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The protein scaffold of the lipocalin odorant-binding protein is suitable for the design of new biosensors for the detection of explosive components

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Abstract

The detection of hazard exposure is a current priority, including the detection of traces of explosive molecules in different environments like luggage storage rooms and public places, and is becoming a major requirement for homeland security. In the present study we carried out a preliminary investigation on the binding capacities of four forms of the lipocalin odorant-binding protein (OBP) for the detection of explosive components such as diphenylamine, dimethylphthalate, resorcinol and dinitrotoluene. The experimental results, showing that OBP binds these compounds with affinity constants ranging between 80 nM and 10.6 mM, indicate that this protein can be used as a probe for the realization of a biosensor to sense explosive compounds.

1. Introduction

Control of the illicit trade in explosives and hazardous compounds represents one of the most effective tools for preventing illegal actions by terrorists and criminal organizations.

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Explosives are complex mixtures of deflagrating and detonating agents, additives and taggants whose detection depends on either animals (dogs) (Harper *et al* 2005) trained for the olfactory perception of the components of explosive mixtures, or on conventional analytical techniques (Steinfeld and Wormhoudt 1998, Mathis and McCord 2003, Zhang *et al* 2006). In the first case detection is made directly by the examination of luggage and goods by security personnel. Limitations are mainly due to the subjective responses of single animals. In the second case, results of analysis are not immediate and must be obtained by trained personnel on collected samples inside a properly equipped laboratory.

An alternative approach to the detection of these substances is the use of biosensors, which are analytical devices based on biological recognition elements (Shriver-Lake *et al* 2003). Biosensors, in fact, can be easily miniaturized and it is possible to produce configurations suitable for specific applications in the field. Their performance is strictly dependent on the affinity and specificity of the recognition element, and antibodies and enzymes are usually employed for this purpose.

In the case of explosive components, most of which are non-immunogenic synthetic organic low-molecular-weight (MW) molecules (140–250 Da), the choice of biological recognition elements derived from the lipocalin (Nygren and Skerra 2004, Schlehuber and Skerra 2005) protein family seems more appropriate than the use of antibodies. The proteins belonging to this family in fact bind small hydrophobic molecules whose masses and physicochemical properties are comparable to those of explosive components (Flower 1996). Lipocalins are expressed in animals, plants and bacteria, and despite low sequence homology (less than 20%), present a conserved structural frame named lipocalin folding, made up of two domains: an eight-strand beta-barrel connected by a short linker (hinge sequence) to a C-terminal alpha-helix (Flower *et al* 2000). The beta-barrel consists of 70–80% of the total number of amino acid residues and represents the ligand-binding site. The function of the alpha-helix is not currently known (Flower *et al* 2000). During the evolutionary pathway, the structural frame of the lipocalins gave rise to a large variety of forms with different binding specificities. It also has many characteristics of so-called protein scaffolds, defined as structural frames that can acquire new binding specificities when subjected to molecular engineering (Nygren and Skerra 2004). Protein scaffolds may be used for the production of artificial affinity reagents to be employed as an alternative (or complement) to antibodies as recognition elements in biosensors (D'Auria and Lakowicz 2001, Staiano *et al* 2005). Protein scaffolds are characterized by stable frames that do not modify their overall 3D structures in response to multiple substitutions in the amino acid sequence (Nygren and Skerra 2004). Lipocalins have already been subjected to molecular engineering, which has highlighted their use as protein scaffolds for the development of affinity reagents (Skerra 2000, Schlehuber and Skerra 2005), e.g. the bilin-binding protein (BBP), an insect lipocalin whose binding specificity has been optimized for digoxigenin, a molecule of relevant biotechnological interest with structural and physicochemical properties totally different from those of biliverdin, the natural ligand of the native protein (Korndorfer *et al* 2003).

In the present study we investigated whether the lipocalin scaffold has potential for the development of recognition elements to be employed for the design of biosensors for the detection of hazardous compounds. The study analysed the ligand-binding assays of several forms of the lipocalin odorant-binding protein (OBP) (Tegoni *et al* 2000) with the explosive components diphenylamine (*N*-phenylbenzenamine; DFA), dimethyl-phthalate (1,2-benzenedicarboxylic acid, dimethyl ester; DMF), resorcinol (1,3-benzenediol; RES) and dinitrotoluene (1-methyl-2,4-dinitrobenzene; DNT) (Meng and Caddy 1997).

In this study, we considered the following OBP forms: porcine OBP (pOBP), a typical monomeric lipocalin (Spinelli *et al* 1998); bovine OBP (bOBP), a dimer with domain swapping

(Tegoni *et al* 1996); mini-bOBP, a truncated form of bOBP formed by the first 122 amino acid residues (Grolli *et al* 2006); GCC-bOBP, a deswapped monomeric form derived from bovine OBP (Ramoni *et al* 2006). The choice of OBP as an experimental model was based on the following background: 1) bOBP and pOBP are two of the of the best characterized lipocalins (Tegoni *et al* 2000). 2) Bovine and porcine OBP can bind a large number of structurally unrelated ligands, with molecular masses and physicochemical properties similar to those of explosives and explosive components (Vincent *et al* 2000, 2004). 3) The recombinant His-tagged form and mutants of OBP are purified at high yields from *Escherichia coli* soluble extracts (30 mg l^{-1} of medium) (Ramoni *et al* 2002). 4) bOBP tolerates point mutations even when they involve residues playing a key role in protein structure stabilization. Interestingly, functional monomeric bOBP mutants (Ramoni *et al* 2002, 2006) and a functional truncated form of bOBP (residues 1–123), comprising the N-terminal and the beta-barrel (mini-bOBP), have already been produced (Grolli *et al* 2003).

The obtained results show that all the different OBP forms considered bind explosive components with affinity constant values ranging between 80 nM and 10.6 mM, indicating that the protein scaffold can be considered as an appropriate model for the design of mutant forms of OBP to be used as probes for the specific recognition of hazardous compounds.

2. Materials and methods

2.1. Materials

The explosive components DFA, DMF, RES and DNT and the fluorescent OBP ligand 1-aminoanthracene (AMA) were from Sigma Aldrich (Milan, Italy). Working solutions of these molecules were prepared either in ethanol (AMA, DMF, RES and DFA) or in acetonitrile (DNT), and then further diluted in Tris-HCl 20 mM pH 7.8. In the ligand-binding studies, the final concentration of organic solvent never exceeded a value of 0.1% (V/V).

2.2. OBP purification and functionality test with AMA

A 6×His affinity tag was placed at the N-terminal of all the OBP forms by polymerase chain reaction (PCR) using specific primers. The fused cDNAs were subcloned in the expression vector pT7-7 and the expression of the proteins, in BL21-DE 3 *E. coli*, was realized as previously reported for the recombinant forms of porcine and bovine OBP (Ramoni *et al* 2002). The purification of the proteins was obtained by affinity chromatography with a Ni-NTA agarose (Qiagen, Germany) according to the manufacturer's instructions, followed by a second chromatographic step on the anion exchange column Resource Q (Amersham Biosciences, Italy), in fast protein liquid chromatography (FPLC). The purity of each OBP preparation was determined by sodium-dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and protein concentrations were calculated based on the absorbance values at 280 nm (13 000 and 48 000 M^{-1} cm^{-1}, respectively, for porcine and bovine OBP, 18 450 M^{-1} cm^{-1} for mini-bOBP and 19 400 M^{-1} cm^{-1} for GCC-bOBP). Functionality of the different OBP forms was determined by direct titrations using the fluorescent ligand AMA as previously reported (Ramoni *et al* 2001). Briefly, 1 ml samples of 1 μM OBP, in 20 mM Tris-HCl buffer pH 7.8, were incubated overnight at 4 °C in the presence of increasing concentrations of AMA (0.156–10 μM). Fluorescence emission spectra between 450 and 550 nm were recorded with a Perkin-Elmer LS 50 luminescence spectrometer (excitation and emission slits of 5 nm) at a fixed excitation wavelength of 380 nm and the formation of the AMA–OBP complex was followed as an increase of the fluorescence emission intensity at 480 nm. The dissociation

Table 1. Dissociation constants (μM) of the binding complexes between OBP and explosive components.

	K_d AMA	K_d DMF	K_d resorcinol	K_d DFA	K_d DNT
pOBP	1.2	32	2100	0.112	18
bOBP	0.6	800	5000	0.12	10 560
GCC-bOBP	1	1210	5300	0.08	260
Mini-bOBP	0.9	1200	5200	0.95	3 480

constants of the AMA–OBP complexes were determined from the hyperbolic titration curves using the nonlinear fitting program of Sigma Plot 5.0 (CambridgeSoft Corp., Cambridge, MA, USA). The concentrations of the AMA–OBP complexes were determined on the basis of emission spectra obtained incubating AMA (0.1–10 μM) with saturating amounts of both OBP forms.

2.3. Determination of the dissociation constant of the 'explosive components–OBP' complexes

The dissociation constants of complexes between the OBP forms and the different explosive components were determined by competitive binding tests with the fluorescent ligand AMA, as previously described for other ligands (Vincent *et al* 2000, 2004). Each OBP form (0.5 μM in the case of bOBP and 1 μM for the other forms) was dissolved in 20 mM Tris-HCl buffer pH 7.8 and pre-incubated at room temperature for 20 min with AMA (0.7 μM). 1 ml samples of these solutions were then poured into different tubes containing increasing amounts of each explosive component. The samples were incubated for another 30 min at room temperature and the binding of the competitors was followed as a decrease of the fluorescence emission of the AMA–OBP complex at 480 nm upon excitation at 380 nm (excitation and emission slits 5 nm). The apparent K_d values for the binding complexes were determined from the competition curves analysed as two-parameter hyperbolic decays with the nonlinear fitting function of the software Sigma Plot 5.0. The true K_d values were then calculated from the following formula (Ramoni *et al* 2001):

$$K_d^{true} = K_d^{app} \times \frac{1}{1 + (1/K_d^{AMA} \times [AMA])} \quad (1)$$

where K_d^{AMA} is the dissociation constant of the AMA–OBP complex.

3. Results

3.1. Protein purification and functional characterization

SDS-PAGE of the purified forms of OBP gave the bands at the expected molecular weights of the protein subunits. The binding capacity of the protein was determined employing the fluorescent ligand AMA. The hyperbolic titration curves (not shown) gave K_d values (table 1) in agreement with those of functional preparations of the different OBP forms (Ramoni *et al* 2002).

3.2. Binding of AMA and explosive components

The affinities for DMF, RES, DNT and DFA were determined by measuring the progressive chasing of AMA bound to OBP, in response to increasing concentrations of each compound. The competition curves, in the case of pOBP, are reported as an example in figure 1 and the

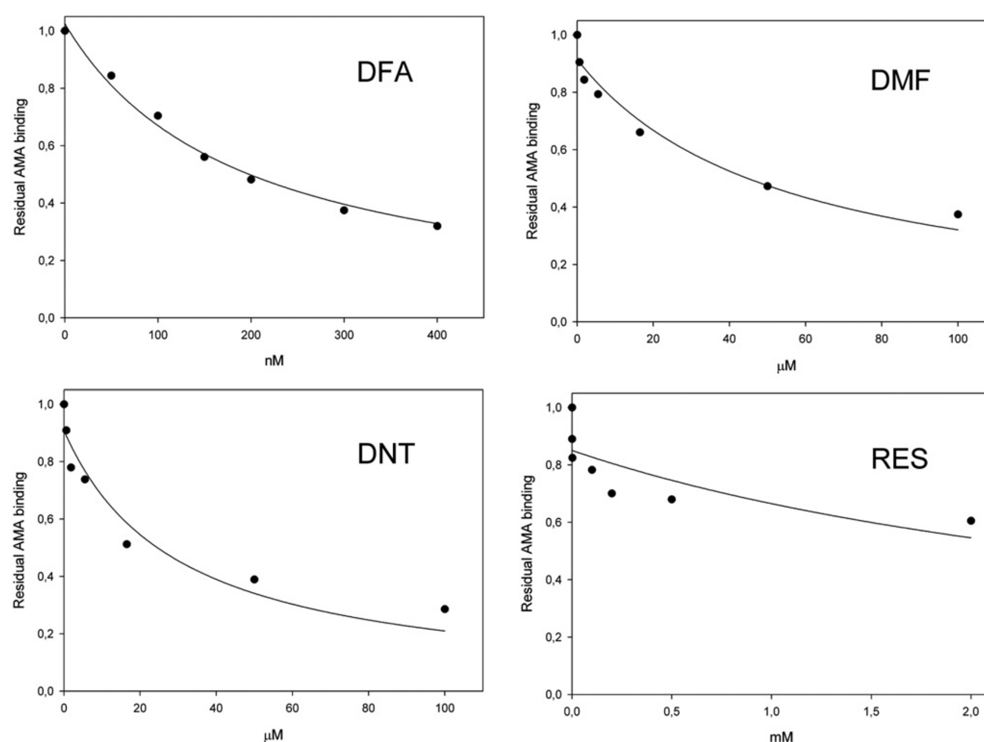

Figure 1. Competitions between AMA bound to pOBP and DFA, DMF, DNT and RES. Protein samples were incubated with a fixed amount of AMA (1 μM) and increasing levels of the explosive components. Each point on the y-axis shows the concentration of AMA still bound per OBP monomer relative to the initial value, on a scale of 0–1, versus the concentration of each competitor.

values of the dissociation constants for all the OBP forms are listed in table 1. When the concentrations of the competitors corresponded to their limits of solubilization in aqueous solutions, in some cases the displacement of AMA did not even reach 50% of the initial value. Consequently, statistical analysis of the data points was restricted to the concentration levels of competitors that gave a measurable decrease of fluorescence due to AMA released from OBP. In spite of this experimental limit, all the competition curves could be treated as two-parameter hyperbolic decays, with R factors ranging between 0.97 and 0.99, and showed that all the OBP forms bind the explosive components considered with different affinity constant values. The K_d values reported in table 1 are very heterogeneous, ranging between 80 nm and 10.6 mM, and show significant differences in the binding properties between pOBP and the group of tested protein that includes the bOBP and its mutants. This difference is remarkable in the case of DMF and DNT, molecules that pOBP binds with much higher affinity (two to three orders of magnitude) than the other OBP forms. It must be underlined that the dissociation constant value is in the same range as that obtained for odorant compounds and other putative ligands *in vivo* by pOBP (Vincent *et al* 2000, 2004), especially in the case of the explosive agent DNT that gave a K_d value of 18 μM. While the dissociation constant of RES indicates weak binding to all the OBP forms, the high affinity observed for DFA is peculiar. In fact, dissociation constants in the submicromolar and nanomolar range were not previously observed in the case of OBP ligands (Vincent *et al* 2000, 2004).

4. Discussion

In the present study we investigated whether the protein scaffold of the lipocalin OBP is suitable for the preparation of affinity reagents for the detection of explosive components. To this aim we determined the binding properties of four different OBP forms with respect to four explosive components with mass and physicochemical properties similar to those of the ligands of this type of protein. The molecules that we considered belong to the following classes of explosive components: detonating agents (DNT), plasticizers (DMF) and additives (DFA and RES) (Meng and Caddy 1997). The experimental results showed that all the OBP forms can bind all the molecules tested and suggest that the protein scaffold of OBP can be considered to be promising platform for the production of biological recognition elements to be employed in biosensors for the detection and identification of explosive substances. In particular, the high affinity that DNT, DMF and DFA show for pOBP suggests that the binding site of this form should be primarily considered for the development of mutants for the detection of these hazardous compounds. The production of protein affinity reagents is usually performed through a molecular engineering approach based on the preparation of random mutants, and on the selection of the protein forms with improved selectivity and affinity for a specific ligand (Korndorfer *et al* 2003). It must be underlined that, in the case of OBP, this approach could be associated with a rational design of the structure of the ligand-binding site because the three-dimensional structures at the atomic level are available for several forms of OBP, included pOBP (Spinelli *et al* 1998), bOBP (Tegoni *et al* 1996) and the GCC-bOBP mutant, which were subjects of the present investigation (Ramoni *et al* 2006).

The number of OBP forms currently available from different animal species is quite large, including bovine, porcine, rat (Lobel *et al* 2002), human (Briand *et al* 2002), rabbit (Garibotti *et al* 1997), elephant (Lazar *et al* 2002) and porcupine (Ganni *et al* 1997). Since there are remarkable differences in their binding spectrum, it might be appropriate to extend the experimental approach adopted in the present work to other forms of OBP. This would allow us to determine the best OBP scaffold for virtually all types of molecules included in the formulations of explosive mixtures. The compounds we tested here represent only a partial selection of the explosive components that, on the basis of molecular mass and physicochemical properties, could be bound by OBPs. In particular, our study was restricted to some of the explosive components which are freely available from chemical suppliers. In the future we intend to extend the present investigation to molecules which are peculiar for specific explosive formulations (deflagrating and detonating agents, taggants, etc), that can only be purchased and manipulated with the permission and under the control of national security agencies. The final aim of our molecular engineering approach would be the availability of a large number of OBP mutants, with single specificities, to set up protein arrays that might be employed as ‘multichannel recognition elements’ in biosensors for the detection of different types of explosive formulations.

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