasing temperature (Table 4.1). The value of \( k_1 \) decreased to 0.005 and 0.002 \( \mu \text{M}^{-1} \text{min}^{-1} \) while \( k_2 \) decreased to 0.002 and 0.001 \( \mu \text{M}^{-1} \text{min}^{-1} \) for the experiments at 14 \(^\circ\)C and 4 \(^\circ\)C, respectively. The lower reaction rates compared to the experiments at 23 \(^\circ\)C led to higher half-lives of \( t_{1/2}(k_1) = 6.4 \) and 15.6 min and \( t_{1/2}(k_2) = 13.5 \) and 56.6 min for 14 \(^\circ\)C and 4 \(^\circ\)C, respectively (Table 4.1). The results show that the ligand exchange reaction between humic acid and mb was much slower at temperature conditions found in many natural environments than compared to conditions at room temperature. The activation energy of the ligand exchange reaction was calculated using the Arrhenius Equation 4.2,

\[
k = A \cdot \exp \left( \frac{-E_a}{R \cdot T} \right)
\]

where \( k \) is the complexation rate, \( A \) a pre-exponential factor, \( E_a \) the activation energy, \( R \) the gas constant, and \( T \) the absolute temperature (Appendix C Fig. C.3). For the environmentally important slow reaction phase, we calculated an \( E_a \) of 116 kJ mol\(^{-1}\).

The activation energy may be used to predict half-lives of ligand exchange reactions for natural conditions. Although mb has not been measured in natural samples up to now, the concentrations are presumably low. Assuming an average Cu concentration of 5.5 \( \mu \text{mol/g} \) HA [Nriagu, 1979] and a total HA concentration of 0.07 g L\(^{-1}\) HA, as used in the experiments,
the CuHA concentration is about 0.4 µM. Presuming a total mb concentration of 0.38 µM,
the corresponding half-life \( t_{1/2}(k_2) \) increases to 185 min at 23 °C and to 1800 min at 10 °C.
Consequently, the ligand exchange reaction would be slow at environmental conditions.
4.3 Results and Discussion

4.3.4. Competition between humic acid and methanobactin for copper binding

In a previous study, three different types of Cu-mb complexes were found by metal titrations and model calculations. These complexes can undergo protonation reactions, depending on Cu and mb concentrations [Pesch et al., 2012]. Cu-mb dimers (mb$_2$H$_4$Cu and mb$_2$H$_3$Cu) form at low Cu-to-mb ratios. The monomers (mbHCu and mbCu) were formed with increasing Cu concentrations and mb bound two Cu ions (mbHCu$_2$ and mbCu$_2$) at Cu-to-mb ratios above 1. The different mb complexes were separated by SEC-ICP-MS. Cu-free mb was found to be eluted from the SEC column after 22.2 min. Cu-bound mb complexes had longer elution times whereby monomer mb-Cu complexes appeared after 27 min and mbCu$_2$ complexes after 29 min [Pesch et al., 2012] (Appendix C Fig. C.4), as indicated with dotted lines in Figure 4.4.

![Figure 4.4: Separation of Cu-free and Cu-bound methanobactin (mb) in the presence of 0.1 g L$^{-1}$ humic acid (HA) at pH 8. The absorbance of eluates at 280 nm is plotted as a function of elution time. The elution times of the different mb complexes are indicated as dotted lines [Pesch et al., 2012].](image-url)
Table 4.2.: Competition between humic acid (HA) and methanobactin (mb) for Cu binding at varying total concentrations of mb, Cu, and HA in 0.01 M KCl and 0.005 M MOPS at pH 8 or 0.005 M MES at pH 6 as determined with size-exclusion chromatography. Model calculations were conducted with the speciation program ECOSAT using previously determined stability constants for Cu binding by mb [Pesch et al., 2012] and HA [Christl et al., 2001].

<table>
<thead>
<tr>
<th>pH 8, 0.1 g L(^{-1}) HA</th>
<th>Initial concentrations</th>
<th>Cu-free mb</th>
<th>mb-Cu</th>
<th>mb-Cu(_2)</th>
<th>total measured conc.</th>
<th>Model calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu(_{tot})</td>
<td>mb(_{tot})</td>
<td>Cu(<em>{tot})/mb(</em>{tot}) mol/mol</td>
<td>Cu (\mu)M</td>
<td>mb (\mu)M</td>
<td>Cu/mb mol/mol</td>
</tr>
<tr>
<td>20.6 15.9 1.30</td>
<td>0.9 0.6</td>
<td>12.1 12.6 0.96</td>
<td>0.3 0.0</td>
<td>14.1 13.5 1.04</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>8.3 30.9 0.27</td>
<td>0.5 17.5 0.03</td>
<td>7.2 9.2 0.78</td>
<td>0.3 0.0</td>
<td>8.5 29.5 0.29</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>20.6 30.9 0.67</td>
<td>0.2 4.3 0.05</td>
<td>20.8 25.1 0.83</td>
<td>0.2 0.0</td>
<td>21.7 31.8 0.68</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>40.8 30.9 1.32</td>
<td>2.0 2.7</td>
<td>22.9 25.1 0.91</td>
<td>0.6 0.0</td>
<td>27.5 30.6 0.90</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>40.9 15.9 2.58</td>
<td>1.7 1.0</td>
<td>12.8 13.6 0.94</td>
<td>0.5 0.0</td>
<td>16.0 15.2 1.06</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>pH 8, 0.5 g L(^{-1}) HA</td>
<td>20.9 15.9 1.32</td>
<td>0.7 0.0</td>
<td>9.6 9.4 1.02</td>
<td>0.0 0.0</td>
<td>10.8 9.2 1.17</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>8.7 30.9 0.28</td>
<td>0.4 19.2 0.02</td>
<td>4.9 7.4 0.66</td>
<td>0.3 0.0</td>
<td>5.9 29.5 0.20</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>20.9 30.9 0.68</td>
<td>0.6 3.2 0.18</td>
<td>15.9 19.3 0.83</td>
<td>0.5 0.0</td>
<td>17.6 24.8 0.71</td>
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<tr>
<td></td>
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<td>1.5 1.4</td>
<td>24.8 26.8 0.93</td>
<td>0.6 0.0</td>
<td>28.4 30.1 0.94</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>42.1 15.9 2.65</td>
<td>1.8 0.0</td>
<td>8.8 9.2 0.96</td>
<td>0.9 0.0</td>
<td>8.8 9.2 0.96</td>
<td>0.8</td>
</tr>
<tr>
<td>pH 6, 0.1 g L(^{-1}) HA</td>
<td>20.1 15.5 1.30</td>
<td>1.3 2.1 0.60</td>
<td>12.4 12.1 1.02</td>
<td>0.6 0.0</td>
<td>15.4 16.6 0.93</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>8.1 30.0 0.27</td>
<td>0.5 15.1 0.03</td>
<td>8.2 10.6 0.78</td>
<td>0.5 0.0</td>
<td>9.7 29.4 0.33</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
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<td>0.4 0.0</td>
<td>21.0 29.8 0.70</td>
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<tr>
<td></td>
<td>39.9 30.1 1.32</td>
<td>2.3 4.3 0.54</td>
<td>24.3 25.9 1.02</td>
<td>0.8 0.0</td>
<td>29.1 32.0 0.91</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>39.9 15.5 2.58</td>
<td>0.0 2.4 0.00</td>
<td>13.7 13.1 1.04</td>
<td>0.7 0.0</td>
<td>14.4 18.0 0.80</td>
<td>0.7</td>
</tr>
<tr>
<td>pH 6, 0.5 g L(^{-1}) HA</td>
<td>20.4 15.5 1.32</td>
<td>1.6 2.5 0.61</td>
<td>12.5 12.9 0.98</td>
<td>0.6 0.0</td>
<td>15.8 18.0 0.88</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>8.4 30.1 0.28</td>
<td>0.6 15.2 0.04</td>
<td>6.4 8.3 0.77</td>
<td>0.5 0.0</td>
<td>8.0 26.5 0.30</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
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<td>20.7 23.0 0.90</td>
<td>0.5 0.0</td>
<td>22.5 31.9 0.71</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>41.1 30.1 1.37</td>
<td>2.7 5.0 0.55</td>
<td>25.8 26.0 0.99</td>
<td>0.8 0.0</td>
<td>30.9 34.7 0.89</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
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<td>12.6 11.7 1.08</td>
<td>1.0 0.0</td>
<td>18.0 16.6 1.08</td>
<td>0.5</td>
</tr>
</tbody>
</table>
To investigate the speciation of mb in the presence of Cu-humic acid in more detail, samples containing varying Cu and HA concentrations were analyzed with the same SEC-ICP-MS setup at pH 6 and 8. In the following, the results obtained at pH 8 are discussed first, and are then compared to the findings at pH 6. The SEC elution profile of 0.1 g L\(^{-1}\) HA without mb showed that the majority of HA was eluted with the void volume of the column (Fig. 4.4). However, it took 50 min to completely elute smaller molecular size fractions of the HA. Mixed solutions containing 0.1 g L\(^{-1}\) HA and 30 µM mb in the presence and absence of Cu were loaded onto the column. The results showed that Cu-free mb and mb-Cu were eluted after the same time as if injected without HA. Thus, neither in the presence nor in the absence of Cu a significant fraction of mb was sorbed to HA. These findings are consistent with the observed additivity of the UV–vis spectra (*vide supra*). Furthermore, mb appeared to effectively complex Cu from HA. The formation of the mb-Cu\(_2\) was, however, not observed, indicating a strong complexation of the first Cu ion but only a weak complexation of the second ion as previously proposed [Pesch et al., 2012].

For all experiments, the total amounts of Cu complexed by mb were slightly lower in the presence of HA compared to the reference samples where Cu was added in a soluble form (Figure 4.5A and Table 4.2). The amount of Cu bound by mb decreased with increasing concentration of HA. In experiments with initial Cu-to-mb ratios of about 1.3, the majority of mb complexed Cu by forming 1:1 complexes, regardless of the HA concentration. In contrast to the samples without HA, the formation of a mb complex which binds a second Cu ion (mb-Cu\(_2\)) was not observed in the presence of HA. When an excess of mb was added to HA (Cu/mb = 0.3), the remaining Cu-free mb concentration increased from 8 µM to 17 and 19 µM mb in the presence of 0.1 and 0.5 g L\(^{-1}\) HA, respectively. Likewise, the amount of mb-Cu decreased with increasing HA concentration, indicating that HA can partially compete with mb at high HA concentrations. Concurrently, the Cu-to-mb ratio decreased. The Cu-to-mb ratio of less than 1 suggests that mb dimer complexes (mb\(_2\)-Cu), which are expected to form at these experimental conditions, may be eluted at same time as the monomer complexes. These findings are in accordance with the elution of mb\(_2\)-Cu complexes in the absence of HA [Pesch et al., 2012]. Furthermore, the decrease in complexation efficiency with higher HA concentrations indicates that mb may be
less competitive if Cu is bound to the high affinity sites of humic acids.

At pH 6, similar findings were observed as compared to pH 8 (Fig. 4.5B and Table 4.2). When the Cu-to-mb was about 1.3, an effective Cu complexation by mb was observed combined with the formation of 1:1 mb-Cu species. Analogously, a similar binding behavior as for pH 8 was observed when an excess of mb was added. In contrast to the experiments performed at pH 8, however, the complexation did not depend on the concentration of HA at pH 6.

For each experiment, theoretical Cu-to-mb ratios were calculated with ECOSAT based on the stability constants for Cu binding by mb [Pesch et al., 2012] and the NICA-Donnan model describing Cu binding to HA [Christl et al., 2001]. A comparison of the theoretical Cu loadings of mb (given as Cu\textsubscript{mb}-to-mb ratios) with the ratio calculated from the total Cu complexed by mb (Table 4.2) indicated excellent agreement with experimentally determined Cu-to-mb ratios at pH 8. At lower total mb concentrations, the calculated Cu-to-mb ratios are 40 % lower than the experimentally determined ratios. The difference may result from the fact that already minor inaccuracies in the determination of concentrations lead to a large difference in the calculated Cu/mb ratios. At pH 6, the calculated ratios are 10 to 40 % lower than the observed ratios, especially at a HA concentration of 0.5 g L\textsuperscript{-1}. The high UV–vis-absorbance of 0.5 g L\textsuperscript{-1} HA solutions may slightly affect the quantification of the mb concentration. Most importantly, the Cu binding constants for both, mb and HA, were fitted to datasets sweeping larger ranges of conditions and concentrations. It is to be expected that small discrepancies as observed here will occur at any specific concentration or condition. Thus, we consider the observed discrepancy acceptable. Consequently, the previously determined model parameters are suitable to establish predictions on the competitive behavior of mb and HA for Cu binding.

### 4.4. Conclusions

The results obtained in this study demonstrated that mb effectively complexes Cu from humic substances. The kinetics of ligand exchange showed that mb-Cu complexes are formed by a fast ligand exchange reaction from low and high affinity sites with corresponding differences
Figure 4.5.: Cu bound by methanobactin as analyzed by size-exclusion chromatography at (a) pH 8 and (b) pH 6 with 16 and 31 μM mb, in the absence or in the presence of 0.1 or 0.5 g L⁻¹ HA equilibrated with increasing total Cu concentrations. The reference data for Cu binding in the absence of HA (squares) were taken from Pesch et al. [2012].
in kinetic lability. The equilibrium experiments showed that mb is competitive with HA for Cu binding and forms strong 1:1 Cu:mb complexes. Additionally, SEC chromatography proved that 1:2 mb-Cu complexes do not form in the presence of HA. Thus, 1:2 mb-Cu complexes do not play an important role for Cu binding in natural environments.

At pH 6, no difference between Cu complexation by mb at different HA concentrations was observed, whereas at pH 8, Cu bound by mb slightly decreases with increasing HA concentrations. Free Cu$^{2+}$ activities of the analyzed samples ranged from $10^{-7.3}$ to $10^{-10.6}$ at pH 6 and from $10^{-9.4}$ to $10^{-13.0}$ at pH 8 leading to the conclusion that mb is capable of mobilizing Cu complexed to NOM in natural environments even if Cu$^{2+}$ activities are low. Additionally, neither Cu-free nor Cu-bound mb was sorbed to humic acids. Consequently, mb is available to methanotrophic bacteria after its release to the extracellular phase and holds the potential to act as a member of a Cu acquisition system.

Considering the fast complexation and the effective complexation of Cu, as determined in this study by kinetics and equilibrium experiments, respectively, mb appears to be very efficient for Cu acquisition in organic-rich systems. In many natural environments, however, the kinetics of ligand exchange are expected to be slowed down by lower temperatures and by the presence of low mb and Cu concentrations considerably increasing the half-lives of the ligand exchange reaction. This observation points out that methanotrophic Cu acquisition in organic-rich systems may be kinetically limited by slow ligand exchange reactions at low temperatures and Cu loadings. Hence, it may be interesting to study adjunctive pathways of direct ligand attack of Cu:HA complexes at very low Cu loadings in the future. In the equilibrium experiments carried out in this study, Cu-to-HA ratios ranged from 20 to 470 µmol Cu g$^{-1}$ HA, whereas an average concentration of 5.5 µmol Cu g$^{-1}$ HA has been reported for natural systems [Nriagu, 1979]. Model calculations using this average Cu-to-HA ratio and the stability constants determined for Cu binding by mb [Pesch et al., 2012] and HA [Christl et al., 2001] revealed that the theoretical loading of mb (Cu$_{mb}$-to-mb) would decrease to 0.1. Thus, the amount of Cu complexed by mb may decrease with decreasing total Cu-to-HA ratios.

Although, in natural systems mb concentration are expected to be low, higher concentrations might be found locally in the vicinity of mb releasing methanotrophic bacteria allowing an
efficient supply of the essential nutrient to the cells. Methanobactin was found to enhance
the growth of the methanotroph *Methylosinus trichosporium* OB3b when Cu was supplied as a
sparingly soluble mineral, whereas a smaller effect was observed when soluble Cu concentrations
were higher [Chi Fru et al., 2011]. This implies that mb plays a major role in the mobilization
of Cu when Cu is strongly bound, e.g., to NOM or mineral surfaces. Thus, mb production
and release is essential in weakly acidic to circumneutral organic-rich environments, such as
peatlands, lake sediments, and soils enriched in organic matter.

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References


5. Conclusions

This thesis demonstrates that methanobactin released by methanotrophic microorganisms effectively mobilizes Cu from natural organic matter. Therefore, methanobactin can play a major role in Cu acquisition in organic-rich environments. In the following, the key findings of the present research are briefly summarized, before discussing the significance of Cu binding by methanobactin for methanotrophic growth in natural systems. Furthermore, suggestions for future research are presented.

During the first part of this project (Chapter 2), the chalkophore methanobactin produced by *Methylosinus trichosporium* OB3b has been successfully isolated from the nutrient medium. In contrast to literature suggesting a simple resin extraction, spectroscopic analyses and mass balance calculation revealed that methanobactin was present in the extract, but contributed only 32 % to the total isolated organic carbon. Thus, a second purification step is indispensable in addition to resin extraction in order to obtain pure Cu-free methanobactin which is prerequisite for investigations of the biogeochemical functions of chalkophores.

A chemical speciation model describing proton and copper binding by methanobactin and the formation of methanobactin complexes has been successfully established (Chapter 3). Methanobactin was found to have a high affinity for Cu. Cu binding by methanobactin can mainly be explained by the formation of three types of Cu-methanobactin complexes which can undergo protonation reactions, additionally. High initial affinity resulted from the occurrence of Cu-methanobactin dimer complexes, mainly mb$_2$H$_4$Cu and mb$_2$H$_3$Cu with log $K$ values of 58 and 52, respectively at Cu-to-methanobactin ratios (Cu/mb) below 0.5. With increasing Cu-to-methanobactin ratio (Cu/mb > 0.5), methanobactin bound Cu as monomer (mbHCu
and mbCu), whereas at elevated Cu activities (Cu/mb > 1) methanobactin bound two Cu ions (mbHCu₂ and mbCu₂). The occurrence of the different Cu-methanobactin complexes has been spectroscopically confirmed after separation of the complexes with size-exclusion chromatography. Furthermore, the results demonstrate that Cu binding by methanobactin is pH-dependent and the optimized stability constants for proton and Cu binding can be used to predict the behavior of methanobactin in natural environments. Model calculations suggested that monomer Cu-methanobactin complexes prevail under natural conditions.

Methanobactin was found to acquire Cu from humic acid by a fast initial ligand exchange reaction, followed by a slightly slower ligand exchange reaction when Cu is complexed from high affinity sites of humic acid (Chapter 4). Methanobactin effectively mobilizes Cu from humic substances by forming strong 1:1 Cu:methanobactin complexes even if soluble Cu concentrations are low. Methanobactin was not sorbed to humic acid and is, therefore, still available to the methanotrophs after its release to the extracellular phase. Considering the fast complexation kinetics and the effective complexation of Cu in equilibrium as shown in this study, methanobactin is very efficient for Cu acquisition in organic-rich environments. Moreover, the experimental results obtained on the complexation of Cu from humic substances agree well with model predictions. Thus, the established speciation model for methanobactin can be applied for predictions in natural systems.

The ability of methanobactin to mobilize Cu in the presence of low Cu activities reflects the functioning of the chalkophore as an external component of a specific nutrient acquisition system. Methanobactin may play a major role in aerobic weakly acidic to neutral organic-rich environments where Cu bioavailability is largely controlled by the presence of natural organic ligands. Under circumneutral pH conditions, up to 70 % of the total Cu can be sorbed to iron and manganese oxides [Blume et al., 2010] and thereby represents an important additional Cu source for methanotrophic microorganisms. Methanotrophic activity of methanotrophs grown on Cu minerals depends on the solubility of the used minerals [Chi Fru et al., 2011]. Thus, in natural systems the growth of methanotrophs and methane oxidation rates may depend on the one hand on the availability Cu determined by the solubility of the Cu minerals or the binding
capability of organic and mineral sorbents in question and on the other hand on the capability of methanobactin to mobilize Cu from the respective Cu pool in order to provide the essential micro-nutrient to the cells. In environmental systems where different Cu reservoirs are present, Cu will first be acquired from the pool of weakly sorbed Cu before Cu minerals exhibiting low solubility and slow dissolution kinetics are gaining relevance.

Recent studies proved that methanotrophic bacterial populations are subject to biogeographical and spatial dynamics [Lueke et al., 2010; Chi Fru, 2008]. Different factors, such as oxygen, methane, and nitrogen concentration or temperature and soil moisture, influence the development of microbial communities. The impact of Cu and, particularly, of Cu availability on the population structure of methanotrophs is not yet understood. As methane oxidation by pMMO and the regulation of pMMO and sMMO expression can be influenced by the availability of Cu, it seems very likely that the speciation of Cu in natural systems and the ability of some aerobic methanotrophs to enhance Cu uptake by producing methanobactins influence the development of methanotrophic bacterial populations. Since the structures of methanobactins have only been elucidated recently, the focus to understand the link between methanobactin and methane oxidation or population dynamics has only been initiated. The synthesis of methanobactin, however, has been observed in both, α-Proteobacteria and γ-Proteobacteria and seems to be independent of the factor whether the chalkophore-producing methanotrophs may express sMMO beside pMMO expression [Semrau et al., 2010]. Although first studies indicated that the production of methanobactin is widespread among methanotrophs, only few chalkophores have been isolated so far. Consequently, the characteristics of the environments populated by methanobactin-releasing methanotrophs have hitherto not been identified. Marine environments, where Cu activities are extremely low due to alkaline pH conditions, show a low methanotrophic microorganisms diversity. Nevertheless, strains that are adapted to such conditions need a specific Cu mobilization strategy. Analogously, the presence of a high content of natural organic matter may foster methanotrophs capable of mobilizing Cu with an external acquisition mechanism. Methanobactin was found to be unstable under pH conditions below 5, suggesting that methanotrophs in acidic peat bogs, for instance, do not produce methanobactin. In addition, such a high affinity Cu acquisition system may not be necessary in acidic environ-
ments as Cu availability increases with decreasing pH and, thus, methanobactin production is more beneficial under slightly acid and circumneutral conditions. In cold environments, such as northern wetlands and tundra soils, methanobactin-induced Cu mobilization may be hindered due to the slow kinetics of ligand exchange at lower temperature (Chapter 4). Interestingly, the only methanotroph known not to produce pMMO, *Methylocella*, prevails in these environments [Chi Fru, 2011, and citations therein]. Thus, indices about ecosystems where methanobactin may be potentially released for Cu acquisition can be gained from our knowledge of the chemical properties of methanobactin.

In the present dissertation, Cu binding by methanobactin (Chapter 3) and the potential of methanobactin for Cu acquisition from natural organic matter (Chapter 4) have been investigated. Latter experiments confirmed the ability to predict complexation by methanobactin using the established speciation model. Yet in natural systems, the presence of various other metals influences the binding properties of methanobactin. In order to build up a more comprehensive understanding of the biogeochemical functions of chalkophores, the specificity of methanobactin for Cu binding and ligand exchange reactions with Cu-humic substances have to be assessed in the presence of competing cations such as, e.g., iron and zinc. The determination of the reaction rates of the first phase of the ligand exchange reaction between Cu-humic acid and methanobactin was complicated by the analytical limitations of the used setup (Chapter 4). The importance of the first phase of the ligand exchange reaction, however, increases with the lower temperatures of many natural environments and efforts ameliorating the accuracy of the reaction rates should be promoted. Furthermore, investigations allowing a wider range of the relative concentrations of all reagents are needed to enhance our understanding of the mechanistic pathways of the ligand exchange reaction. Improvements may be obtained by applying rapid mixing methods or by using voltammetric techniques. Additional information about the efficiency of methanobactin for Cu acquisition from natural organic matter could be gained by assessing the influence of Cu availability controlled by humic substances and of methanobactin on cell growth of methanotrophs and on sMMO and pMMO activity. The determination of methanobactin concentrations produced by methanotrophs in the presence of
Cu sources predominant in the environment, such as Cu-humic acid, would provide valuable information for further predictions. Attempts to model the kinetics of ligand exchange and the complexation of methanobactin in equilibrium are restricted due to unknown concentrations of methanobactin in natural systems. Up to now, methanobactin has never been detected in the environment, a gap that needs to be filled.
References


A. Supporting Information to Chapter 2

Isolation and purification of Cu-free methanobactin from *Methylosinus trichosporium* OB3b
A.1. Chromatograms of isolated methanobactin

Figure A.1.: The stability of isolated methanobactin was assessed with HPLC analysis. Freeze-dried isolates (fraction 3 of resin extracts) were dissolved in deionized water and analyzed using the HPLC procedure which was used for fractionation of resin extracts. Absorbance of eluates is shown for copper-free methanobactin at (A) 390 nm and (B) 280 nm and for methanobactin after stabilization with CuCl\textsubscript{2} at (C) 390 nm and (D) 280 nm.
B. Supporting Information to Chapter 3

Copper complexation of methanobactin isolated from *Methylosinus trichosporium* OB3b:
pH-dependent speciation and modeling
Appendix B. Supporting Information to Chapter 3

B.1. Determination of methanobactin concentration

The method applied to determine the concentration of methanobactin (mb) stock solutions has previously been described by El Ghazouani et al. [2011]. All solutions were prepared under a N$_2$ atmosphere (O$_2$ < 1 ppm) in a glovebox (Braun) with anoxic deionized water. Cu(I) solution was prepared by dissolving tetrakis(acetonitrile)copper(I)-hexafluorophosphate ([Cu(CH$_3$CN)$_4$]PF$_6$) in de-oxygenated acetonitrile and subsequently diluted in 20 mM HEPES buffer at pH 7.5. The concentration of the Cu(I) solution was checked by atomic absorption spectrometry. Cu(I) was added to a quartz cuvette containing an mb solution in the presence of bicinchoninic acid (BCA) in 20 mM HEPES at pH 7.5. For each titration step, Cu was added inside the glovebox and the solution was equilibrated for 10 min before UV–vis spectra were recorded from 220 to 650 nm with a spectrophotometer (Cary50 Bio, Varian). As mb binds Cu(I) much stronger than BCA, the added Cu is bound to mb up to a Cu to mb ratio of 1. Further Cu additions result in the formation of [Cu(BCA)$_2$]$^{3-}$ complexes which have a strong absorption at 562 nm. Thus, the concentration of Cu(I) added to the solution before the onset of the absorption increase at 562 nm corresponds to the concentration of mb in solution (Fig. B.1). A molar extinction coefficient of 7.8 mM$^{-1}$ cm$^{-1}$ was used to calculate the concentration of [Cu(BCA)$_2$]$^{3-}$ [El Ghazouani et al., 2011].
B.1 Determination of methanobactin concentration

Figure B.1.: Determination of methanobactin concentration. Formation of the $\text{[Cu(BCA)\textsubscript{2}]^{3+}}$ complex upon Cu(I) additions to a mixture of 16 µM methanobactin and 50 µM BCA in 20 mM HEPES at pH 7.5.
B.2. Proton and Copper binding by methanobactin: Additional figures

Figure B.2.: UV–vis absorption spectra of Cu-free methanobactin from pH 4 to 11 in 0.01 M NaCl. Arrows indicate the direction of the spectral change with increasing pH.
Figure B.3.: Cu-methanobactin speciation in 0.01 M NaCl at 25 ±1 °C as a function of Cu^{2+} activity (A) at pH 6 and (B) at pH 7 as calculated with the optimized stability constants given in Table 3.1.
C. Supporting Information to Chapter 4

Competitive ligand exchange between Cu-humic acid complexes and methanobactin
C.1. Kinetics of ligand exchange: Additional figures

Figure C.1.: UV–vis absorption at 392 nm of a 20 µM methanobactin (mb) solution titrated with CuCl₂ at pH 6. A linear decrease in absorption per mol Cu-methanobactin (ΔAbs/mb-Cu) of -0.011 was observed up to molar Cu-to-mb ratio of 1.1.
Figure C.2.: Half-lives for ligands exchange reactions as a function of Cu activity at pH 8 (squares) and pH 6 (diamonds) for $k_1$ (A) and $k_2$ (B). Dashed lines solely serve to illustrate the trends.
Appendix C. Supporting Information to Chapter 4

Figure C.3.: Temperature-dependence of the ligand exchange reaction rate $k_2$. Based on the values of $k_2$, an activation energy $E_a$ of 116 kJ mol$^{-1}$ and a pre-exponential factor $\ln A$ of 42.5 were determined for the second exchange phase by linear regression using the Arrhenius equation

$$\ln k = \ln A - \frac{E_a}{R} \cdot T^{-1}$$

where $R$ is the gas constant and $T$ the absolute temperature.

$slope = \frac{E_a}{R}$
C.2. Ligand exchange in equilibrium: Additional figure

Figure C.4.: Separation of Cu-free and Cu-bound methanobactin (mb) complexes by size-exclusion chromatography. The absorbance of eluates at 280 nm is plotted as a function of elution time. The results are taken from Pesch et al. [2012].
References

D. Cultivation of Methylosinus trichosporium OB3b and methanobactin extraction
Appendix D. Cultivation of *Methylosinus trichosporium* OB3b and methanobactin extraction

**D.1. Methylosinus trichosporium OB3b cultures**

*Methylosinus trichosporium* OB3b cells were grown at 30°C on agar plates containing 15 g L⁻¹ agar, nitrate minimal salts medium (NMS) [Whittenbury et al., 1970], and 20 µM CuCl₂. The composition and preparation procedure of NMS are detailed in Table D.1. The inoculated agar plates were kept in a vented container (GasPak™ 100 Systems, BD Diagnostics). Half of the air volume was removed from the container with a vacuum pump and replaced with methane (Methan 4.5, PanGas). The gas phase was exchanged at least every third day.

**D.2. Methanobactin production and extraction**

50 mL NMS with 0.2 µM were inoculated with cells from the culture plates in a 250 mL Erlenmeyer flask. The cells were grown in a mixture of air and methane in a ratio of 1:1 (v/v) at 30°C and continuously shaken on a horizontal shaker at 100 rpm. After 24 hours, the 50 mL batch was transferred to 100 mL NMS in a 500 mL Erlenmeyer flask and grown for 48 hours. The gas phase was daily exchanged.

Two flasks with cell cultures showing visible cell growth were used to inoculate the final NMS media in a BIOSTAT® A plus bioreactor system (Sartorius). The cultures were grown in batch mode at 30°C, continuously stirred at 250 rpm and purged with a mixture of air and methane in a ratio of 2:1 (v/v) at a flow rate of 60 mL min⁻¹. A pH of 6.8 and total copper concentrations of 0.2 µM were maintained to obtain maximum methanobactin yield. Subsamples of the cultures were daily taken from the bioreactor to measure the optical density of the culture at a wavelength of 600 nm with a UV–vis spectrometer (Cary50 Bio, Varian). When the cultures reached an optical density of 0.8–1 at a wavelength of 600 nm, 90% of the culture was harvested and replaced by fresh medium. The harvested medium was centrifuged twice at 9000 g for 30 min and immediately vacuum-filtered through a 0.2 µm PTFE filter (Millipore) combined with a glass microfiber pre-filter (Whatman®) to remove the cells. The filtrate was then loaded onto a 4 x 30 cm Diaion HP-20 column (Supelco). Subsequently, the column was washed with two column volumes of deionized water prior to elution with 60% methanol. The eluate was freeze-dried immediately.
D.3. Column preparation

Prior to use, the resin (Diaion HP-20, Supelco) was washed with repeated soxhlet extraction for 4 weeks with acetonitrile and rinsed with high-purity deionized water (MilliQ, Millipore, \( \geq 18.2 \text{ M} \Omega \text{ cm} \)). Before loading the filtered *Methylosinus trichosporium* OB3b NMS medium onto the column, the resin was consecutively rinsed with 1 L of 0.1 M NaOH and 0.1 HCL and then re-equilibrated with high-purity deionized water.
Appendix D. Cultivation of *Methylosinus trichosporium* OB3b and methanobactin extraction

Table D.1.: Composition of the nitrate minimal salts medium as proposed by Whittenbury et al. [1970]. After autoclaving NMS, 10 mL L\(^{-1}\) of the phosphate stock solution (C) and 10 mL L\(^{-1}\) of the vitamin stock solution (D) are added. The trace element stock solution and the vitamin stock solution are filtered-sterilized before adding to NMS. The phosphate stock solution is autoclaved before adding to NMS.

<table>
<thead>
<tr>
<th>A. Nitrate Minimal Salts Medium (NMS)</th>
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<tbody>
<tr>
<td>MgSO(_4) \cdot 7 H(_2)O</td>
<td>1.0 g L(^{-1})</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>1.0 g L(^{-1})</td>
</tr>
<tr>
<td>CaCl(_2) \cdot 2 H(_2)O</td>
<td>0.2 g L(^{-1})</td>
</tr>
<tr>
<td>3.8%(w/v) solution FeEDTA</td>
<td>0.1 mL L(^{-1})</td>
</tr>
<tr>
<td>0.1%(w/v) solution Na(_2)MoO(_4) \cdot 2 H(_2)O</td>
<td>0.5 mL L(^{-1})</td>
</tr>
<tr>
<td>Trace element solution (B)</td>
<td>1.0 mL L(^{-1})</td>
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<table>
<thead>
<tr>
<th>B. Trace Element Solution for NMS</th>
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<tbody>
<tr>
<td>FeSO(_4) \cdot 7 H(_2)O</td>
<td>500 mg L(^{-1})</td>
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<tr>
<td>ZnSO(_4) \cdot 7 H(_2)O</td>
<td>400 mg L(^{-1})</td>
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<tr>
<td>MnCl(_2) \cdot 7 H(_2)O</td>
<td>20 mg L(^{-1})</td>
</tr>
<tr>
<td>CoCl(_2) \cdot 6 H(_2)O</td>
<td>50 mg L(^{-1})</td>
</tr>
<tr>
<td>NiCl(_2) \cdot 6 H(_2)O</td>
<td>10 mg L(^{-1})</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>15 mg L(^{-1})</td>
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<tr>
<td>EDTA</td>
<td>250 mg L(^{-1})</td>
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<th>C. Phosphate Stock Solution for NMS</th>
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<tr>
<td>KH(_2)PO(_4)</td>
<td>26 g L(^{-1})</td>
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<tr>
<td>Na(_2)HPO(_4) \cdot 7 H(_2)O</td>
<td>62 g L(^{-1})</td>
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<tr>
<th>D. Vitamin Stock Solution for NMS</th>
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<tbody>
<tr>
<td>Biotin</td>
<td>2.0 mg L(^{-1})</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.0 mg L(^{-1})</td>
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<tr>
<td>Thiamine HCl</td>
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<td>Ca pantothenenate</td>
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<tr>
<td>Vitamine B12</td>
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<tr>
<td>Riboflavin</td>
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<tr>
<td>Nicotiamide</td>
<td>5.0 mg L(^{-1})</td>
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References

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