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Research Paper

Functional and evolutionary characterization of Ohr proteins in eukaryotes reveals many active homologs among pathogenic fungi

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ABSTRACT

Ohr and OsmC proteins comprise two subfamilies within a large group of proteins that display Cys-based, thiol dependent peroxidase activity. These proteins were previously thought to be restricted to prokaryotes, but we show here, using iterated sequence searches, that Ohr/OsmC homologs are also present in 217 species of eukaryotes with a massive presence in Fungi (186 species). Many of these eukaryotic Ohr proteins possess an N-terminal extension that is predicted to target them to mitochondria. We obtained recombinant proteins for four eukaryotic members of the Ohr/OsmC family and three of them displayed lipoyl peroxidase activity. Further functional and biochemical characterization of the Ohr homologs from the ascomycete fungus *Mycosphaerella fijiensis* Mf_1 (MfOhr), the causative agent of Black Sigatoka disease in banana plants, was pursued. Similarly to what has been observed for the bacterial proteins, we found that: (i) the peroxidase activity of MfOhr was supported by DTT or dihydrolipoamide (dithiols), but not by β-mercaptoethanol or GSH (monothiols), even in large excess; (ii) MfOhr displayed preference for organic hydroperoxides (CuOOH and *t*BOOH) over hydrogen peroxide; (iii) MfOhr presented extraordinary reactivity towards linoleic acid hydroperoxides ($k = 3.18 (\pm 2.13) \times 10^8\ M^{-1}\ s^{-1}$). Both Cys^{87} and Cys^{154} were essential to the peroxidase activity, since single mutants for each Cys residue presented no activity and no formation of intramolecular disulfide bond upon treatment with hydroperoxides. The pK_a value of the Cys_p residue was determined as 5.7 ± 0.1 by a monobromobimane alkylation method. Therefore, eukaryotic Ohr peroxidases share several biochemical features with prokaryotic orthologues and are preferentially located in mitochondria.

1. Introduction

Organic hydroperoxide resistance (Ohr) proteins are Cys-based, thiol dependent peroxidases that belong to a family of proteins called Ohr/OsmC. OsmC (Osmotically inducible protein) are structurally related to Ohr enzymes [1] and together define two subfamilies that have their peroxidase activities well characterized within Ohr/OsmC family [2–4]. Members of a third group remain poorly characterized [1].

The physiological role played by Ohr and OsmC has been linked to the defense against organic hydroperoxide insults [1,5–11]. Ohr and OsmC are structurally distinct from peroxiredoxin (Prx) and glutathione-peroxidase (Gpx) enzymes [1,2], although all are Cys-based, thiol dependent peroxidases. While Prx and Gpx enzymes are ubiquitously distributed in all domains of life, Ohr/OsmC proteins were thought to be present only in Archaea and Eubacteria [2,6]. Contrary to Ohr enzymes, most Prx enzymes are highly reactive towards H_2O_2. One exception is Tpx from *E. coli* that similarly to Ohr enzymes also display higher specificity to organic peroxides over H_2O_2 [12], although the large majority of these peroxiredoxins are found in bacteria [13].

The catalytic mechanism of hydroperoxide reduction by Ohr and OsmC proteins is centered on a pair of redox-active cysteines, named peroxidatic (C_p) and resolving (C_r) cysteines, resembling that of the atypical 2-Cys Prxs. Ohr and OsmC are functionally dimeric and the cysteine residues are positioned in each monomer as part of two identical active sites located at opposing sides [2]. Two other residues of the active site also participate in catalysis: an arginine (Arg) and a glutamic acid (Glu) [3,14]. The peroxidase cycle starts with the nucleophilic attack of Cys_p towards the hydroperoxide. Upon hydroperoxide reduction to its corresponding alcohol, the C_p is oxidized to the sulfenic acid (Cys-SOH) intermediate, which readily reacts with C_r, giving rise to an intramolecular disulfide bond [14]. A new cycle begins

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when the disulfide bond is reduced back to the dithiolic form. Lipoyl groups covalently attached to some proteins are the biological reductants of these intramolecular disulfides [4]. Recently, we demonstrated that Ohr enzymes display high specificity for fatty acid hydroperoxides and peroxynitrite as oxidizing substrates [11].

Here, based on an in-depth sequence analysis, we describe the occurrence and distribution of Ohr and OsmC peroxidases in the Eukarya domain. OsmC proteins were only found in Dictyostelia, whereas Ohr members are predominantly present in Fungi (mainly Ascomycota and Basidiomycota). Four recombinant eukaryotic proteins from the Ohr/OsmC family were purified, three of which displayed thiol peroxidase activity. One of these, namely Ohr from the ascomycota fungus *Mychosphaerella fijiensis* Mf_1 (MfOhr), was further characterized, and its presence in the mitochondria of this fungus was demonstrated.

2. Material and methods

2.1. Dataset source and sequence extraction

The amino acid sequence from *Xylella fastidiosa* 9a5c strain was used as query for search against NCBI *nr* sequence database using delta-BLAST via NCBI website [15] (June of 2016) and the profile Hidden Markov Model (HMM) iterative method implemented in Jackhmmer 1.9 web server [16]. We conducted the search against NCBI *nr* sequence database using default options until convergence. The searches were restricted to the Eukarya Domain. Redundant entries and truncated sequences (less than 100 amino acids) were removed using CD-HIT software [17].

2.2. Primary sequence clustering

We identified members of the Ohr and OsmC subfamilies in Eukarya using sequence motifs previously described [1,6]. Additional motifs were detected using alignments of eukaryotic Ohr sequences with structurally solved Ohr (PDB: 1ZB8, from *X. fastidiosa*; 1USP, from *Deinococcus radiodurans*; 3LUS, from *Vibrio cholerae*; 1N2F, from *Pseudomonas aeruginosa*) or OsmC (1NYE, from *Escherichia coli*) proteins. We curated the alignments manually, guided by successive multiple alignments runs generated by MAFFT operating with default sets [18]. The input sequences were collected by delta-blast and jackhmmer searches described in the previous section.

2.3. Phylogenetic analysis

Maximum Likelihood (ML) inference of phylogenetic trees was based on the manually curated MAFFT alignment and the RAxML software [19] and applied to all non-redundant sequences retrieved or only sequences from the Ohr subfamily. For inference, we used Whelan-Goldman (WAG) model of amino acid evolution with rate heterogeneity modeled by a GAMMA distribution and 1000 rapid bootstrap resampled estimates of log-likelihood (RELL bootstrap). The resulting phylogeny was prepared for visualization using Tree Editor from the MEGA 7 software [20].

2.4. Strains and growth conditions

E. coli strains were grown in Lysogenic Broth (LB) medium at 37 °C supplemented with ampicillin (100 μg/mL). *Mycosphaerella fijiensis* Mf_1 was grown in Potato Dextrose Medium (PDB) at room temperature supplemented with streptomycin (100 μg/mL) and chloramphenicol (100 μg/mL). *Dictyostelium discoideum* AX4 cells were grown axenically in liquid maltose HL-5 modified medium [21] supplemented with ampicillin (100 μg/mL) and streptomycin (300 μg/mL) at 22 °C.

2.5. Cloning procedures

To amplify *ohr* (MYCFIDRAFT_54770) and *osmC* (DDB_G0268884) genes without introns, samples of total RNA from germinated conidia of *M. fijiensis* Mf-1 and *D. discoideum* AX4 cells, respectively, were extracted using Trizol reagent (Ambion). RNA samples were treated with RNase-Free DNase I (Ambion) and submitted to reverse transcription (SuperScript II) using Oligo-dT to produce cDNA. To clone *Mfohr* into pET15b (Novagen®) and *DdosmC* into pPROEX expression vectors, sequences were amplified from appropriated cDNAs by PCR using the oligo pairs (5′→3′): Fow_TTAG**CATATG**GCTTCCGTAAGAGCATTC/Rev_TTAG**GGATCC**CGTCCCGCTCTATCCAATAA and Fow_AGT**CATATG**AGCATTAGTAATAAAATTATTGGATCAGC/Rev_AGT**GGATCC**CAAAAACAAATGGTGAGAAATCTG, respectively. The restriction sites for *Nde*I and *Bam*HI are depicted by bold letters. Additionally, for *Mfohr* gene, a second PCR was performed using the same conditions described above using forward oligo (5′→3′) TTAG**CATATG**TCCCCGCCATTCTACACAGCCCAT, to produce a version of the protein MfOhr without the first 33 amino acid residues ($MfOhr_{del}$). The *ohr* gene from *Fusarium oxysporum f.* sp. *cubense* (*Foohr*) and Ohr-like (named as *osmC* gene by [22]) from *Trichomonas vaginalis* (*TvosmC*) were commercially synthesized by GenScript USA Inc., containing the sites for *Nhe*I and *BamHI* restriction enzymes in the flanking regions. The fragments that corresponded to the *Foohr* and *Tvosmc* genes were digested from pUC57 using *Nde*I and *Bam*HI restriction enzymes and subcloned into pET15b. Fidelity of all sequences was checked by chain termination sequencing method using T7 promoter and terminator oligonucleotides.

2.6. Protein purification

Expression of recombinant MfOhr, $MfOhr_{del}$, FoOhr, DdOsmC or TvOsmC was induced by 0.1 mM of isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 16 h at 20 °C in exponential culture (OD_{600} 0.5) of *E. coli* BL21 (*DE3*) CodonPlus (Agilent) harboring the appropriate expression vectors with moderate shaking. Then, cells were harvested by centrifugation and resuspended in the lysis buffer (500 mM NaCl, 20 mM sodium phosphate pH 7.4, 0.2 mg/mL lysozyme, 1 mM PMSF and 20 mM imidazole). Cells were disrupted by sonication (ten alternating cycles of 15 s of sonication 30% amplitude and 1 min on ice bath). Cell debris were separated from the supernatant by centrifugation at 15.000 rpm at 4 °C during 40 min. The supernatant was filtered using a 0.45 μm pore membrane and all expressed proteins were affinity purified (Ni-NTA Agarose column, Qiagen) with a peristaltic pump. The charged resin was washed sequentially with 3 column volumes of washing buffer (500 mM NaCl, 20 mM sodium phosphate pH 7.4) containing 50 mM and 100 mM imidazole and eluted with 3 column volumes of elution buffer (500 mM NaCl, 20 mM sodium phosphate pH 7.4 and 500 mM imidazole). Buffer exchange and concentration of purified proteins were performed in an Amicon Centrifugal 10 MW device (Millipore®). Protein purity was checked by SDS-PAGE and protein concentration was spectrophotometrically determined by its absorbance at 280 nm (for MfOhr, ε_{ox} = 9970 and ε_{red} = 10,095; for $MfOhr_{del}$, ε_{ox} = 4595 and ε_{red} = 4470 M^{-1} cm^{-1}; for FoOhr, ε_{ox} = 11,585 and ε_{red} = 11,460 M^{-1} cm^{-1}, for DdOsmC, ε_{ox} = 10,220 and ε_{red} = 9970 and for TvOsmC, ε_{ox} = 15,720 and ε_{red} = 15,470, according to ProtParam tool [23]).

2.7. Reduction of peroxidases with DTT

In some assays, Cys-based peroxidases ($MfOhr_{del}$ or AhpE) were pre-reduced by 50 mM of DTT for 16 h at 4 °C, in the presence of 500 mM NaCl and 20 mM sodium phosphate pH 7.4. Excess of DTT was eliminated by two rounds of size-exclusion chromatography (HiTrap Dessalting, GE HealthCare) in a buffer (500 mM NaCl and 20 mM sodium phosphate pH 7.4) previously purged with N_2. The efficiency of this procedure was ascertained by the DTNB method [24].