Minireview

# Allosteric enzymes as biosensors for molecular diagnosis

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**Abstract Biosensors are hybrid analytical devices that amplify signals generated from the specific interaction between a receptor and the analyte, through a biochemical mechanism. Biosensors use tissues, whole cells, artificial membranes or cell components like proteins or nucleic acids as receptors, coupled to a physicochemical signal transducer. Allosteric enzymes exhibit a catalytic activity that is modulated by specific effectors, through binding to receptor sites that are distinct from the active site. Several enzymes, catalyzing easily measurable reactions, have been engineered to allosterically respond to specific ligands, being themselves the main constituent of new-generation biosensors. The molecular basis, robustness and application of allosteric enzymatic biosensing are revised here.**


*Key words:* Adaptive binding; Biosensor; Diagnosis; Molecular interaction; Protein engineering

## 1. The biosensor concept

Biosensors are hybrid devices that transform chemical information into an analytically useful signal by means of a biochemical mechanism [1]. Biosensors consist of a receptor system, in which a biological component interacts specifically with a given analyte, and a coupled physicochemical transducer that amplifies the signal resulting from such interaction from molecular up to macroscopical level (Fig. 1A). The purpose of these constructs is the identification, quantification and eventual screening of specific molecules, as present in complex mixtures from moderate to very low concentrations. Therefore, biosensors have utility in analytical research but also in clinical diagnosis, food and pharmaceutical industry, environmental control and process monitoring [2]. The interest in biosensor development has partly arisen from the need of fast and routine analysis of a large number of samples, what requires robustness, sensitivity and reproducibility [3,4]. Then, additional features like low cost of production, miniaturization (for instance as microchips), simple instrumentation and automatic sample processing are extremely desirable, especially for everyday food and diagnostic analysis [5].

The integration of the biological, sensing compound and the signal transducer into a biosensor can be approached through multiple ways. In addition, a wide set of available biological receptors and transducer devices, that are appropriate as sensor components, results in a notable diversity of the instrumental platform for biosensing technology [6]. Enzymes, antibodies, membranes, nucleic acids, whole cells and even tissues are the most used biological receptors for analyte recognition. On the other hand, electrodes, semiconductors, optical components and microbalances are among the most used partner transducers. Essentially, these instruments can generate either optical or electric signals which are very convenient for instrument development, and the commercial application of many biosensors in research but also in industry is noticeable [7].

## 2. Allosteric enzyme modulation

Allosteric enzymes exhibit regulatable catalytic activities upon the binding of an effector molecule, to a receptor site of the enzyme that is different from the active site. In some cases, modulation occurs through binding to distinct, alternative sites, either inhibitory or stimulatory [8]. The mechanics of allosteric modulation depends on a plastic enzyme architecture that allows functional fluctuations of the enzyme active site, triggered by relatively distant intermolecular interactions [9,10]. These remote effects are caused by the binding to protein areas with high conformational flexibility [11,12], or by promoting the association or dissociation of oligomeric enzymes [13–15], as they can be more active in either assembled or disassembled forms.

Allosteric enzymes that catalyze the formation of easily detectable products are potential biosensors (Fig. 1B). The receptor site acts as the recognition element, the active site as the transducer element and the whole enzyme integrates both pieces trough its own structure, and transmits the binding signal via conformational changes. Natural allosteric enzymes, however, cannot be directly used as biosensors since most of their modulators are devoid of analytical interest. However, it would be possible to incorporate, by modular engineering, sensing elements to be displayed at specific sites on the enzyme's surface, that being ligands for relevant analytes, would enable the enzyme to catalytically respond to new effectors. However, as discussed below, the allosteric biosensor prototypes that have been constructed up to now, are the result of trial-and-error approaches rather than of rational design.

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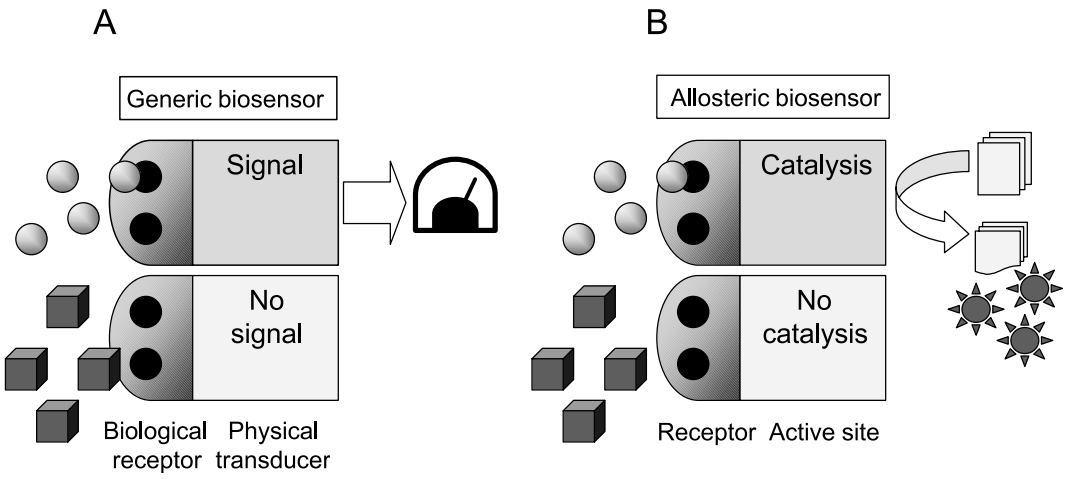


Fig. 1. Generic biosensors (A) are hybrid devices including a biological component acting as the analyte-binding site, and a physicochemical signal transducer that converts the specific interaction with the analyte into a macroscopic signal. Allosteric enzymatic sensors (B) respond to specific effectors through variations (generally up to modulation) of the catalysis rate. In these compact biosensors, the enzyme itself contains both the receptor site and the signal transducer (the catalytic site). The signal is transmitted to the active site by conformational changes triggered by the adaptive binding of the analyte, and the products monitored by standard analytical procedures. In general, the analyte enhances the specific activity from moderate to high. Ideally, the enzyme biosensor should be inactive but fully activable by the effector.

## 3. Enzymes engineered as allosteric biosensors

### 3.1. β-Galactosidase

*Escherichia coli* β-galactosidase hydrolyzes lactose to generate glucose and galactose during the bacterial heterotrophic growth, but also several lactose analogs producing colored, luminescent or fluorescent compounds. The availability of such alternative substrates, specially those that are colorimetric such as 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-gal) or ortho-nitrophenyl β-D-galactopyranoside (ONPG), has strongly supported the use of this enzyme as reporter for analysis of gene expression [16], as a marker for gene cloning and plasmid construction [17] and as partner in fusion proteins for structural stabilization [18], process monitoring [19] and purification [20]. Structurally, it is composed by four identical subunits of 116 000 Da interacting through long and activating interfaces, and displaying four active sites formed by mutual protrusion of the activating interfaces [21]. Among others, the activity of *E. coli* β-galactosidase is modulated by $Mg^{2+}$ [22], by thiol reagents [23] and interestingly by antibodies raised against the enzyme [24], proving a structurally responsible architecture with potential for biosensing.

Among a series of hybrid enzymes in which an antigenic peptide of foot-and-mouth disease virus VP1 capsid protein was displayed in solvent-exposed regions [25,26], two of them showed relevant up-modulation upon binding of anti-peptide monoclonal antibodies. Interestingly, in these two allosteric constructs the viral peptide had been inserted close to the active site and like in other insertional mutants, their specific activity was lower than in the wild-type enzyme. The allosteric enhancement of the hydrolysis rate was titer dependent [27,28] and occurred either through a reduction of $K_m$ or a raise of $k_{cat}$ [29]. Bivalent antibody binding is not required for activation, but the simultaneous interaction with antibodies by more than one enzyme subunit largely expands the catalytic signal [30]. Although the extent of activation promoted by the antibodies was firstly observed between 1.8- and 2-fold, it was enlarged up to more than 4-fold (Table 1) by exploring alternative reaction conditions combined with multiple insertions of the antigenic peptide in the enzyme [31]. These extensively engineered enzymes, displaying up to 12 copies of the peptide, were highly responsive to sera from virus-infected animals [32], proving a high sensitivity and robustness for diagnosis in homogeneous assays. A related series of β-galactosidase sensors, displaying an antigenic peptide from the human immunodeficiency virus (HIV-1) gp41 structural protein, also rendered excellent responses in front of human sera when compared with commercial diagnostic immunoassays [33].

### 3.2. Alkaline phosphatase

*E. coli* alkaline phosphatase is an homodimeric, non-specific

Table 1
Features or engineered allosteric enzymes for homogeneous immunoassays

| Enzyme | Modulation factor | Peptide length[a] | Catalytic modification[b] | Sensing element | Effector | References |
|---|---|---|---|---|---|---|
| β-Galactosidase | 1–4.5 | 15–45 | $\downarrow K_m$; $\uparrow k_{cat}$ | viral antigenic sites | antibodies | [26–28,32] |
| Alkaline phosphatase | 0.5–3 | 13–15 | $\downarrow\uparrow k_{cat}$ | viral antigenic sites | antibodies | [33,34] |
| TEM-1 β-lactamase | 0.06–1.7 | 3–6 | steric hindrance and $\downarrow\uparrow k_{cat}$ | random mimotopes | antibodies | [35] |
| TEM-1 β-lactamase | 0.77–1.43 | 6–12 | steric hindrance and $\downarrow\uparrow k_{cat}$ | random paratopes | streptavidin, ferritin and β-galactosidase | [37] |

[a]In some cases, replacements or partial replacements were done rather than solely insertions. Lengths indicated here refer exclusively to the incorporated foreign peptide sequences.
[b]Proven or suspected.

phosphomonoesterase whose activity is highly appreciated for analytical purposes because of its colorimetric detection. The insertion of an HIV peptide from the structural protein gp120 in the vicinity of the active site rendered a fully active enzyme, but the presence of anti-peptide antibodies inhibited the catalytic rate up to 40–50% [34]. The introduction of either two independent point mutations in the enzyme, however, inverted the sensing response by promoting enzyme activation upon antibody binding (Table 1). The performance of both up- and down-responsive biosensors was further confirmed by using an antigenic peptide from hepatitis C virus, the resulting constructs responding consistently with those carrying the HIV epitope [35].

### 3.3. β-Lactamase

A bacteriophage-transported TEM-1 β-lactamase was submitted to random insertional mutagenesis to explore permissive sites for foreign peptide display, and the resulting libraries are screened by biospanning on immobilized monoclonal antibodies against the prostate specific antigen [36]. The activity of the isolated enzymes, containing mimotopes for those antibodies, was tested upon antibody binding, showing a general and quantitatively important down-regulation by steric hindrance of the active site. However, the clone P66L4-06 responded to the monoclonal antibody PSA66 by enhancing the hydrolysis rate of the substrate Centa up to 1.7-fold and by a slower catalysis of substrate PADAC (35% of the wild-type enzyme). The behavior of this particular construct was probably allosteric, since its enzymatic response was comparable to those of β-galactosidase and alkaline phosphatase constructs [37]. Further insertional mutagenesis of TEM-1 β-lactamase generated a set of peptide-displaying, hybrid enzymes responsive, although at much lesser extent, to non-antibody ligands such as streptavidin, ferritin and β-galactosidase [38].

### 3.4. Green fluorescent protein (GFP)

*Aequorea victoria* GFP was modified to accommodate the complete sequence of TEM-1 β-lactamase, in a predefined, solvent-exposed region in the vicinity of the fluorophore [39]. This construct responded to the binding of the β-lactamase inhibitory protein BLIP by a dramatic increase in fluorescence, without changes in the emission spectrum. An end terminal fusion of GFP carrying a complete β-lactamase was not initially stimulated by BLIP, but after random mutagenesis several responsive variants were isolated. In general, directed evolution procedures have been proven useful to improve the performance of these GFP-derived biosensors [40].

### 3.5. Neural protease

A recent and rare example of rational design in enzymatic biosensor development has been inspired in the principle of intrasteric regulation that rules some natural enzymes [41]. In a partially synthetic construct containing the *Cereus* neural protease, its phosphoramidite inhibitor has been covalently attached through a short and flexible single-stranded (ss)DNA hinge, thus blocking the enzyme activity [42]. The presence of complementary DNA molecules, hybridizing with the hinge DNA segment and restricting its flexibility, promotes the release of the inhibitor and switches on the protease. Its activity, which results then to be dependent on the concentration of DNA molecules with specific sequence, can be detected by fluorescence through the hydrolysis of peptidic substrates containing both a fluorophore and a quencher.

## 4. Ribozymes and allosteric biosensing

Nucleic acids exhibit variations in their catalytic activity upon binding of different ligands, especially small organic effectors [43,44]. This allosteric property can be further enhanced by appropriate engineering and exploited for biosensor and biochip construction, mainly based on ribozymes, as shown in recent particular examples and reviews [45–47]. Although a solely modular rational approach seems to be not sufficient for successful biosensor performance, in combination with directed molecular evolution it has resulted into fascinating prototype molecules [48].

## 5. Concluding remarks

Allosteric enzymatic regulation is very promising as the basis for fast and reliable diagnosis via targeted molecular sensing. Both allosteric and non-allosteric enzymes can be engineered to respond to specific effectors, through variations (desirably up-modulation) of their specific activity. Interestingly, the enzymatic sensing is ideal for homogeneous assays and suitable for miniaturization and automatic processing. Although the up-to-now available prototypes have been mainly obtained by trial-and-error approaches, there are increasing examples of successful rational design. Furthermore, both straight protein engineering in the nearby of the active site, and directed molecular evolution have been proven as useful technologies to improve the robustness and sensitivity of enzymatic biosensors. Antibodies, being critical analytes in the diagnosis of most infectious diseases, are appropriate targets for allosteric sensing, since their adaptive binding efficiently triggers the structural transitions required for enzymatic modulation. However, analytes other than antibodies can also be allosterically detected, proving the enormous potential and wide spectrum of applications of the enzyme-based biosensing.

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## References

[1] Thévenot, D.R., Toth, K., Durst, R.A. and Wilson, G.S. (2001) Biosens. Bioelectron. 16, 121–131.
[2] Nakamura, H. and Karube, I. (2003) Anal Bioanal. Chem., in press.
[3] Wollenberger, U., Schubert, F., Pfeiffer, D. and Scheller, F.W. (1993) Trends Biotechnol. 11, 255–262.
[4] Scheller, F.W., Pfeiffer, D., Hintsche, R., Dransfeld, I., Wollenberger, U. and Schubert, F. (1990) Ann. N. Y. Acad. Sci. 613, 68–78.
[5] Madou, M. and Tierney, M.J. (1993) Appl. Biochem. Biotechnol. 41, 109–128.
[6] Huber, A., Demartis, S. and Neri, D. (1999) J. Mol. Recognit. 12, 198–216.
[7] Rich, R.L. and Myszka, D.G. (2002) J. Mol. Recognit. 15, 352–376.
[8] Yeo, G.F. and Madsen, B.W. (1998) Biochim. Biophys. Acta 1372, 37–44.
[9] Yon, J.M., Perahia, D. and Ghelis, C. (1998) Biochimie 80, 33–42.
[10] Hammes, G.G. (2002) Biochemistry 41, 8221–8228.

[11] Hoischen, C., Levin, J., Pitaknarongphorn, S., Reizer, J. and Saier Jr., M.H. (1996) J. Bacteriol. 178, 6082–6086.
[12] Weyand, M., Schlichting, I., Herde, P., Marabotti, A. and Mozzarelli, A. (2002) J. Biol. Chem. 277, 10653–10660.
[13] Traut, T.W. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 125–163.
[14] Traut, T.W., Ropp, P.A. and Poma, A. (1991) Adv. Exp. Med. Biol. 309B, 177–180.
[15] Kurganov, B.I. (1983) J. Theor. Biol. 103, 227–245.
[16] Casadaban, M.J., Martinez-Arias, A., Shapira, S.K. and Chou, J. (1983) Methods Enzymol. 100, 293–308.
[17] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Yang, S., Veide, A. and Enfors, S.O. (1995) Biotechnol. Appl. Biochem. 22 (Pt. 2), 145–159.
[19] Benito, A., Valero, F., Lafuente, J., Vidal, M., Cairo, J., Sola, C. and Villaverde, A. (1993) Enzyme Microb. Technol. 15, 66–71.
[20] Ullmann, A. (1984) Gene 29, 27–31.
[21] Jacobson, R.H., Zhang, X.J., DuBose, R.F. and Matthews, B.W. (1994) Nature 369, 761–766.
[22] Martinez-Bilbao, M. and Huber, R.E. (1996) Biochem. Cell Biol. 74, 295–298.
[23] Golan, R., Zehavi, U., Naim, M., Patchornik, A., Smirnoff, P. and Herchman, M. (2000) J. Protein Chem. 19, 123–128.
[24] Celada, F. and Strom, R. (1983) Biopolymers 22, 465–473.
[25] Benito, A., Mateu, M.G. and Villaverde, A. (1995) Biotechnology (NY) 13, 801–804.
[26] Feliu, J.X. and Villaverde, A. (1998) FEBS Lett. 434, 23–27.
[27] Feliu, J.X., Ramirez, E. and Villaverde, A. (1998) FEBS Lett. 438, 267–271.
[28] Benito, A., Feliu, J.X. and Villaverde, A. (1996) J. Biol. Chem. 271, 21251–21256.
[29] Feliu, J.X., Ramirez, E. and Villaverde, A. (2000) FEBS Lett. 473, 123.
[30] Alcalá, P., Ferrer-Miralles, N. and Villaverde, A. (2002) Biotechnol. Lett. 24, 1543–1551.
[31] Cazorla, D., Feliu, J.X., Ferrer-Miralles, N. and Villaverde, A. (2002) Biotechnol. Lett. 24, 467–477.
[32] Feliu, J.X., Ferrer-Miralles, N., Blanco, E., Cazorla, D., Sobrino, F. and Villaverde, A. (2002) Biochim. Biophys. Acta 1596, 212–224.
[33] Ferrer-Miralles, N., Feliu, J.X., Vandevuer, S., Muller, A., Cabrera-Crespo, J., Ortmans, I., Hoffmann, F., Cazorla, D., Rinas, U., Prevost, M. and Villaverde, A. (2001) J. Biol. Chem. 276, 40087–40095.
[34] Brennan, C., Christianson, K., Surowy, T. and Mandecki, W. (1994) Protein Eng. 7, 509–514.
[35] Brennan, C.A., Christianson, K., La Fleur, M.A. and Mandecki, W. (1995) Proc. Natl. Acad. Sci. USA 92, 5783–5787.
[36] Legendre, D., Soumillion, P. and Fastrez, J. (1999) Nat. Biotechnol. 17, 67–72.
[37] Ferrer-Miralles, N., Feliu, J.X. and Villaverde, A. (2000) Biochem. Biophys. Res. Commun. 275, 360–364.
[38] Legendre, D., Vucic, B., Hougardy, V., Girboux, A.L., Henrioul, C., Van Haute, J., Soumillion, P. and Fastrez, J. (2002) Protein Sci. 11, 1506–1518.
[39] Doi, N. and Yanagawa, H. (1999) FEBS Lett. 453, 305–307.
[40] Doi, N. and Yanagawa, H. (2002) Methods Mol. Biol. 183, 49–55.
[41] Kobe, B. and Kemp, B.E. (1999) Nature 402, 373–376.
[42] Saghatelian, A., Guckian, K.M., Thayer, D.A. and Ghadiri, M.R. (2003) J. Am. Chem. Soc. 125, 344–345.
[43] Soukup, G.A. and Breaker, R.R. (2000) Curr. Opin. Struct. Biol. 10, 318–325.
[44] Soukup, G.A. and Breaker, R.R. (1999) Trends Biotechnol. 17, 469–476.
[45] Soukup, G.A. and Breaker, R.R. (1999) Struct. Fold Des. 7, 783–791.
[46] Soukup, G.A. and Breaker, R.R. (1999) Proc. Natl. Acad. Sci. USA 96, 3584–3589.
[47] Steele, D., Kertsburg, A. and Soukup, G. (2003) Am. J. Pharmacogenomics 3, 131–144.
[48] Breaker, R.R. (2002) Curr. Opin. Biotechnol. 13, 31–39.