FEBS Letters 583 (2009) 1189–1195

FEBS Letters

journal homepage: www.FEBSLetters.org

X-ray structure of a two-domain type laccase: A missing link in the evolution of multi-copper proteins

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ARTICLE INFO

Article history:
Received 19 December 2008
Revised 2 March 2009
Accepted 4 March 2009
Available online 11 March 2009

Edited by Hans Eklund

Keywords:
Crystal structure
Laccase
Metagenome
Molecular evolution
Multi-copper protein
Nitrite reductase

ABSTRACT

A multi-copper protein with two cupredoxin-like domains was identified from our in-house metagenomic database. The recombinant protein, mgLAC, contained four copper ions/subunits, oxidized various phenolic and non-phenolic substrates, and had spectroscopic properties similar to common laccases. X-ray structure analysis revealed a homotrimeric architecture for this enzyme, which resembles nitrite reductase (NIR). However, a difference in copper coordination was found at the domain interface. mgLAC contains a T2/T3 tri-nuclear copper cluster at this site, whereas a mononuclear T2 copper occupies this position in NIR. The trimer is thus an essential part of the architecture of two-domain multi-copper proteins, and mgLAC may be an evolutionary precursor of NIR.

1. Introduction

Copper is a metal essential for life, and copper-containing proteins play various roles in biosystems, such as in electron transfer (e.g., azurin and plastocyanin), oxygen transfer (e.g., hemocyanin), and catalysis (e.g., laccase, ascorbate oxidase, tyrosinase, and galactose oxidase). These proteins use cupredoxin-like domains as structural units. The number of domains in a subunit, and the pattern of subunit assembly, varies from protein to protein, which, together with point mutations, appear to promote the functional evolution of copper proteins [1]. For example, ascorbate oxidase (AO) is a monomeric protein consisting of three cupredoxin domains [2]. Ceruloplasmin (CP) is also monomeric, but contains six cupredoxin domains [3]. Nitrite reductase (NIR) is composed of three identical subunits, each of which contains two cupredoxin domains [4]. Each cupredoxin domain contains a single copper-binding motif, in which various types of copper ions are included. Copper is classified into types I, II, and III (T1, T2, and T3) based on spectroscopic properties. T1 copper has an absorption peak at ~600 nm and a narrow hyperfine coupling in EPR (electron paramagnetic resonance) spectroscopy. This copper is generally coordinated by three strong ligands (one cysteine and two histidines) and one weaker ligand, typically methionine. In some fungal laccases, the methionine is replaced by non-ligating residues leucine or phenylalanine. T2 copper has a much weaker absorption, broader hyperfine interactions, and is generally coordinated with 4–5 histidines and water–oxygen ligands. T3 is a copper pair, antiferromagnetically coupled, usually coordinated by three histidines per copper and a bridging moiety [5]. High conservation of copper-binding motifs in cupredoxin domains allows for easy identification of copper proteins based solely on the amino acid sequence.

Taking advantage of the rapid accumulation of bacterial genomic sequences, Nakamura et al. [6] identified numerous protein sequences that possess cupredoxin-like domains. Based on the topology of the copper-binding motifs, they classified the proteins into several categories and proposed a possible evolutionary scheme, as shown in Fig. 1. In addition to well-characterized proteins, such as NIR, AO, and CP, there are numerous other proteins, designated as [X], [Y], [A], [B], and [C] in Fig. 1. Most of these

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.03.008

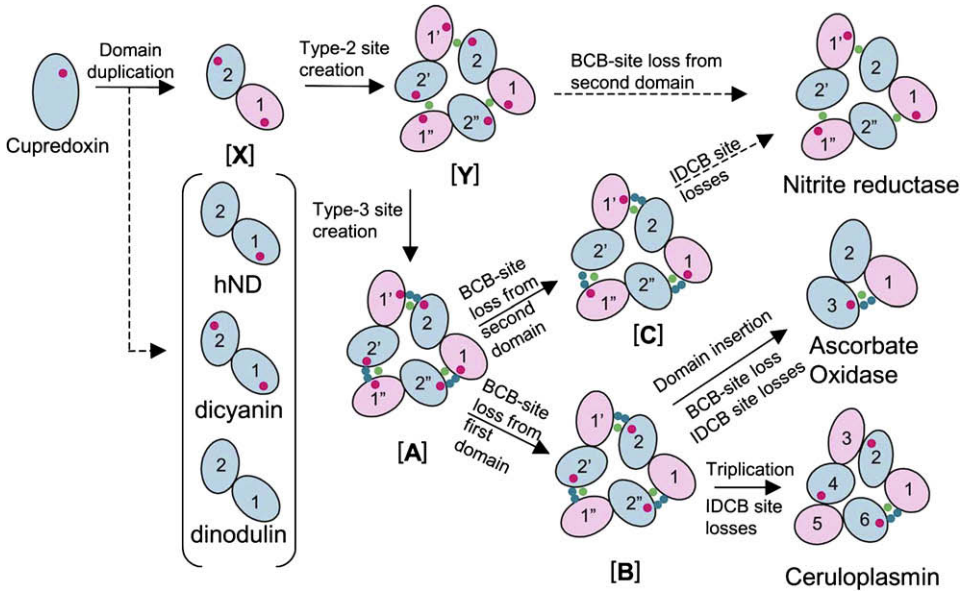

Fig. 1. Schematic representation of the domain organization of copper proteins and their hypothetical common ancestors. This figure was taken from Fig. 2 in Nakamura et al. [6] with the authors' permission.

proteins varied between simple cupredoxin to well-established, multi-domain proteins. Although these "missing links" are uncharacterized, some of them have been studied. SLAC [7] and EpoA [8,9] are type [B] proteins composed of two cupredoxin domains. These proteins were identified in *Streptomyces* and have laccase activity. Recently, the crystal structure of SLAC was determined [10,11]. The enzyme formed trimer and resembled NIR [4] and CP [3]. A type [C] protein has also been reported. More than 20 years ago, a "blue copper oxidase" was purified from *Nitrosomonas europaea* [12]. Biochemical studies revealed that the enzyme was trimeric and displayed both laccase and NIR activities. Recent genetic studies [13,14] have confirmed that the enzyme is a type [C] protein.

In this paper, we report the crystal structure of a novel type [C] laccase, designated mgLAC, which was identified from our in-house metagenomic sequence database [15–18]. We determined the three-dimensional structure of mgLAC to better understand the molecular basis for the evolution of multi-copper proteins.

2. Materials and methods

2.1. Enzyme activity assays

mgLAC was prepared as previously described [18]. Enzyme activities were measured from the decrease in the concentration of dissolved molecular oxygen at 25 °C in McIlvaine buffer (pH 4.5 for ABTS and 8.5 for others), containing 1 mM substrate on a PreSens oxygen meter (Microx TX3).

2.2. Spectroscopic measurements

The UV–visible absorption spectrum of mgLAC (~13 μM in 20 mM Tris–HCl, pH 8.0) was recorded at 25 °C on a Jasco UV/VIS spectrophotometer (model V-550). The EPR spectrum was recorded on a Bruker ESP350E spectrometer at 77 K using 0.13 mM enzyme in 20 mM Tris–HCl (pH 8.0). The inductively coupled plasma-atomic emission spectrometry (ICP-AES) measurement was carried out using 0.13 mM enzyme in 20 mM Tris–HCl (pH 8.0) on a SII NanoTechnology SPS4000.

2.3. Bioinformatics tools

A BLAST search was carried out in the non-redundant database of GenBank, via the internet at http://www.ncbi.nlm.nih.gov/BLAST/ [19]. Multiple sequence alignment was carried out using a web-based version of ClustalW (http://www.ebi.ac.uk/clustalw/), with default parameter settings [20]. To analyze protein domain structures, the simple modular architecture research tool (SMART) was used (http://smart.emblheidelberg.de/) [21]. For identification of signal peptides, the SignalP facility in SMART was used [22].

2.4. X-ray crystallographic analysis

Crystallization and preliminary X-ray crystallographic results are described as previously noted [18]. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters of $a = 74.67$, $b = 100.95$, and $c = 124.11$ Å. The structure was solved by the single-wavelength anomalous diffraction (SAD) technique using Cu atoms. The heavy-atom refinement, density modification, and initial structure modeling were performed using autoSHARP [23]. Further model building and structure refinement were carried out using the COOT [24] and refmac [25] programs. The progress and validity of the refinement process were checked by monitoring the *R*-free value for 5% of the total reflections [26]. Model geometry was analyzed using the MOLPROBITY program [27]. The data collection and refinement statistics are summarized in Table 1. The root-mean-square deviation was calculated using the LSQMAN program [28]. Domain interfaces were analyzed using the Protein–Protein Interaction Server [29]. The figures were prepared by the PyMOL program (http://pymol.sourceforge.net), using the coordinates from PDB files 2BW4 (NIR from *Achromobacter cycloclaster*), 1AOZ (AO from *Cucurbita pepo* var. *melopepo*), and 2J5W (CP from human).

3. Results and discussion

3.1. Identification of a two-domain laccase in a metagenomic library

In silico screening of our in-house metagenomic sequence database identified an open reading frame containing two copper-binding motifs [18]. The protein, designated mgLAC, is comprised of