Ostreopixin: A hemopexin fold protein from the oyster mushroom, Pleurotus ostreatus

Katja Ota a, Miha Mikelj a, Tadeja Papler a, Adrijana Leonardi b, Igor Križaj b,c,d, Peter Maček a,*

a Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia
b Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia
c Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Alkerčeva 5, SI-1000 Ljubljana, Slovenia
d Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins, Jamova 39, SI-1000 Ljubljana, Slovenia

1. Introduction

Proteins of the hemopexin family (Pfam: PF00045), which was previously known as the “pexin family” [1], have various numbers of hemopexin-like repeats (HX-repeats), and are widely distributed among organisms. The heme-binding glycoprotein hemopexin, vitronectin and extracellular matrix metalloproteinases are the best known representatives of HX-repeat proteins [2–5]. In plants, several hemopexin-like proteins have been discovered in legumes, and most recently in rice [6–11]. These proteins have been suggested to have roles in polyamine metabolism and in the protection from heme-induced oxidative stress in legumes [9,11], and to participate in chlorophyll degradation in rice [11]. The large majority of the hemopexin family members are putative proteins, including photopexins A and B from Photorhabdus fluorescens, the first bacterial proteins predicted to have HX-repeats [12]. Such proteins have also been predicted for fungi; e.g., in Uncinocarpus reesii, Coccidioides immitis, Coccidioides posadasii and Postia placenta. Search of the Joint Genome Institute genomic databases [13] reveals a putative protein (JGI: Protein ID: 1113759) with HX-repeats also in the edible oyster mushroom Pleurotus ostreatus. However, none of these have been described at the protein level. Here, we present the isolation and identification of a native protein from P. ostreatus that has four HX-repeats, which we have named ostreopxin (Opx). We detected this ~26 kDa protein as it co-eluted with HX-repeats also in the edible oyster mushroom Pleurotus ostreatus. Functional studies using recombinant Opx reveal here for the first time that fungi can produce four-bladed hemopexin-like proteins that are very similar to orthologs from plants. We show that Opx binds heme but not polyamines.

2. Material and methods

2.1. Materials

The NdeI and XhoI restriction enzymes, Rapid DNA ligation kits, GeneJET™ PCR purification kits; GeneJET™ gel extraction kits, TransformAid™ bacterial transformation kits, GeneJET™ plasmid miniprep kits, and PageRuler™ prestained protein ladder were from Fermentas (Germany). Thrombin and the plasmid pET1570-9639.$ – see front matter © 2013 Elsevier B.V. All rights reserved.

http://dx.doi.org/10.1016/j.bbapap.2013.03.027
from Novagen (Millipore, USA). Oligonucleotide primers and the gene coding for rOpx-H6 were synthesized by MWG Operon (Germany). PD-10 desalting columns, Mono Q anion-exchange columns and Biacore C5 chips were from GE Healthcare (Sweden). Ni²⁺-NTA-agarose was dissolved in 50 mM Tris–HCl, titrated between 35% and 60% ammonium sulfate saturation was dissolved using solid ammonium sulfate. The protein fraction that precipitated using a Sephadex G-75 column at 4 °C, and eluted with 50 mM Tris–HCl, was centrifuged at 26,323 × g, before and 2 min after the addition of the thrombin cleavage buffer (20 mM Tris–HCl, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.4) on a PD-10 desalting column. After the thrombin cleavage, the sample buffer was exchanged for 20 mM Tris–HCl, pH 8.0, to purify the rOpx on a Mono Q anion-exchange column. The protein was eluted with a 0–100 mM NaCl gradient in the same buffer.

2.5. Protein primary structure analysis

Edman degragation of the intact nOpx from size-exclusion chromatography that had been electro-transferred from the SDS-PAGE gel to a polyvinylidene difluoride membrane (Millipore) was not effective, most likely due to a blocked terminal amino group. Therefore, the protein was isolated first on a C₄-RP HPLC column using a 0% to 90% acetonitrile gradient in 0.1% (v/v) TFA, and then hydrolyzed using sequencing grade trypsin (Sigma, USA). The resulting tryptic peptides were separated on a C₄-RP HPLC column using a 0% to 90% acetonitrile gradient in 0.1% (v/v) TFA. The four most abundant peptides were N-terminally sequenced by automated Edman degradation on a Procise 492A protein sequencing system (Applied Biosystems).

2.6. ESI–MS and LC–MS/MS amino acid sequencing

The proteins were analyzed using a 1200 series HPLC-Chip/LC/MS Trap XCT Ultra mass spectrometer (Agilent Technologies, Germany) and a MALDI-TOF UltraflexExtreme III mass spectrometer (Bruker, USA). Prior to MS analysis, the proteins were additionally purified on a C₄-RP HPLC column using a 0% to 90% acetonitrile gradient in 0.1% (v/v) TFA, reduced, carboxamidomethylated, and fragmented with proteomics grade trypsin (Sigma, USA). Mass spectrometry data were analyzed using Spectrum Mill software Rev A03.03.084 SR4 (Agilent Technologies, Santa Clara, CA, USA) on the NCBI protein database and the JGI raw genome data (P. ostreatus PC15 v2.0) [13]. To align the amino-acid sequences, the on-line ClustalW [17] or MAFFT software [18] was used.

2.7. Modeling of three-dimensional structure

Homology modeling of nOpx and searching for similar three-dimensional (3D) structures were performed with on-line I-TASSER [19], and the figures were rendered using CHIMERA [20].

2.8. Protein–hemin interactions

Interactions of hemin with rOpx or with bovine serum albumin (BSA) as the control, were studied using UV/VIS absorption, steady-state fluorescence spectroscopy, and surface plasmon resonance refractometry.

The absorption spectra (250–480 nm) of both rOpx and BSA (20 μM each, in PBS) before and 2 min after the addition of the final 10 μM Hn.Cl were recorded in a 1-cm light path quartz cuvette using a Shimadzu UV2101PC UV–VIS scanning spectrophotometer.

Steady-state fluorescence measurements were performed on a Jasco FP750 spectrophotometer in a thermostated cell holder at 20 °C, equipped with a magnetic stirrer. The emission and excitation bandwidths were set to 10 nm. The sample volume was 1.5 mL in a 1-cm path length quartz cuvette. Intrinsic fluorescence of rOpx and BSA (2 μM, in 140 mM NaCl, 20 mM Tris–HCl, pH 7.4) was measured by selective excitation of the fluorescence of tryptophan residues at 295 nm wavelength. The emission spectra were recorded in the 300 nm to
450 nm range, before and after 10 min incubations with HnCl (2, 4, 10 μM). In fluorimetric titration experiments, aliquots of 100 μM HnCl in PBS were added to 2 μM rOpx or BSA, to reach final concentrations of 0.2 μM to 18.9 μM HnCl. The fluorescence emission intensity was monitored at a wavelength of 330 nm, at 20 °C. The final HnCl concentrations were corrected for each volume increment. The HnCl quenching constant was evaluated using the Stern–Volmer plot [21]:

\[ F_0/F_{corr} = 1 + K_{SV} \cdot [Q] \]  

(1)

where, \( F_0 \) and \( F_{corr} \) are the fluorescence intensities of the proteins in the absence and presence of a given concentration of the quencher, \([Q]\). \( F_{corr} \) is the fluorescence intensity corrected for the inner filter effect, and \( K_{SV} \) is a Stern–Volmer quenching constant. With respect to the geometry of the measuring cell, the fluorescence intensity was corrected for primary and secondary inner filter effects according to Lakowicz [21–23] as:

\[ F_{corr} = F_{obs} \cdot 10^{(A_{295}+A_{330})/2} \]  

(2)

where, \( F_{obs} \) is the observed fluorescence upon titration of the protein with HnCl at a wavelength of 330 nm, and \( A_{295} \) and \( A_{330} \) are the absorbances of HnCl at 295 nm (protein excitation wavelength) and 330 nm (fluorescence emission wavelength), respectively.

The kinetics of the interactions were monitored on BioCore X and BioCore T100 surface-plasmon-resonance-based refractometers, and the data were processed using the BIAevaluation software (GE Healthcare, Sweden). rOpx or BSA was coupled to a CMS chip at up to 3000 RU using a standard N-hydroxysuccinimide/N-ethyl-N-

\[ \alpha \] -[3-dimethyl aminopropyl]-carbodiimide coupling procedure, as recommended by the manufacturer. HnCl solutions at 0.2 μM to 1.0 μM in PBS were injected at a flow rate of 30 μL/min for 60 s in PBS as a running buffer, at 25.0 °C. Non-specific interactions were minimized by adding 0.005% P20 surfactant to the running buffer. Sensorgrams were corrected for the reference flow-cell response (nonderivatized surface), and fitted to standard kinetic models provided by the BIAevaluation software package. Similarly, as described above, we additionally checked the interactions of the immobilized rOpx with polyamines as analytes: spermidine, spermine, putrescine and cadaverine (all at 0.2 mM or 2 mM, in PBS).

3. Results and discussion

SDS-PAGE analysis of the peak P3 from the size-exclusion chromatography of the dissolved 35% to 60% ammonium sulfate protein precipitate resulted in a ~26 kDa protein band (Supplementary Fig. S1, nOpx) and the ~16 kDa osteolyisin A. N-terminal amino-acid sequencing of the intact ~26 kDa protein that had been electro-transferred to polyvinylidene difluoride membranes failed, probably due to a blocked N-terminal amino group. Hence, this protein with an ESI–MS-determined molecular mass of 25,101.0 Da was isolated on a C4-RP HPLC column and fragmented with trypsin. Due to the complexity of the protein, it was fragmented with trypsin, with fresh trypsin, with the eluted protein having a molecular mass of 25,101.0 Da, which is identical to nOpx. Pristane extracts from C. posadasii, C. immitis and C. Reed were subjected to trypsin degradation. After tryptic degradation, the radiolabeled protein eluted at pH 4.5. (a) Lane 1, total protein extract; lane 2, protein not adsorbed to Ni2+-NTA-agarose; (flow-through); lane 3, nOpx from P. ostreatus eluted at pH 4.5. (b) Lane 4, rOpx-H6; lane 5, rOpx (after removal of H6 tag); M, molecular mass markers, as indicated on left of (a) in kDa.
domain, RGD motif, and heparin binding domains, enables this protein to operate multi-functionally [4,28]. In contrast to these vertebrate proteins, the function and biological roles of bacterial, fungal and plant hemopexin-like proteins remain little known. Recently, hemopexin-like proteins isolated from legume seeds were shown to bind various ligands. The garden pea (P. sativum) PA2 protein has been reported to bind thiamine [29], while a very similar protein from Lens culinaris binds both thiamine and hemin [10]. The chickpea (Cicer arietinum) seed albumin PA2 can interact with hemin [6]. The grass pea (L. sativus) protein LS-24 can bind spermine or hemin mutually [8], but CP4 from cow pea (V. unguiculata), a protein with a 41% sequence similarity to LS-24, interacts with spermine only [9]. Moreover, the binding of hemin has also been reported for a rice plant (Oryza sativa) protein, OsHFP, which has a 3D structure very similar to LS-24 [11].

Our finding that nOpx binds tightly to Ni$^{2+}$-NTA-agarose columns suggested that it is a bivalent-metal-binding protein. Indeed, further assays have shown that rOpx can interact with hemin, but in contrast to LS-24 and CP4, rOpx gave no surface plasmon resonance response with polyamines (Supplementary Fig. S4). The rOpx-induced increase in hemin absorbance is lower than that compared to BSA, which suggests that affinity of rOpx for hemin is lower than that of BSA.

Next we explored the hemin binding characteristics in more detail using quenching of the protein tryptophan fluorescence by hemin, and surface plasmon resonance kinetics analysis of the protein–hemin interaction. In agreement with the absorbance data, the hemin quenching of BSA was more efficient compared to rOpx (Fig. 3). The fluorescence emission spectra and their wavelength maxima ($\lambda_{max}$) in Fig. 3a suggest that two BSA tryptophan residues are more solvent-exposed, and are also more efficiently quenched by hemin than the same four in rOpx. We obtained a virtually linear Stern–Volmer plot only after correction of fluorescence intensity for inner filter effects (Fig. 3b). Using uncorrected data, we observed that hemin concentrations even above ~1 μM produced significant upward curving of the Stern–Volmer plot, as has been observed already before for serum albums [32]. This might explain why our quenching constant, $K_{SV}$, of 4.75 $\times$ 10$^4$ L/mol determined for BSA is markedly smaller than that of 1.4 $\times$ 10$^5$ L/mol determined previously for BSA [32], although this was reported for uncorrected fluorescence intensities. Comparably, our $K_{SV}$ of 2.62 $\times$ 10$^4$ L/mol determined for rOpx is lower, but in the range of that of BSA. We suggest that both values for $K_{SV}$ are largely accounted for by a static quenching component that results from binding of hemin to the protein probes, with little due to dynamic hemin quenching of the tryptophan residues [21].

Steady-state fluorimetric titration is one of the most used techniques for the evaluation of the binding of small ligands to proteins. However, determination of reliable binding constants can be complex, and this can be hampered by a variety of factors (reviewed in [22]). This is also true here, primarily due to the solubility properties of hemin, and to unknown (non)equivalent binding sites/protein molecule, or even to various classes of binding sites [22]. Specifically, when hemin and other metalloprotoporphyrins IX are above ~1 μM in solution, it is well-known that they form dimers, and then they aggregate to form micelles if their concentration exceeds 4 μM [15]. These dimerization and aggregation processes might influence the binding of the metalloporphyrins to proteins, as has been shown for hemopexin and other serum proteins, including albumin. This thus substantially complicates any postulation of binding models and any determination of kinetics and binding parameters [30,31]. However, if all of these
constraints are ignored, and with the assumption that the contribution of the dynamic quenching component is small, our fluorimetric titration will result in an apparent dissociation constant of 38 μM for the hemin–rOpx interaction. This estimated value is comparable to those determined for hemin interactions with the C. arrietum [6] and L. culinaris [10] PA2 proteins: 36 μM and 32 μM, respectively.

To reduce the ambiguities discussed above, < 1 μM hemin was used in the surface plasmon resonance investigations. The fitting of real-time kinetics curves revealed that for the BSA–hemin interaction, neither 1:1 binding nor other standard kinetics models can be applied (Fig. 4). We suggest that BSA might have more binding sites for hemin, as already reported for a variety of other BSA ligands [22]. Fitting of the hemin interaction with rOpx resulted in a bivalent-ligand-binding model with high statistical significance (χ² = 0.691). These kinetics predict that one hemin can bind two rOpx molecules with different affinities. Only the first association constant could be calculated, as 3.82 × 10⁷ L/mol (Kd ~ 2.62 μM). Our estimate of the rOpx dissociation constant for hemin, as Kd ~ 2.62 μM, appears lower than those determined for the PA2 proteins. However, the hemin: protein binding stoichiometry of ~1:2 found for these legume proteins is similar to that of the hemin interaction with rOpx. Of note, despite a lower affinity for hemin, the mode of the hemin interaction with rOpx and the legume proteins resembles that of hemin binding to hemopexin to some extent. Hemopexin has a high affinity hemin binding site located between two four-bladed β-propeller domains and the interdomain linker [33].

Compared to other hemin-binding proteins, like hemopexin [34], glutathione transferase [35], myosins [36] and β-lactoglobulin [37], the hemin affinity for rOpx appears considerably lower. However, in addition to iron, other metal (I or III) chelates remain as putative ligands of these fungal hemopexin-like proteins as found for hemopexin in the example [38,39]. Our observation that rOpx binds tightly to Ni²⁺–NTA-agarose supports this suggestion.

4. Conclusions

We have provided evidence here that P. ostreatus produces a four-HX-repeat protein, named as ostreopexin, that has a moderate affinity for hemin, but not for polyamines. This single domain hemopexin-like protein is also predicted for some genomes of Ascomycota and Basidiomycota species, and it is structurally and functionally very similar to the plant hemopexin-like proteins, PA2 albumins, in legume seeds and rice.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2013.03.027.

References


Fig. 4. Sensorgrams of rOpx (a) and BSA (b) interactions with Hn.Cl (0.2, 0.4, 0.6, 0.8 and 1.0 μM in phosphate buffered saline, pH 7.4). Hn.Cl was injected over rOpx or BSA that were immobilized on the CM5 chip, at a flow rate of 30 μl/min for 60 s, at 25 °C. Sensorgrams corrected for reference flow-cell response are shown as thick lines, and best fits as thin lines.


Supplementary Material

Ostreopexin: A hemopexin fold protein from the oyster mushroom
Pleurotus ostreatus

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Supplementary figure captions

Figure S1. Sephadex G-75 size-exclusion chromatography of the P. ostreatus 35% to 60% ammonium sulphate protein fraction. $V_e$ and $V_t$ are the elution volume and total column volume, respectively. Inset: Representative SDS-PAGE on 12% polyacrylamide gel, stained with Coomassie blue. Lane 1, proteins from 35% to 60% ammonium sulphate fraction; lane P3, peak fraction from chromatography, as indicated; M, molecular mass marker (in kDa); nOpx, native ostreopexin; nOlyA, ostreolysin A.

Figure S2. Identification of the P. ostreatus native hemopexin-repeat protein (nOpx). (a, b) Amino-acid sequence alignments. (a) Edman_pept_nOpx(a), tryptic peptides through internal Edman micro-sequencing of nOpx protein from Sephadex G-75. Pleos_jgi_pHX/nOpx(a) and Pleos_jgi_pHX/nOpx(b), nOpx protein from Sephadex G-75 and Ni$^{2+}$-NTA chromatography, respectively, showing matching of MS/MS peptides (underlined) with the amino-acid sequence of P. ostreatus predicted protein Pleos_jgi_pHX (not shown) (jgi|PleosPC15_2|1113759|estExt_Genemark1.C_080166, Protein ID 1113759). Also shown: sequences of predicted HX-repeat containing proteins from the fungi Uncinocarpus reesii (UniProt: C4JWF3) and Coccidioides posadasii (UniProt: E9DA27), and albumin-2 from the plants Pisum sativum (UniProt: P08688) and Lathyrus sativus (UniProt: D4AEP7). (b) Consensus amino acid sequence of nOpx. Asterisks, identical residues. The four HX-repeat sequences are in bolded. (c) Structure of rOpx-H₆ with a linker (shaded) and the thrombin cleavage site sequence. (d) Structure of rOpx after cleavage of the H₆-tag with thrombin.

Figure S3. Three-dimensional structural model of nOpx obtained through the I-TASSER online server. The four HX-repeats are indicated by 1-4; N and C show N- and C-termini. The nOpx structure is most similar to plant PA2 albumins from Vigna unguiculata (PDB ID: 3OYO) [9] with a RMSD of 1.18 Å, and Lathyrus sativus (PDB ID: 3LP9) [8] with a RMSD of 1.20 Å.

Figure S4. Sensorgrams of rOpx interactions with cadaverine, putrescine, spermidine, and spermine (all 2 mM in phosphate buffered saline, pH 7.4). Polyamines were injected over rOpx that was immobilized on the CM5 chip, at a flow rate of 30 µL/min for 60 s, at 25 °C. Sensorgrams were corrected for reference flow-cell response.
Figure S2

a) Edman_pept_nOpx (a) 

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b) nOpx (consensus) 

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<td>231</td>
</tr>
<tr>
<td>Latsa_Alb-2_D4AEF7</td>
<td>TNEVYFFGKDYARVTVTPGTADQ1MDGVKRLDYWSLRGLIPLEN</td>
<td>231</td>
</tr>
</tbody>
</table>

c) rOpx-H6 = consensus nOpx-GSEGKSSGLVPRGSHHHHHH

d) rOpx = nOpx-GSEGKSSGLVPR
Figure S3

3  
N  C  4

2  
N  C  1

90°
Figure S4

![Graph showing the response (RU) over time (s) for different treatments. The graph compares the effects of 2 mM cadaverine, 2 mM putrescine, 2 mM spermidine, and 2 mM spermine on the response.](image-url)