Control of domain swapping in bovine odorant-binding protein

Roberto RAMONI*, Florence VINCENT†, Alison E. ASHCROFT‡, Paolo ACCORNERO*¹, Stefano GROLLI*, Christel VALENCIA†, Mariella TEGONI† and Christian CAMBILLAU†²

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

†Architecture et Fonction des Macromolécules Biologiques, UMR 6098, CNRS, IFR1, 31 Chemin Joseph Aiguier, 13402 Marseille CEDEX 20, France,

and ‡Mass Spectrometry Facility, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

As revealed by the X-ray structure, bovine odorant-binding protein (OBPb) is a domain swapped dimer [Tegoni, Ramoni, Bignetti, Spinelli and Cambillau (1996) *Nat. Struct. Biol.* 3, 863–867; Bianchet, Bains, Petosi, Pevsner, Snyder, Monaco and Amzel (1996) *Nat. Struct. Biol.* 3, 934–939]. This contrasts with all known mammalian OBPs, which are monomers, and in particular with porcine OBP (OBPp), sharing 42.3% identity with OBPb. By the mechanism of domain swapping, monomers are proposed to evolve into dimers and oligomers, as observed in human prion. Comparison of bovine and porcine OBP sequences pointed at OBPp glycine 121, in the hinge linking the β -barrel to the α -helix. The absence of this residue in OBPb might explain

why the normal lipocalin β -turn is not formed. In order to decipher the domain swapping determinants we have produced a mutant of OBPb in which a glycine residue was inserted after position 121, and a mutant of OBPp in which glycine 121 was deleted. The latter mutation did not result in dimerization, while OBPb-121Gly+ became monomeric, suggesting that domain swapping was reversed. Careful structural analysis revealed that besides the presence of a glycine in the hinge, the dimer interface formed by the C-termini and by the presence of the lipocalins conserved disulphide bridge may also control domain swapping.

Key words: protein structure, olfaction, domain swapping.

INTRODUCTION

Domain swapping was first demonstrated by Mazzarella et al. [3], by comparing monomeric pancreatic RNase with dimeric bovine seminal RNase (BS-RNase). By this mechanism monomers are proposed to evolve into dimers and oligomers, often gaining a function [4,5]. In the oligomer, the swapped domain interacts with an identical protein chain and has an environment identical with that of the same domain in the monomer [6,7]. Several examples of swapped proteins have been recognised; diphtheria toxin (DT), BS-RNase, cell cycle regulatory protein (CksHs2), antibody fragments [8,9], human prion, and cystatins [7,10–14]. Some hypotheses have been proposed to explain the structural basis of such a phenomenon, for example: (1) shortening of the hinge region connecting the swapped domain to the rest of the molecule, (2) point mutations suppressing or inserting charges which favour an open conformation of the hinge, and (3) the presence of a proline at the point where the swapped domain leaves the protomer [6,7,15]. Moreover, it has been proposed that the new interface created by the dimer formation may provide an additional active site with the eventual gain of a catalytic or regulatory function, as frequently found in oligomeric proteins [6,7,16]. In some cases, where the structure of the monomer and the dimer exist in nature, the forces triggering the stabilization of the dimer have been determined. Examples include, the pH shift in the case of DT [10] and RNase [17], the presence of metal ions in CksHs2 [18], and the length of the hinge loop in the transcription protein of λ -phage [19], staphylococcal nuclease [20], and pancreatic RNase [4].

We [1] and others [2] solved the three-dimensional structure of bovine odorant-binding protein (OBPb), an unusual lipocalin

since it is dimeric and does not possess cysteine residues. The disulphide bridge between the C-terminal and the β -barrel, otherwise strictly conserved in lipocalins, was absent in OBPb. Furthermore, the α -helix domain (residues 124–141), instead of flanking the β -barrel (residues 9–118) of the same monomer, matched the other monomer. The polypeptide chain in the hinge region (residues 119–123) was extended through the dimer interface, stretching out the α -helix to lie over the side of the second monomer (Figure 1A). A classical lipocalin could be restored using computer graphics to reassemble the two structural elements of different monomers. In contrast, porcine OBP (OBPp) is a monomer [21], carrying two cysteines involved in the conserved disulphide bridge and sharing a very high sequence identity with OBPb (42.3%), an uncommon feature in the lipocalin family (Figure 1B). The structure of OBPp, however, revealed a classical lipocalin fold [21]. Careful examination of residues 117–126 in OBPp and OBPb revealed that Gly121 might play a crucial role in the formation of the turn in OBPp (Figure 1B) [21]. Structural comparison of the two linkers superimposed reinforced the conclusion of the sequence alignment, confirming that Gly121 is at the position where domain swapping takes place in OBPb and, moreover, that it shifts the alignment of the structural elements (Figure 1A). We proposed that the absence of Gly121+, the lack of cysteines and, therefore, of disulphide bridges, made it possible for the domain swapping to occur in OBPb.

The interest in elucidating the mechanism of domain swapping induced us to clone OBPb and OBPp, and design a mutant of OBPb, mimicking the sequence of OBPp, with a glycine residue after position 121. By this means, we expected domain swapping to be reversed. Symmetrically, we have produced a mutant of

Abbreviations used: OBP, odorant-binding protein; OBPb, bovine OBP; OBPp, porcine OBP; OBPp- Δ 121, OBPb deletion 121 mutant; DT, diphtheria toxin; BS-RNase, bovine seminal RNase; CksHs2, cell cycle regulatory protein; RT-PCR, reverse transcription-PCR; LB, Luria–Bertani; AMA, 1-aminoanthracene; IEF, isoelectric focusing; SAL, salivary lipocalin; EMS, electrospray MS; DLS, dynamic light scattering; K_D , partition coefficient.

¹ Present address: Dipartimento di Anatomia, Farmacologia e Medicina Legale, Università di Torino, Corso Massimo D’Azeglio 52, 10126, Torino, Italy.

² To whom correspondence should be addressed (e-mail cambillau@afmb.cnrs-mrs.fr).

Figure 1 Comparison of bovine and porcine X-ray structures and sequences

(A) Porcine OBp structure (pink) is superimposed on the bovine OBp structure, with the two monomers differently coloured (green and yellow). Zoom view (inset) of the hinge regions: the turn of OBp 115–134 (pink), the two extended stretches of OBp 116A–123A (green), and 121B–135B (yellow). (B) Sequence alignment of OBp and OBp. Identical residues are in red boxes. Secondary structure elements are indicated by arrows.

OBp lacking glycine 121, with the aim of inducing domain swapping. Recombinant OBpb and OBpp have the same aggregation state and comparable affinity for a fluorescent probe as the native proteins. The OBpb deletion 121 mutant (OBpp-Δ121) displays the same monomeric state as the native protein. In contrast, the insertion of Gly121+ into OBpb produces a monomeric protein which is still functional, suggesting that domain swapping has been reversed. Modelling, with computer graphics, of the dimer interface formed by the C-termini of OBpb and comparison with that of the swapped OBpp model suggest that, besides the presence of the glycine in the hinge, the rearrangement capacity of the C-termini, and hence, the presence of the conserved disulphide bridge, also controls the domain swapping phenomenon.

MATERIALS AND METHODS

RNA purification and reversed transcription (RT)-PCR

Bovine and porcine nasal mucosa were taken from freshly slaughtered animals. Aliquots (100–500 mg) of tissue were frozen in liquid nitrogen, and then homogenized in the presence of Tri reagent (Sigma). RNA was purified according to the procedure indicated by the manufacturer.

Four degenerated primers (Gibco BRL) were constructed to correspond to N- and C-terminal ends of porcine and bovine OBp amino acid sequences. The 5' sense and 3' antisense bovine OBp primers were: 5'-GCCAGGARGARGAGGCGYAGCARAA-3' (16 mer; aa sequence: AQEEEEAEQN) and 5'-CTCGGGGTGGGGRTGRTCYTCRTTYTC-3' (32 mer; aa sequence: ENENHPHPE). The 5' sense and 3' antisense porcine OBp primers were: 5'-GAGGAGCCCCAGCCCGARCARAYCC-3' (8 mer; aa sequence: EEPQPEQDP) and 5'-GGCGGGGCAGTCRTCYCTYTCRAT-3' (16 mer; aa sequence: IERDDCPA).

RNA (5–6 µg) was reverse transcribed with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Uppsala, Sweden) in the presence of a poly(dT) primer in a total volume of 33 µl, following the manufacturer's instructions. An aliquot (4 µl) of each RT reaction was amplified by PCR (hot start 95° for 5 min, annealing 60 °C for 1 min; denaturing 94 °C for 1 min, extension 72 °C for 1.5 min, using Boehringer Mannheim Taq polymerase).

Agarose gel electrophoresis revealed a single band just below the 507–516 bp marker. After elution from the gel, OBp cDNA was subcloned into p-GEM T-easy vector (Promega, Madison, U.S.A.). After transformation into competent *Escherichia coli*

Mutant	DNA Template	Type of mutation	Primers
M3	OBPb	Insertion of Gly121 +	Ins. Gly121 + 5'-CGGACTGTTTGTTAA <u>AGGC</u> CTGAATGTGAAGATG-3' STUI
M4	OBPp	Deletion of Gly121	3'-GCCTGACAAACAATTCCGGACTTACAACCTTCTAC-5' Del. Gly121 5'-CTGTTGGGCAAA—ACTGACATTGAAGATCAAGATTGGAG-3' SAU3A-I 3'-GACAACCCGTTT—TGACAGTAACTTCTAGTTCTA AACCTC-5'

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Gel permeation

Gel permeation experiments were performed on an FPLC Superdex 75 column (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 7.8. The molecular masses of the OBP isoforms were determined both in the absence and in the presence of NaCl (150 and 250 mM). Molecular mass calibration curves at the different ionic strengths were the result of individual runs of bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (23 kDa), soybean trypsin inhibitor (21.5 kDa) and ribonuclease (13.7 kDa) (Pharmacia low molecular mass calibration kit). Each determination was carried out by loading 0.5 ml of protein solution on to the column at a concentration of 1 mg/ml. The molecular mass determination of the complex OBP-thymol was carried out after equilibrating the column with 10 volumes of 1 mM thymol in 20 mM Tris/HCl buffer, pH 7.8, containing 250 mM NaCl. The complexes were formed by pre-incubating the proteins (0.5 ml of 30 μ M OBPb, OBPP and OBPb-M3 solutions) overnight with 100 μ M thymol in the same buffer.

Native PAGE and isoelectric focusing (IEF)

Recombinant proteins were analysed by native PAGE on 15% acrylamide gels [24]. Mutated OBPb was loaded with native OBPb and boar salivary lipocalin (SAL). Proteins were visualized by staining with Coomassie Blue. Albumin and ovalbumin were loaded as internal references. Analytic IEF was carried out with the NuPAGE[®] Novex system (Invitrogen, Groningen, The Netherlands) using a pH 3–7 precast gel and following the manufacturer's instructions. IEF markers were carbonic anhydrase pI 5.9, carbonic anhydrase II pI 5.4, α -lactoglobulin pI 5.1, trypsin inhibitor pI 4.6, and glucose oxidase pI 4.2.

Electrospray MS (EMS)

Samples were analysed on a Q-T of orthogonal acceleration quadrupole-time-of-flight MS equipped with nano-electrospray ionization (Micromass UK Ltd., Manchester, UK). The proteins were concentrated at 6 mg/ml in 20 mM sodium acetate buffer, pH 5.5.

Samples were analysed in (1:1, v/v) aqueous acetonitrile with 0.05% formic acid or water alone, as specified. An aliquot of this solution (2–3 μ l) was used to fill a gold-plated, borosilicate nanospray vial which was placed inside the ionization source of the MS. Positive ionization was used for the sample analyses, with a capillary voltage of 900 V and a sampling cone voltage of 40 V. Nitrogen was employed as the drying gas. The micro-channel plate detector was set at 2400 V. Data were acquired over the appropriate m/z range and spectra processed using the MassLynx software supplied with the MS. An external calibration was applied to the data using horse heart myoglobin (M_r 16951.5) as reference.

Dynamic Light Scattering (DLS) mass determination

DLS analyses were carried out with a Dynapro-MS800 instrument (Protein Solution, Lakewood, NJ, U.S.A.). The measurements were performed by injecting 15 μ l of protein in Tris (20 mM, pH 7.8) at 1.5 mg/ml for the M3 mutant of OBPb, 2.5 mg/ml sample for the native OBPb wild type, and 3.6 mg/ml for the recombinant native OBPb. Before measurement, the proteins were filtered through a Millipore 4 mm syringe unit. All calculations were carried out using the software provided with the instrument.

Modelling

A domain-swapped model of OBPp was constructed with TURBO-FRODO [25] (SGI Octane workstation; Silicon graphics Geometry Partners Directory, Mountain View, CA, U.S.A.) by superimposing the display two monomers of OBPp onto the swapped OBPb X-ray structure [1]. The Protein Data Bank (<http://www.rcsb.org/pdb/>) reference is 1OBP. Glycine 121 was deleted on both monomers of the display and the extended swapped junctions were modelled following those of OBPb.

RESULTS

Cloning and expression of OBPb and OBPP

The nucleotide sequences of OBPb and OBPP cDNAs are reported in Table 1, where the sequences of the primers used for RT-PCR cloning are underlined. The primary structure, determined on the basis of the cDNA, shows few discrepancies with respect to the sequences of the native proteins [21,26]. In the case of OBPP an Arg residue was found at position 50 instead of Lys. In OBPs, position 50 is occupied by an external residue which does not seem to be involved in the stabilization of the structure of the protein. Position 50 is conserved in some lipocalin subclasses, for example, Ser in pheromones binding proteins, Asn or Asp in von Ebner gland proteins and vomeronasal secretory proteins where it might possibly be involved in the interactions with receptors, but is not conserved in OBPs [27]. On this basis we assume that the two chemically similar residues found in position 50 of the recombinant proteins probably reflect a variability inside the species and are not due to sequencing error.

The purification protocol followed for the recombinant proteins was basically the same as for the native proteins [1,21], except for the substitution of the Bakerbond column with a much faster and more selective ResourceQ column. In all the purification steps, recombinant OBPs behaved like the corresponding native proteins. The expression yield of the recombinant proteins was in the range of 8–15 mg/l of culture.

Molecular mass determination and AMA binding assay of the recombinant native OBPs

The physico-chemical and binding properties of native and recombinant OBPs were compared by gel permeation, native and denaturing PAGE, and binding tests with the fluorescent ligand AMA.

Molecular mass determination

The molecular masses of OBPs, under native conditions, were determined by gel-permeation in 20 mM Tris/HCl, pH 7.8, 250 mM NaCl (Figure 2A). Native and recombinant OBPb were eluted at the same molecular mass of 40.2 kDa, in reasonably good agreement with the molecular mass values of an OBP dimer calculated on the basis of the protein sequence (36.8 kDa). Also, native and recombinant OBPP were eluted at the same apparent molecular mass of 23.9 kDa, which was significantly higher than the molecular mass calculated from the amino acid sequence (17.7 kDa). This result is not surprising since for OBPP, higher molecular mass values in gel permeation have already been observed [28]. Indeed, ambiguity in the molecular mass has been described also for the denatured form, as determinations in the 24–28 kDa range have been reported in SDS/PAGE [21], and the expected molecular mass of 17.7 kDa was obtained only

Figure 2 Molecular mass determination on Superdex 75 at different ionic strengths

(A) 20 mM Tris/HCl, pH 7.8, and 250 mM NaCl; (B) 20 mM Tris/HCl, pH 7.8, and 150 mM NaCl; (C) 20 mM Tris/HCl, pH 7.8. Native (empty triangle) and recombinant (black triangle) OBPs, native (empty square) and recombinant (black square) OBPs, OBp-M4 (empty circle) and OBp-M3 (black diamond). Molecular mass standards are also reported (black dots). The regression equation and the linear correlation coefficients are: ($y = -0.032x + 6.3$, $r = 0.998$) ($y = -0.0339x + 6.39$, $r = 0.996$) ($y = -0.05277x + 7.16$, $r = 0.99$) for (A), (B) and (C), respectively.

upon increasing to 5.0% (w/v) the SDS concentration in the denaturing buffer [29]. Native and recombinant OBPs were then run in denaturing PAGE at low and high concentration of SDS. Indeed at high SDS concentration, the molecular mass of the recombinant protein was also shifted at about 18 kDa (not shown).

AMA titration

The affinity of the recombinant OBPs for the fluorescent ligand AMA was tested in comparison to that of the native OBPs (Figures 3A and 3B). Within the experimental errors, the dissociation constants and maximal saturation values of the recombinant OBPs and OBPs are very similar to those of the corresponding native proteins (Table 2).

As shown above, the experimental data were consistent with identical physico-chemical properties of the recombinant and native OBPs.

Site-directed mutagenesis and purification of OBP mutants

Point mutations were introduced into the hinge of bovine and porcine recombinant OBPs with a view to transforming OBPs into a classical lipocalin and OBPs into a domain swapped one:

a Gly residue was inserted after position 121 of OBPs (121+, OBPs-M3), and the Gly residue at position 121 of OBPs was deleted (121d, OBPs-M4). The mutations were carried out on the OBP cDNAs directly in the expression vector pT7-7.

The purification protocol was the same as in the case of the recombinant native proteins. Among the different mutants, only OBPs-M3 could be purified with a satisfactory yield (5–15 mg/l of culture). At all purification steps OBPs-M3 behaved like the native recombinant OBPs, except for a lesser interaction with the ion-exchange resin (elution at 200 mM NaCl instead of 280 mM). OBPs-M4 could be purified exactly like porcine recombinant OBP, but the extract from BL21DE(3) contained a very low amount of soluble protein and the final yield was about 1 mg/l of cell culture. Crystals of the mutated protein M3 were obtained. They diffracted weakly, however, and could not lead to an exploitable data set.

Molecular mass determination and AMA binding assay of OBP mutants

Molecular mass determination

The molecular masses of OBPs-M3 and OBPs-M4 were determined in gel permeation under the same conditions as the native and the recombinant proteins (Figure 2A). OBPs-M3 gave a

Figure 3 Titrations of OBP isoforms with the fluorescent ligand AMA
(A) Native (triangles) and recombinant (circles) OBpb, **(B)** native (triangles) and recombinant (circles) OBpp, and **(C)** OBpb-M3 (Gly121 +) OBpb.

Table 2 AMA affinity

OBP isoform	K_{diss} (μ M)	Max. saturation level (moles of AMA/mole of OBP)
Native OBpb	0.66	1.65
Recombinant OBpb	0.71	1.54
M3 Gly121 +	0.54	0.78
Native OBpp	1.14	0.81
Recombinant OBpp	1.06	0.84

value of 19.1 kDa, that closely matches the molecular mass of the monomer of OBpb (18.4 kDa), whereas OBpp-M4 was eluted at the same molecular mass as the native and the recombinant

Figure 4 IEF and native-PAGE OBPs gels
(A) IEF gel (pI 3-7): OBpb (lane 2), SAL (lane 3), IEF markers (lane 4), OBpb-M3 (lane 5), native OBpb (lane 6). An unrelated protein is shown in lane 1. **(B)** Native-PAGE (15% acrylamide): native OBpb (lane 1), OBpb-M3 (lane 2), SAL (lane 3) albumin and ovalbumin (lane 4).

OBpp (23.9 kDa). No further experiments were carried out with OBpp-M4 since it behaved as the native monomeric OBpp.
The influence of the ionic strength on the aggregation state of OBpb-M3 was tested by running the same gel permeation at 150 mM and in the absence of NaCl (Figures 2B and 2C). The molecular masses estimated for OBpb-M3 are 21.8 and 20.0 kDa, which are slightly higher than that found at 250 mM NaCl (19.1 kDa), but within the error limits for such a determination. Indeed, native OBpb and OBpp run as controls also gave somewhat different molecular masses, with confidence limits comparable to OBpb-M3 in the case of OBpb (20.45 ± 1.35 kDa and 40.15 ± 1.45 kDa) and higher in the case of OBpp (26.75 ± 2.85 kDa). These differences have to be ascribed to small changes in the interaction of the proteins with the resin particles at different ionic strength, as may be seen in the calibration curves

Figure 5 Electrospray mass spectra of OBPs

(A) Native OBp under denaturing conditions, (B) native OBp in water, and (C) the mutant Gly121 + (OBp-M3) in water.

obtained under the three conditions. In conclusion, OBp-M3 has been shown to be monomeric in the range of ionic strengths tested (0–250 mM NaCl).

The molecular mass of OBp-M3 was also determined in the presence of 1 mM thymol; native OBp and OBp were loaded

under the same conditions as controls. All the proteins were eluted at the same apparent molecular mass as the previous gel-permeation experiment (results not shown) indicating that the aggregation state is not significantly modified by the presence of a ligand in the hydrophobic pocket.

AMA titration

The binding affinity for AMA was tested for the mutant OBPb-M3 (Figure 3C). The partition coefficient (K_D) and the saturation values (Table 2) indicate that the mutant form of OBPb maintains the ligand-binding capacity with a maximum stoichiometry clearly indicating a monomer state (0.78 moles of AMA/moles of OBPb-M3) and that the affinity for the fluorescent probe is comparable to that of the native OBPb dimer (K_D 0.54 and 0.71 μ M for OBPb-M3 and OBPb, respectively).

IEF and native electrophoresis

IEF of the OBPb-M3 was carried out with other lipocalins of known pI value; monomers, like SAL (pI 5.07) and OBPp (pI 4.18), and the native OBPb (calculated pI 4.94), which is a dimer. SAL was taken as internal reference, as it is a monomer with a pI value (5.07) close to that of OBPb. OBPp migrates very low at pH 4.1, not very different from the expected pI, and SAL migrates at pI approx. 4.8, slightly lower than expected (Figure 4A, lanes 2 and 3). Although the migration appears somewhat distorted, SAL, OBPb-M3 and native OBPb behave similarly on the gel and migrate roughly at the same pH value (Figure 4A, lanes 3, 5 and 6).

In native-PAGE, native OBPb migrates less than OBPb-M3 (Figure 4B, lanes 1 and 2); SAL, which is similar to OBPb-M3 in terms of aggregation state, migrates further than the OBPb dimer (Figure 4B, lanes 1 and 3) and significantly closer to OBPb-M3 (Figure 4B, lanes 2 and 3). The different migration of native OBPb and OBPb-M3 has to be attributed to a difference in molecular mass, as the charge of the two proteins is identical. Accordingly, since OBPb-M3 and SAL have a very similar charge (pI 5.07 and 4.94, respectively), the lower migration of OBPb-M3 might be rationalized on the basis of the slight difference of molecular mass (18.5 and 20 kDa for OBPb-M3 and SAL, respectively).

MS

Mass spectra were recorded for the native OBPb in denaturing (methanol and formic acid pH 2) and non-denaturing conditions (water). The mass-converted spectra show a single peak, centred at 18370 Da in denaturing conditions and 36762 Da in non-denaturing conditions (Figures 5A and 5B). The theoretical mass of the monomer of the native enzyme calculated on the basis of the amino acid sequence is 18431 Da. Thus, the spectra indicate that OBPb is a dimer under non-denaturing conditions, and a monomer under denaturing conditions. The difference observed between the theoretical mass of the monomer (18431 Da) and the experimental mass (18370 Da) is 61 Da, which cannot be accounted for by proteolysis of one amino acid and might be explained by the existence of isoproteins.

The mass spectrum recorded on OBPb-M3 in water shows a major peak corresponding to a population of proteins of 18487 Da (Figure 5C). The theoretical mass of the mutant is that of the native enzyme (18431 Da) with the addition of the N-terminal methionine residue (131 Da) and of the glycine residue (57 Da), leading to a value of 18619.6 Da. We therefore conclude that under non-denaturing conditions OBPb-M3 is mainly in a monomeric form. The difference observed between the major peak experimental mass and the theoretical value is 132.6 Da, a value close to the mass of a Met residue (131.2 Da). This indicates that this residue has probably been removed by proteolysis. A minor peak observed at 18030 Da (Figure 5C) may possibly be ascribed to a cleaved protein, depleted of

Figure 6 Distribution of the molecular mass (%) as a function of the gyration radius (nm)

(A) M3 mutant of OBPb (1.5 mg/ml) and (B) native OBPb (2.5 mg/ml).

the first Met and of the following 4 amino acids, namely Ala, Gln, Glu, Glu, leading to a calculated mass of 18,031 Da. Another minor peak at 18544 Da (Figure 5C) can be explained by the formation of an adduct between the major protein species (Met depleted; 18487.8 Da) and an acetate anion of the dialysis buffer (theoretical mass of 18544.8 Da).

DLS

Four independent measurements of 20 acquisitions were carried out for each protein. The peaks obtained were symmetrical and thin, indicating the presence of unique species. The determined gyration radii calculated were very different for the two proteins, mutant M3 (2.075 ± 0.125 nm), and native OBPb (2.70 ± 0.10 nm) (Figure 6). Molecular masses of 18.75 ± 1.25 kDa for the mutant M3 and 34.7 ± 2.2 kDa for native OBPb were deduced from the measurements. The recombinant native OBPb has also been submitted to DLS. Its gyration radius and molecular mass were found to be close to that of the native wild type, at 2.65 ± 0.05 nm and 32.5 ± 0.5 kDa, respectively (results not shown). These values are in agreement with the respective values of the protein masses. The low mass values observed for the dimeric OBPb (34.7 kDa or 32.5 kDa versus 38 kDa) arise from their significant deviation from globularity as compared to the monomeric form.

Modelling

In the absence of experimental structural data, modelling of a swapped OBPp dimer has been carried out. The monomer model has been cut into three sections, namely the β -barrel domain (11–117), the hinge (118–123) and the α -helix domain (124–157). The dimer has been modelled by superimposing the isolated structural elements onto the OBPb dimer, and introducing a

Figure 7 Consequence of domain swapping at the C-termini of OBPs

(A) Model of a domain swapped OBPP dimer. In the dimer, the two monomers are yellow and magenta. The two C-termini clash with each other (red arrows), and cannot rearrange, due to the constraint of the disulphide bridges (in green, indicated by the blue arrows). (B) X-ray structure of OBPP (green) with a second dimer superimposed on the first one (magenta). The two extended C-termini would clash (red arrows) if the rearrangement of the C-terminus of monomer B did not occur. The absence of disulphide bridges makes this rearrangement possible (green arrow).

deformation of the linker analogous to that observed in OBPP. The disulphide bridges of OBPP kept their place, although formed by different monomers. Visual analysis of this model revealed that besides the crossed-junction, the only contacts at the interface of the OBPP dimer involve four residues at the C-terminus (Figure 7A), and are repulsive, as they produce several atom clashes at less than 1 Å (where 1 Å = 0.1 nm) distance. Therefore, the C-termini conformation is not favourable to the formation of a stable domain swapped OBPP dimer. Different conformations of the C-termini had been observed in OBPP [1]. As the reason for this is still unexplained, we have superimposed one OBPP dimer on to another, crossing the A and B monomers. In this structure we observe a clash of the extended C-termini on top of each other (Figure 7B). Thus, in OBPP the remodelling of the C-terminus of subunit B is determinant for maintaining the domain swapping.

DISCUSSION

The phenomenon of domain swapping, or arm-exchange, has been shown to occur in many proteins [6,7,10–14]. The first, and obvious, effect of domain swapping in dimers or oligomers is the increase in contact area and the resulting stabilization. Very seldom can a new function be directly related to its presence, however. It seemed, therefore, interesting to investigate how easily a domain swapped dimer could be transformed in a non-swapped species (dimer or monomer) and *vice versa*. On the basis of sequence and structure comparisons between porcine and bovine OBPs, we have postulated that the lack of a glycine residue in the turn between the β -barrel domain and the α -helix was responsible for domain swapping. We hypothesized that this shorter turn could not accommodate the constraint produced by the deletion, and would lead to an extended structure favouring the domain swapping. We therefore designed experiments to validate this hypothesis: (1) by introducing a glycine after Lys-121 in OBPP (mutant Gly121+), we hoped to remove the constraint in the turn, making it possible for the lipocalin α -helix

to fold back on the β -barrel, and (2) by removing Gly-121 of OBPP, thus introducing a constraint in the turn, which may lead to a domain swapped structure.

The MS and molecular mass determination carried out on the mutant OBPP-M3 (Gly121+) indicated that, as a result of the insertion of Gly121+, the dimeric structure of OBPP was destroyed, leading to monomers in solution or in the gas phase. The exact structure of the OBPP-M3 mutant monomer could not be elucidated, however, due to the poor quality of the crystals. We can imagine that, once the domain swapped dimer disrupted, the α -helix and the strand- β_9 at the C-terminal remain extended and do not fold back on the barrel. This hypothesis was not totally absurd, as the disulphide bridge usually locking the α -helix to the β -barrel in lipocalins is absent in OBPP. However, it has been pointed out that in domain swapped systems the 'domains interface' is the same in the monomer and in the dimer [6,7]. In OBPP, this domain interface accounted for 940 Å², which is relatively large to maintain the domains together in the monomer.

The experiments with OBPP in solution clearly indicated that the protein remained monomeric, as in the native protein. This indicated that the OBPP structure was able to accommodate the constraint introduced by the shorter turn (Gly121d); indeed the monomer may have been stabilized by the disulphide bridge 63–155. It may also indicate that the nature of the residues facing each other at the putative 'dimer interface' (the inter-monomer interface, not the inter-domain interface mentioned above), is not compatible with the formation or the maintenance of the dimer. Computer modelling of an OBPP dimer presenting a domain swapping, indicated clearly that swapped junctions can readily be constructed which are similar to those observed in native OBPP. However, several clashes result from the interaction of the C-termini at the interface of the two monomers. Furthermore, due to the constraints of the disulphide bridges (Cys 63–155) which, in the model, were indeed established between two different monomers, the C-termini cannot rearrange in a conformation compatible with dimerization.

A similar clash of C-termini was not observed in the OBPb dimer [1]. Amazingly, when the structure was solved, the C-termini showed different conformations for the same sequence; in subunit A, the C-terminus was found to be extended, while in subunit B, it formed a short α -helix of 7 residues. This observation remained unexplained at the time but may now prove to be the reason that domain swapping is possible. We suggest that the rearrangement of subunit B of OBPb in a helical conformation, occurs to prevent C-termini clashing and to establish favourable contacts between them. This rearrangement can occur in OBPb and not in OBPP because the former does not possess cysteines and, hence, no constraining disulphide bridge can be established.

Conclusion

We have shown that OBPb domain swapping is made possible by the absence of a glycine after position 121, which normally constrains the turn between the β -barrel and the α -helical domain, and leads to an elongated linker. In all OBPs, introduction of domain swapping should probably result in a similar dimer geometry, due to topological constraints, and should bring the two C-termini in forbidden close contact. In classical lipocalins, these contacts cannot be released by conformational rearrangements, due to the presence of a disulphide bridge blocking the C-terminal ends. Among eukaryotic lipocalins, OBPb is the only one devoid of cysteine residues [27]. The rearrangement of the C-terminus of monomer B in an helical conformation is therefore possible, leading to favourable contacts between the two C-terminal segments. To date, the phenomenon of domain swapping is, therefore, limited to OBPb. Most prokaryotic lipocalins, however, do not possess cysteines or have only one cysteine in the lipocalin framework. The absence of strong homology of prokaryotic lipocalins with OBPb does not allow us to determine with confidence if the turn between the β -barrel and the α -helical domain is constrained. The absence of disulphide bridges in these lipocalins may indicate, however, that if some constraints exist in the turn, domain swapping may become possible, thus extending this phenomenon to a wide variety of lipocalins.

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