Biosensors and Bioelectronics 26 (2011) 4184–4190

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Reagentless fluorescent biosensors from artificial families of antigen binding proteins

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ARTICLE INFO

Article history:
Received 9 February 2011
Received in revised form 14 April 2011
Accepted 15 April 2011
Available online 22 April 2011

Keywords:
Antibody
Antigen
Binding protein
Biosensor
Fluorescence
Solvatochromic fluorophore

ABSTRACT

Antibodies and artificial families of antigen binding proteins (AgBP) are constituted by a connected set of hypervariable (or randomized) residue positions, supported by a constant polypeptide backbone. The residues that form the binding site for a given antigen, are selected among the hypervariable residues. We showed that it is possible to transform any AgBP of these families into a reagentless fluorescent biosensor, specific of the target antigen, simply by coupling a solvatochromic fluorophore to one of the hypervariable residues that have little or no importance for the interaction with the antigen, after changing this residue into cysteine by mutagenesis. We validated this approach with a DARPin (Designed Ankyrin Repeat Protein) and a Nanofitin (also known as Affitin) with high success rates. Reagentless fluorescent biosensors recognize their antigen in an immediate, quantitative, selective and specific way, without any manipulation of the sample to analyze or addition of reagent.

1. Introduction

Reagentless fluorescent (RF) biosensors can be obtained by integrating a biological receptor, which is directed against the target analyte, and a solvatochromic fluorophore, whose emission properties are sensitive to the nature of its local environment, in a single macromolecule. The fluorophore transduces the recognition event into a measurable optical signal. The use of extrinsic fluorophores, whose emission properties differ widely from those of the intrinsic fluorophores of proteins, tryptophan and tyrosine, enables one to detect and quantify the analyte in complex biological mixtures. The integration of the fluorophore must be done in a site where it is sensitive to the binding of the analyte without perturbing the affinity of the receptor (Altschuh et al., 2006; Loving et al., 2010).

The possibility of obtaining, for any antigen considered as an analyte, RF biosensors which respond to the binding of the antigen by a variation of fluorescence, would have numerous applications in micro- and nano-analytical sciences. Antibodies and artificial families of antigen binding proteins (AgBP) are well suited to provide the recognition module of RF biosensors since they can be directed against any antigen. A general approach to integrate a solvatochromic fluorophore in an AgBP when the atomic structure of the complex with its antigen is known, and thus transform it into a RF biosensor, has been described recently (Brient-Litzler et al., 2010). A residue of the AgBP is identified in the neighborhood of the antigen in their complex. This residue is changed into a cysteine by site-directed mutagenesis. The fluorophore is chemically coupled to the mutant cysteine. When the design is successful, the coupled fluorophore does not prevent the binding of the antigen, this binding shields the fluorophore from the solvent, and it can be detected by a change of fluorescence.

The variable fragments (Fv) of antibodies comprise a polypeptide backbone, which is conserved both in sequence and structure, and six loops of hypervariable residues, which are grafted onto the backbone and form the antigen binding site (paratope). The artificial families of AgBPs are similarly constructed. For example, one may start from a natural family of binding proteins and either design a canonical polypeptide backbone or select a representative member from this family (Binz et al., 2003; Drevelle et al., 2009; Famm et al., 2008; Mouratou et al., 2007; Urvoas et al., 2010). The residue positions that contribute to antigen binding in the various elements of the natural family, are identified through a careful anal-

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doi:10.1016/j.bios.2011.04.030

ysis of the available structural and functional data (Arcus, 2002; Mosavi et al., 2002; Theobald et al., 2003). Generally, these positions form a connected set on one side of the canonical protein. The corresponding residues are then randomized at the genetic level to constitute a random library of genes, coding for an artificial family of AgBPs. We refer to the positions of the randomized residues as hypervariable, by analogy with antibodies. The elements of a random family that bind a target antigen, are selected in vivo or in vitro by methods of display that physically link a gene and its product, e.g. phage, ribosome or yeast display (Beste et al., 1999; Binz et al., 2004; Heyd et al., 2003; Jespers et al., 2004; Mouratou et al., 2007; Nord et al., 1997).

The methods for the selection of AgBPs from artificial families imply that the residues that form structural or energetic contacts with the antigen, are mainly located at hypervariable positions. Antibodies and AgBPs generally use only a subset of the residues at the hypervariable positions to bind their target antigen and the hypervariable positions that are not used to form contacts with the antigen, are located in its neighborhood (MacCallum et al., 1996).

Here, we explored the possibility of deriving RF biosensors from any element of artificial families of AgBPs, in the absence of specific structural data, by using their peculiar method of construction. Our strategy consisted in individually changing the residues of the hypervariable positions into cysteine at the genetic level, in chemically coupling a solvatochromic fluorophore with the mutant cysteine, and then in ordering the resulting conjugates through their relative sensitivity s_r, that involves both their affinity for the antigen and their relative variation of fluorescence signal. To validate this approach, we used two different AgBPs, for which no specific structural data is available: H4S, a Nanofitin (also known as Affitin) which is directed against hen egg-white lysozyme (HEL) (Cinier et al., 2009; Pecorari and Alzari, 2008), and MBP3_16, a DARPin which is directed against the MalE protein from *Escherichia coli* (Binz et al., 2004).

2. Materials and methods

2.1. Buffers and genetic constructions

Buffer A was 500 mM NaCl, 50 mM Tris–HCl, pH 8.0; buffer B, as buffer A but pH 7.5; buffer C, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4; buffer D, 0.005% (v/v) Tween 20, 0.1 mg/mL BSA in buffer C; buffer E, 5 mM dithiothreitol (DTT) in buffer D; buffer F, 0.005% (v/v) Tween 20 and 5 mM DTT in buffer C.

The *E. coli* strains NEB-Express-I^q (New England Biolabs), XL1-Blue (Bullock et al., 1987) and AVB99 (Smith et al., 1998) have been described. Plasmid pH4S codes for H4S, a Nanofitin which is directed against hen egg-white lysozyme (HEL) (Cinier et al., 2009). The sequence of H4S is identical to that previously published, except that residue Cys29 has been changed into Ser29 (Pecorari and Alzari, 2008). The numbering does not take an engineered extension NH2-MRGSHHHHHHG into account (Fig. S1 in Appendix A). Plasmid pQEMBP3_16 codes for MBP3_16, a DARPin which is directed against MalE (GenBank AY326426) (Binz et al., 2004). All the recombinant proteins carried a hexahistidine tag (H6). Changes of residues were introduced by mutagenesis of the expression plasmids as described (Brient-Litzler et al., 2010).

2.2. Production and characterization of proteins and conjugates

The parental protein H4S(wt) and its mutant derivatives were produced in the cytoplasm of the recombinant strain NEB-Express-I^q(pH4S) and derivatives. The MalE protein was produced in the cytoplasm of XL1-Blue(pQEMBP), bt-MalE in AVB99(pAT224) and MBP3_16 and its mutant derivatives in XL1-Blue(pQEMBP3_16) and derivatives as described (Binz et al., 2004). The proteins were purified by affinity chromatography on a column of fast flow Ni-NTA resin (Qiagen) and eluted with imidazole, in buffer A or B according to their pI value. The analysis of the purification fractions by SDS-PAGE in the presence or absence of 2.5% (v/v, 0.4 M) 2-mercaptoethanol, the quantification of the protein bands, and the measurement of the protein concentrations by absorbance spectrometry were performed as described (Brient-Litzler et al., 2010). The pure fractions (>98% homogeneous in reducing conditions), were pooled and kept at −80 °C. The conjugates between N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester; Invitrogen) and the cysteine mutants of either H4S or MBP3_16 were prepared essentially as described (Section S1 in Appendix A) (Brient-Litzler et al., 2010). The conjugate between 2-mercaptoethanol and the IANBD ester was prepared by mixing the two molecules in stoechiometric amounts and then incubating the mixture for 30 min at 25 °C. In the following paragraphs, all the characterizations of proteins and conjugates were performed at 25 °C. In addition, those of the cysteine mutants were performed in the presence of 5 mM DTT to reduce any intermolecular disulfide bond.

2.3. Fluorescence variation and antigen binding: theory

A conjugate (or biosensor) B and antigen A form a 1:1 complex B:A, with a dissociation constant K_d, according to the reaction:

$$B + A \leftrightarrow B:A \tag{1}$$

The total concentration $[B]_0$ was kept constant and the total concentration $[A]_0$ was varied in titration experiments. The fluorescence intensity F of the conjugate at a given value of $[A]_0$ satisfies the following equation:

$$\frac{(F - F_0)}{F_0} = \frac{\Delta F}{F_0} = \left(\frac{\Delta F_\infty}{F_0}\right)\left(\frac{[B:A]}{[B]_0}\right) \tag{2}$$

where F_0 and F_∞ are the values of F at zero and saturating concentrations of A, $\Delta F = (F - F_0)$ and $\Delta F_\infty = (F_\infty - F_0)$ (Renard et al., 2003). The values of $\Delta F_\infty/F_0$ and K_d were determined by fitting Eq. (2), in which [B:A] is deduced from the equations of equilibrium and mass conservation, to the experimental values of $\Delta F/F_0$, measured in a titration experiment as described (Eq. (S4) in Appendix A) (Brient-Litzler et al., 2010; Renard et al., 2003).

The sensitivity s and relative sensitivity s_r of a conjugate can be defined by the following equations for the low values of $[A]_0$, i.e. in the initial part of the titration curve:

$$\Delta F = s[A]_0 \tag{3}$$

$$\frac{\Delta F}{F_0} = \frac{s_r[A]_0}{[B]_0} \tag{4}$$

s and s_r can be expressed as functions of characteristic parameters of the conjugate:

$$s_r = \left(\frac{\Delta F_\infty}{F_0}\right)\left(\frac{[B]_0}{(K_d + [B]_0)}\right) \tag{5}$$

$$s = f_b s_r \tag{6}$$

where $f_b = F_0/[B]_0$ is the molar fluorescence of the free conjugate (Renard and Bedouelle, 2004). The lower limit of detection $\delta[A]_0$ of the conjugate is linked to the lower limit of measurement of the spectrofluorometer δF by the following equations:

$$\delta[A]_0 = s^{-1}\delta F = s_r^{-1}[B]_0\left(\frac{\delta F}{F_0}\right) \tag{7}$$