Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1784 (2008) 651–657
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbapap

Deswapping bovine odorant binding protein

Roberto Ramoni [b], Silvia Spinelli [a], Stefano Grolli [b], Virna Conti [b], Elisa Merli [b], Christian Cambillau [a,*], Mariella Tegoni [a,*]

[a] *Architecture et Fonction des Macromolécules Biologiques, UMR 6098 CNRS and Universités Aix-Marseille I & II, Campus de Luminy, Case 932, 13288 Marseille Cedex 09, France*

[b] *Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Università degli Studi di Parma, Via del Taglio 8, 43100 Parma, Italy*

Received 17 September 2007; received in revised form 30 November 2007; accepted 9 January 2008
Available online 29 January 2008

Abstract

The X-ray structure of bovine Odorant Binding Protein (bOBP) revealed its association as a domain swapped dimer. bOBP, devoid of any cysteines, contrasts with other mammalian OBPs, which are monomeric and possess at least one disulfide bridge. We have produced a mutant of bOBP in which a glycine residue was inserted after position 121. This mutation yielded a monomeric bOBP-121Gly+ in which domain swapping has been reverted. Here, we have subsequently introduced two mutations, Trp64Cys and His155Cys, in view to stabilize the putative monomer with a disulfide bridge. We have determined the crystal structure of this triple mutant at 1.65 Å resolution. The mutant protein is monomeric, stabilized by a disulfide bridge between Trp64Cys and His155Cys, with a backbone superimposable to that of native bOBP, with the exception of the hinge and of the 10 residues at the C-terminus. bOBP triple mutant binds 1-amino-anthracene, 1-octen-3-ol (bOBP co-purified ligand) and other ligands with μM Kd values comparable to those of the swapped dimer.

Keywords: Crystal structure; Olfaction; Odorant binding protein; Domain swapping

1. Introduction

Odorant Binding Proteins (OBPs) belong to the lipocalin family of proteins [1–3] generally involved in the transport of hydrophobic ligands [4]. OBPs are located primarily in nasal mucus and mucosa [5,6] and were therefore proposed to function as molecular shuttle between the air mucus interface and the olfactory receptors binding site. In contrast with insects OBPs [7–9], the function of mammalian OBPs is still a matter of debate since no direct proof of their role as odorant carrier has been established, yet. They bind odorants with ~μM affinity [10–13] and broad specificity, excepted for rat OBPs which were shown to discriminate between three different classes of compounds [12,13]. The 3D structures of bovine OBP (bOBP) and porcine OBP (pOBP), alone [14–17] or in complex with odorant molecules [10,11] have been obtained, and show a lack of specific interactions in binding.

Among OBPs and lipocalins, bOBP is the most peculiar representative: it is devoid of disulfide bridge and displays the C-terminal helix swapped between the two monomers in the dimer [17], possibly providing interdependent properties to the two subunits and supposedly at the origin of anti-cooperative binding of IBMP reported in the literature [18]. OBP isolated from bovine nasal mucus has been shown to harbour a naturally occurring ligand, 1-octen-3-ol, a typical component of bovine breath and an extremely potent olfactory attractant for many insect species [19].

Comparisons of the sequences and 3D structures of bOBP and pOBP led to the hypothesis that bOBP domain swapping might originate from a shorter hinge segment linking the β-barrel and the C-terminal α-helix, due the absence of the

Abbreviations: AMA, 1-amino-anthracene; OBP, odorant binding protein; OBP, porcine OBP; bOBP, bovine OBP; rmsd, root mean square difference; PBP, pheromone binding protein; ESRF, European Synchrotron Radiation Facility; IPTG, isopropyl β-D-thiogalactoside

* Corresponding authors. Tel.: +33 491 82 55 90; fax: +33 491 266 720.
E-mail addresses: cambillau@afmb.univ-mrs.fr (C. Cambillau), tegoni@afmb.univ-mrs.fr (M. Tegoni).

1570-9639/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2008.01.010

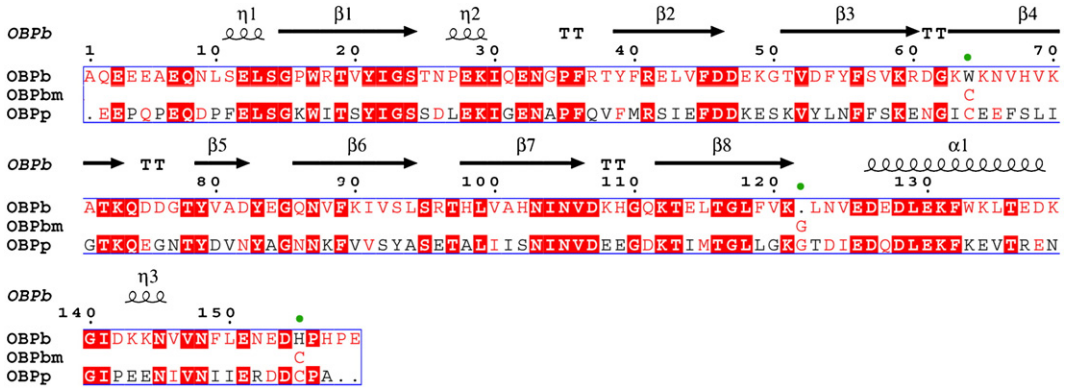

Fig. 1. Sequence alignments of bOBP and pOBP (made by MULTALIN; http://prodes.toulouse.inra.fr/multalin/multalin.html). Red shading highlights conserved amino acids. The 3 green dots highlight the Glycine present in pOBP and inserted in the mutant (Gly 121+) and the two Cysteine mutations.

glycine residue in comparison to pOBP (Fig. 1) [14]. We have generated a mutant of bOBP, named M3, in which a glycine residue was inserted after Lysine 121 and shown that the resulting gene product is a monomer able to bind odorants similarly to the native protein [20]. Crystals of this mutant diffracted weakly and could not lead to a decent data set. This led us to suspect that some domain mobility might occur due to the lack of a disulfide bridge. In this paper, we describe the crystal structure and ligand binding characterization of the unswapped bOBP monomeric (GCC-bOBP) obtained by introducing a Gly after position 121 and two cysteine residues at positions 64 (Trp) and 155 (His), corresponding to the cystine bridge positions in pOBP [14].

2. Materials and methods

2.1. Site-directed mutagenesis and expression in Escherichia coli BL21 (DE3) of GCC-bOBP

Two point mutations, Trp 64 Cys and His 156 Cys, were introduced in the sequence of the M3 mutant of bovine OBP that presents the insertion of a Gly residue in position 121+. The new mutant was named GCC-bOBP. The mutations were introduced in the M3 DNA cloned in the expression vector pT7-7 by using the 'Quick Change Site-Directed mutagenesis kit' from Stratagene, as previously reported [20] and the following oligos: 5′-CGGGATGGAAAATGCAAGAATGTACATGTC-3′ and 5′-CTTGGAAAACGAAGACTGCCCCCACCCC-3′. The sequence of the mutated plasmid was verified. The mutated plasmid was introduced into competent *E. coli* BL21 (DE3) cells and several single colonies of transformants, selected on LB ampicillin (50 μg/ml) agar plates, were then cultured in liquid ampicillin (50 μg/ml) LB medium (10ml) at 37°C to an OD_{600} of 1.2–1.4. Aliquots of these cultures were further amplified in LB at 37° to an OD_{600} of 0.7 and then the protein expression was induced by the addition of 1mM IPTG. The time course of the expression of GCC-bOBP was followed for 5h by SDS/PAGE analysis of culture samples taken at 1 h intervals.

2.2. Purification of recombinant and mutated OBPs

The GCC-bOBP was purified from soluble extracts of *E. coli* BL21 cell cultures transformed with the expression vector pT7-7 containing the GCC-bOBP cDNA, as previously reported [20], with some modifications. Briefly, after bacterial culture, cell disruption and dialysis in 20 mM Tris–HCl, pH 7.8, the soluble protein extract was loaded onto a FPLC Resource Q Column (1 ml, Amersham Biosciences) equilibrated with the same buffer and eluted with a linear (0–0.5M) NaCl gradient. The OBP containing fractions were pooled and further purified on a FPLC MonoQ column (Amersham Biosciences) applying the same linear (0–0.5M) NaCl gradient.

2.3. Direct and competitive binding titration

The fluorescent OBP ligand 1-amino-anthracene was from Fluka (Switzerland). Odorants were purchased from Aldrich (2-iso-butyl-3-metoxy-pyrazine (IBMP) and 1-octen-3-ol (OCT)) and Fluka (benzophenone (BZP), dihydromyrcenol or 2,6-dimethyl-7-octen-2-ol (DHM) and undecanal (UND)). Stock solution (2mM) of the odorants were prepared in ethanol, then further diluted in Tris–HCl 20 mM pH 7.8, ethanol 0.2% (v/v) (Tris-ethanol buffer). The influence of the concentration of ethanol on the chasing process of AMA was tested and found negligible up to 1% (v/v) [11]. In the binding studies, the odorant solutions in Tris-ethanol buffer were prepared just before each experiment and used only one time.

The functionality of GCC-bOBP was determined with a direct binding test with the fluorescent ligand 1-amino-anthracene. The experimental procedure was as previously reported [19–21] with minor modifications. Briefly, 1 ml samples of OBP (0.5 μM) in Tris–HCl 20 mM, pH 7.8, were incubated for 1 h at room temperature with different concentrations of AMA (0.05–12.8 μM). Upon excitation at 380nm, fluorescence emission spectra (between 450 and 550 nm) were recorded with a Perkin Elmer LS 50 luminescence spectrometer (excitation slit 5nm, emission slit 5nm, excitation pathway 2mm, emission pathway 10mm) and the formation of the AMA-OBP complex was monitored as an increase of the fluorescence emission intensity at 480nm. The saturation level was evaluated on the basis of a calibration curve obtained by incubating AMA (0.1–10 μM) with saturating amounts of GCC-bOBP (50 μM). The K_{diss} of the AMA-OBP complex was determined from the hyperbolic titration curve ($Y = (B_{max} \times X) / (K_{diss} + X)$ where B_{max}=maximal binding value) for one binding site, using Sigma Plot 5.0 (Cambridge Soft Corp., Cambridge, MA, USA).

Competitive titrations between AMA and several odorants were set up to investigate the binding properties of GCC-bOBP. The competitive bindings were realized following the protocol used with the native bovine and porcine OBPs [11,19,20]. The protein sample (1.0 μM) was incubated with a fixed amount of AMA (1.0 μM) and increasing concentrations of competitors: 1-octen-3-ol (0–100 μM), benzophenone (0–50 μM), dihydromyrcenol (0–50 μM), undecanal (0–25 μM) and 2-iso-butyl-3-metoxy-pyrazine (0–150 μM). The competitive titration with 2-iso-butyl-3-metoxy-pyrazine in the concentration range 0–150 μM was also set up with the dimeric swapped recombinant bovine OBP, for comparison. The release of AMA bound to the proteins was monitored as a decay of fluorescence intensity at 480nm upon excitation at 380nm. The resulting curves were analyzed as hyperbolic decay with the Sigma Plot 5.0 software for the determination of the apparent dissociation constants (K_{diss} app.). The true K_{diss} were calculated from the apparent K_{diss}, with the formula: K_{diss} true = K_{diss} app × 1 / [1+(1 / K_{diss} AMA × [AMA])], which takes into account the K_{diss} for AMA and the concentration of AMA [19].

2.4. Gel permeation

Gel permeation experiments were realized as for the recombinant bovine and porcine OBPs and the mutant M3 [20], by loading the different OBP samples