Biochimica et Biophysica Acta 1854 (2015) 1853–1859

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Multicopper manganese oxidase accessory proteins bind Cu and heme

Cristina N. Butterfield [a,1], Lizhi Tao [b], Kelly N. Chacón [a,2], Thomas G. Spiro [c], Ninian J. Blackburn [a], William H. Casey [d], R. David Britt [b], Bradley M. Tebo [a,*]

[a] Division of Environmental and Biomolecular Systems, Institute of Environmental Health, Oregon Health & Science University, Portland, OR 97239, United States
[b] Department of Chemistry, University of California, Davis, CA 95616, United States
[c] Department of Chemistry, University of Washington, Seattle, WA 98195, United States
[d] Department of Geology, University of California, Davis, CA 95616, United States

ARTICLE INFO

Article history:
Received 1 July 2015
Received in revised form 24 August 2015
Accepted 27 August 2015
Available online 29 August 2015

Keywords:
Multicopper oxidase
Metallo-subunit
Electron paramagnetic resonance spectroscopy
Type 2 copper
X-ray absorption spectroscopy
Protein oligomerization

ABSTRACT

Multicopper oxidases (MCOs) catalyze the oxidation of a diverse group of metal ions and organic substrates by successive single-electron transfers to O_2 via four bound Cu ions. MnxG, which catalyzes MnO_2 mineralization by oxidizing both Mn(II) and Mn(III), is unique among multicopper oxidases in that it carries out two energetically distinct electron transfers and is tightly bound to accessory proteins. There are two of these, MnxE and MnxF, both approximately 12 kDa. Although their sequences are similar to those found in the genomes of several Mn-oxidizing *Bacillus* species, they are dissimilar to those of proteins with known function. Here, MnxE and MnxF are co-expressed independent of MnxG and are found to oligomerize into a higher order stoichiometry, likely a hexamer. They bind copper and heme, which have been characterized by electron paramagnetic resonance (EPR), X-ray absorption spectroscopy (XAS), and UV–visible (UV–vis) spectrophotometry. Cu is found in two distinct type 2 (T2) copper centers, one of which appears to be novel; heme is bound as a low-spin species, implying coordination by two axial ligands. MnxE and MnxF do not oxidize Mn in the absence of MnxG and are the first accessory proteins to be required by an MCO. This may indicate that Cu and heme play roles in electron transfer and/or Cu trafficking.

1. Introduction

Many metalloproteins that conduct electron transfer and redox processes are composed of oligomeric protein complexes whose subunits play essential roles in function. MoFe-protein from nitrogenase [1–3], β and γ carbonic anhydrase [4–6], ammonia and particulate methane monooxygenase [7,8], ribonucleotide reductase [9], and cytochrome c oxidase [10] are all examples of multi-subunit proteins whose catalytic functions rely on the redox mechanisms of their metallocenters. Elucidating these mechanisms begins with the characterization of individual metal binding centers in isolated subunits.

Multicopper oxidases (MCOs), on the other hand, have previously only been isolated as monomers and homopolymers. MCOs catalyze the oxidation of a variety of substrates, including phenolic compounds and metals, as well as the reduction of O_2 to H_2O [11,12]. They facilitate numerous physiological functions including morphogenesis, stress defense, and lignin degradation [11]. All MCOs have at least four canonical Cu atoms bound within three spectroscopically distinct centers. The three spectroscopically distinct centers are a type 1 center, which is comprised of one S(Cys), one S(Met), and two N(His), a type 2 center with three N(His) ligands, and a type 3 binuclear center which coordinates Cu using two N(His) ligands per Cu atom. Electron transfer commences at the substrate site, proximal to the type 1 Cu. A network of peptide bonds guide electron flow to a trinuclear cluster, composed of one type 2 Cu and a type 3 binuclear center, which are ~9 Å from the type 1 Cu [13]. The final electron acceptor, O_2, binds at the trinuclear center, forming two water molecules after four successive one-electron transfers. MCOs are arranged in two, three, or six domain structures, with the Cu centers being formed by ligands across protein domains. Until the discovery of the Mnx complex in the marine *Bacillus* species PL-12 [14], MCOs were heterologously expressed as single gene products and no MCO required additional subunits for function.

Mnx is a multi-protein complex that is located on the outer spore surface and has been shown to catalyze Mn(II) oxidation and form MnO_2 mineral [15,16]. While the role MnO_2 formation plays in spore function is unclear, a large cellular energy investment is made in order to produce Mnx. The expression of soluble and active Mn oxidase

Abbreviations: MCO, multicopper oxidase; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption spectroscopy; UV–vis, UV visible; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ICP-OES, inductively coupled plasma-optical emission spectrometry; MnxEF, refers the product of the co-expression of genes *mnxE* and *mnxF*; MnxEF3, refers to the H21A, H80A, and H82A modified protein.

* Corresponding author.
E-mail address: tebob@ohsu.edu (B.M. Tebo).
[1] Present affiliation: Department of Earth and Planetary Science, University of California, Berkeley, CA 94720, United States.
[2] Present affiliation: Reed College, Department of Chemistry, Portland, OR 97202, United States.

http://dx.doi.org/10.1016/j.bbapap.2015.08.012
1570-9639/

requires the co-expression of at least three genes on the *mnx* gene cluster: *mnxG*, the multicopper oxidase (MCO), and *mnxE* and *mnxF*, small proteins of unknown function [14]. The three proteins co-purify into a complex of roughly 230 kDa in size, and reported to bind anywhere from 6.4–15 Cu atoms per protein complex [14,17]. Like all MCOs, MnxG contains a type 1 Cu and carries out substrate oxidation, but since no other MCO requires the co-expression of subunits, the specific roles of MnxE and MnxF have been unclear.

Characterizing the metal binding features of the Mnx complex subunits is essential to piecing together its mechanism. The MnxE and MnxF subunits are small, ~12 kDa proteins and are well-conserved among the Mn oxidizing *Bacillus* spp. that have been sequenced, yet they do not contain any conserved domains from which one could predict function. MnxF is reported to possess a potential Cu-binding site [16]; there are several histidine and cysteine residues in MnxE and MnxF that could potentially bind Cu. Importantly, the MCO MnxG cannot be expressed in a soluble active form if it is not synthesized with MnxE and MnxF in the cell, but the latter can be separately purified as an oligomer, MnxEF. We made site-directed mutations at three potential Cu binding sites and utilized electron paramagnetic resonance (EPR), X-ray absorption spectroscopy (XAS), and UV–visible spectrophotometry (UV–vis) to characterize wild type and mutant MnxEF and the complete MnxG(EF) complex. Herein, we characterize the metal cofactors associated with the MnxEF accessory proteins, which provides insights into their possible function in the larger MnxEFG complex and implicates their role as integral metal-facilitated redox partners that contribute to electron transfer during Mn oxidation.

2. Methods

2.1. Alignments

Alignments were carried out with MUSCLE with default parameters on the following URL http://www.ebi.ac.uk/Tools/msa/muscle/ [18,19] with NCBI accession numbers MnxE PL-12 ABP68888, MB-7 ABP68897, and SG-1 EDL64242 and MnxF PL-12 ABP68889, MB-7 ABP68898, and SG-1 EDL64243.

2.2. Cloning and mutagenesis

mnxE and *mnxF* (NCBI accession EF158106) were amplified from *Bacillus* sp. PL-12 (Taxonomy ID: 161537) genomic DNA by the following primers: Fwd 5′-**CCGCGG**TATGCATGACTCGCCATT-3′ and Rvs 5′-**GTCG AC**ATAGTCTTCGAGCTTCG-3′. *mnxDEFG* were amplified by the following primers: Fwd 5′-**CCGCGG**TATGCGTCATTCGGATTATTTGAAAAATTTGT-3′ and Rvs 5′-**GTCGAC**TGCCTTTTCTTCATTGTCCCACC-3′. These two amplicons were ligated into pJet1.2 (Thermo) entry vectors, then cloned by restriction enzyme digestion and ligation into the *Strep*-tag pASK/IBA3plus expression vector using SacII and SalI (sequences in bold). In place of the *mnxF* or *mnxG* stop codon the *Strep*-tag (underlined) was engineered to a linker (italicized) at the C-terminus (*VDLQGDHGLSA*WSHPQFEK). Single amino acid mutations were generated in the pJet1.2/*mnxDEFG* construct with the Stratagene QuickChange® site-directed mutagenesis kit (Agilent). MnxF H21, 80, and 82 were changed to Ala. Then the *mnxEF* H21/80/82A mutant or *mnxDEFG* H21/80/82A were PCR amplified from the pJet1.2/*mnxDEFG* construct and cloned into an empty pJet1.2 plasmid and moved to the pASK/IBA3plus vector as described above.

2.3. Gene expression, in vivo Cu loading, and protein purification

The resulting constructs were transformed into *Escherichia coli* BL21 (DE3) and grown at 37 °C to an OD_{600} ~ 0.5 in Luria–Bertani (LB) broth containing 0.2 mM $CuSO_4$, 10 mM Tris–HCl pH 7.5, and 100 mg/l ampicillin. The temperature was then lowered to 17 °C by cooling the culture on ice or in a refrigerated shaker (for *mnxDEFG* expression) or kept at 37 °C (for *mnxEF* expression) and then 0.2 mg/l anhydrotetracycline was added to induce transcription of the *mnx* genes. The cells continued to shake and express for 16–20 h (for *mnxDEFG*) or for 3 h (for *mnxEF*). $CuSO_4$ was added to a final concentration of 2 mM and the shaking function was stopped for at least 22 h to allow for the microaerobic uptake of Cu ions into the *E. coli* cytoplasm as described in Durao et al. [20]. This Cu loading step was omitted during the protein preparation to obtain the "partially loaded" protein.

The cells were harvested by centrifugation 5000 ×*g* 4 °C 10 min, suspended in *Strep*-Tactin wash buffer (100 mM Tris pH 8.0, 150 mM NaCl) amended with 1 mM $CuSO_4$ and an EDTA-Free SIGMA*FAST*™ Protease Inhibitor Cocktail Tablet, and lysed using a sonication microtip for 1 min/ml cell lysate at 40% amplitude with 10 s on/off pulses on ice. The cell debris was pelleted by centrifugation 15,000 ×*g* 4 °C 30 min and the supernatant was filtered through a 0.4 μm pore PVDF filter. The clarified lysate was then added to 1 ml (for MnxEF protein) or 5 ml (for MnxDEFG protein) column volume (CV) of *Strep*-Tactin Superflow Plus (Qiagen). By peristaltic pump (~1 ml/min flow rate), the unbound protein fraction was removed and the resin was washed with 20 CV Streptactin wash buffer. The protein was eluted with 5 CV wash buffer plus 2.5 mM D-Desthiobiotin and the column was regenerated with 15 CV wash buffer plus 1 mM 2-(4-hydroxyphenylazo) benzoic acid. The eluted protein was concentrated to <1.5 ml on 50 kDa (for MnxEF) or 100 kDa (for MnxDEFG) molecular weight cutoff filtration units (Millipore). The protein was then dialyzed in 50 mM Tris–HCl pH 8 and decreasing NaCl concentrations from 150 to 50 mM NaCl and flash frozen with 20% ethylene glycol for future analyses. The protein was quantified by the Thermo Scientific Pierce bicinchoninic acid (BCA) protein assay.

2.4. Mass spectrometry

Purified protein was run on Tris Glycine 4–15% sodium dodecyl sulfate polyacrylamide electrophoresis gel (Bio-Rad) and stained in Imperial protein stain (Pierce). The protein band that migrated to 12 kDa according to the PageRuler™ Unstained Protein Ladder (Thermo) was excised by a clean razor blade and submitted to the Oregon Health & Science University Proteomics Shared Resource Center for analysis with the *Bacillus* sp. PL-12 *mnxE* and *mnxF* triple mutant sequences. Mass spectrometric analysis was performed as previously described in Butterfield et al. 2013 [14].

2.5. UV–visible spectroscopy

The UV–visible spectra were collected on a SpectraMax M2 in a 50 μl 1 cm path length quartz cuvette.

2.6. Electron paramagnetic resonance (EPR) spectrometry

X-band continuous wave electron paramagnetic resonance (CW EPR) spectra were recorded using a Bruker (Billerica, MA) Biospin EleXsys E500 spectrometer equipped with a cylindrical TE011-mode resonator (SHQE-W). Cryogenic temperatures were achieved and controlled using an ESR900 liquid helium cryostat in conjunction with an Oxford Instrument temperature controller (ITC503) and gas flow controller. All CW-EPR data were collected under non-saturation and slow-passage conditions. The spectrometer settings used were as follows: microwave frequency = 9.374 GHz, microwave power = 0.2 mW, conversion time = 40 ms, modulation amplitude = 8 G, and modulation frequency = 100 kHz. EasySpin computational package was used to simulate the EPR data [21].

2.7. Mn oxidation activity assay

Protein was routinely purified to a concentration of about 5–10 μM in 3 ml before concentrating. A small aliquot of purified protein was