

Protein engineering and the development of generic biosensors

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Biosensors exploit the remarkable specificity of biomolecular recognition to provide analytical tools that can measure the presence of a single molecular species in a complex mixture. A new strategy is emerging in the development of biosensor technologies: molecular-engineering techniques are being used to adapt the properties of proteins to simple, generic detector instrumentation, rather than adapting instruments to the unique requirements of a natural molecule.

In a biosensor, molecular sensing is provided by the interaction between a biological macromolecule, such as a nucleic acid or a protein, and its cognate ligand. Detection occurs at two levels – molecular signal transduction, in which the physical properties of a macromolecule change upon binding, and macroscopic signal determination, to detect this change. The interplay between this molecular signal-transduction event and the detection instrumentation is central to the construction of effective biosensor technologies. The development of most biosensors involves the identification of a naturally occurring macromolecule that provides the desired analyte specificity (typically an enzyme or antibody), the discovery of a suitable signal and the construction of a detector adapted to the macromolecule in question¹. Although effective biosensors have been developed in this way, each device is unique and requires substantial development time and optimization. To overcome this limitation, new approaches are being developed in which protein-engineering techniques are being used to adapt the signal-transduction properties of biological molecules to the detector instrumentation, rather than adapting instruments to the unique requirements of each natural molecule. This is achieved by integrating functional groups that provide simple signal-transduction mechanisms, such as optical or electrochemical changes, directly into the proteins themselves. The aim is to develop a modular molecular-engineering system in which the integrated signal-transduction function and the binding site are so constructed that each can be changed separately without destroying the communication between them. Ultimately, the goal is systematically to isolate families of molecules that vary in their binding site but retain a constant signal-transduction function, thereby creating generic biosensors that share the same detection instrumentation and measure a wide range of analytes.

Ideally, such molecular sensors should be homogeneous (reagentless), so that the measurement does not require a change in the composition of the sensor, unlike heterogeneous systems, which consume either the analyte (enzyme-based systems) or a signal-transduction component (e.g. displacement of a labelled antigen in a competitive immunoassay). The

direct integration of the signal-transducing function into the macromolecule itself fulfils this design parameter. Furthermore, the synthesis of the sensing element should be straightforward. In that respect, the simplest system is one in which the reporter function is completely intrinsic to the molecule and does not need to be introduced by the covalent modification or assembly of several macromolecular components. Finally, there should be a readily accessible method to generate the molecular diversity for the isolation of a panel of binding sites. Again, protein-engineering techniques have been developed that can address this problem, and so it is no longer necessary to rely on natural diversity alone. In this article, we survey newly emerged model systems and attempt to point out how recent advances in protein-engineering techniques will be used to build upon these early results to combine a repertoire of binding sites with optimized, integrated molecular signal-transduction functions.

Basic strategies

Two strategies have been used to design modular protein-engineering systems for biosensor development: (1) find a protein with the appropriate specificity and introduce a signal-transduction function; (2) identify a protein with a particularly well-behaved intrinsic signal-transduction function and construct appropriate binding sites.

The most commonly employed approach is to couple a reporter group, such as a fluorophore, covalently to a protein with the desired binding characteristics. Such prosthetic groups can be introduced by direct incorporation into the polypeptide using chemical synthesis^{2,3} or, more usually, by covalently attaching appropriately functionalized derivatives to site-specifically positioned reactive thiols; these are usually introduced by constructing cysteine point mutations using oligonucleotide-directed mutagenesis^{4–11}, although they can be introduced by substrate-mediated thiolation of the protein¹², thereby avoiding the use of genetically modified proteins. A variety of optical sensors have been constructed by the site-specific incorporation of fluorophores that transduce binding events by changes in either fluorescence-resonance-energy transfer (FRET) between pairs of fluorophores^{2,4–6} or the microenvironment of single fluorophores^{3,7–12}.

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An interesting alternative to the site-specific introduction of reporter functions has been the construction of macromolecular assemblies in which the recognition and signalling functions reside in different molecules. A family of electrochemical sensors has been constructed in this way by combining antibodies and pore-forming transmembrane peptides¹³.

Only a few systems have been identified that possess intrinsic signal-transduction functions suitable for modulation by the introduction of an engineered binding site: α -haemolysin, a bacterial transmembrane pore protein, has been successfully used to construct electrochemical sensors¹⁴; the remarkable properties of the green fluorescent protein (GFP) have been exploited to construct an optical sensor^{15,16}; and alkaline-phosphatase activity has been modulated by inserting antibody-binding epitopes into its active site¹⁷.

The biomolecular sensing elements are frequently immobilized to construct flow cells, to form patterned surfaces by creating multichannel arrays or as a consequence of the detector technology itself. Genetic engineering can be used to introduce functional groups that allow the oriented immobilization of a protein onto an appropriate chemically derivatized surface. These include the attachment of a protein to glass surfaces derivatized with thiols by the introduction of cysteine mutations in the protein¹⁸, to iminodiacetate-derivatized surfaces via a metal-mediated link to oligohistidine peptides fused to the C-terminus of the protein¹⁹, or to surfaces coated with a streptavidin monolayer, to which the protein binds via C-terminal fusions of peptides that mimic biotin binding^{20,21}.

Using these protein-engineering approaches, sensors have been constructed for various metals^{2, 4,14-16,22,23}, phosphate⁸, small organic molecules such as cAMP⁶, maltose^{7,9} and glucose¹⁰, and a variety of macromolecules^{11,13,17}. Some have already been incorporated into analytical instrumentation^{4,13}; others have properties that allow them to be used for real-time, *in vivo* measurements at the cellular level^{9,8,15,16,24,25}. In the following sections, we examine some of these systems in more detail.

Adapting natural binding proteins by introducing reporter groups

Bacterial periplasmic binding proteins

The periplasmic space in *Escherichia coli* and *Salmonella typhimurium* contains a family of ligand-binding proteins that form part of the chemotaxis and nutrient-uptake systems and are specific for a wide variety of ligands with binding constants in the 100 nM range. These proteins have been extensively characterized by genetic, biochemical and structural studies, and found to share little sequence identity, be diverse in molecular weight, but have a similar overall structure (a single chain that folds into two domains connected by a hinge region)²⁶. The ligand-binding site is invariably located in the interface between the two domains. It has been shown in a number of proteins that binding is accompanied by a large conformational change, in which the two domains close around the ligand, involving a bending and twisting motion around the hinge region. This has been exploited to introduce single fluorophores that respond to ligand binding in the phosphate binding protein⁸ (PBP), maltose binding protein^{7,9} (MBP) and glucose/galactose binding protein¹⁰ (GBP). None of these proteins have been incorporated into an analytical device as yet, but the PBP-based sensor has been used to great effect to measure inorganic-phosphate release in muscle^{8,24}.

In each case, the transducing element was provided by the site-specific introduction of microenvironment-sensitive fluorophores coupled to unique cysteines constructed by site-directed mutagenesis. Two approaches have been used to set up the signal-transduction mechanism (Fig. 1). In the first, the fluorophores are positioned in the binding pocket^{7,8,10}, so that changes in fluorescence are a consequence of direct interactions with the bound ligand^{10,27}. Although conceptually straightforward, requiring only that the locations of residues in the binding site are known, it has the disadvantage that it can be accompanied by unfavourable steric interactions between the fluorophore and the ligand, which lower the ligand affinity. In the second approach, the fluorophores are positioned in locations

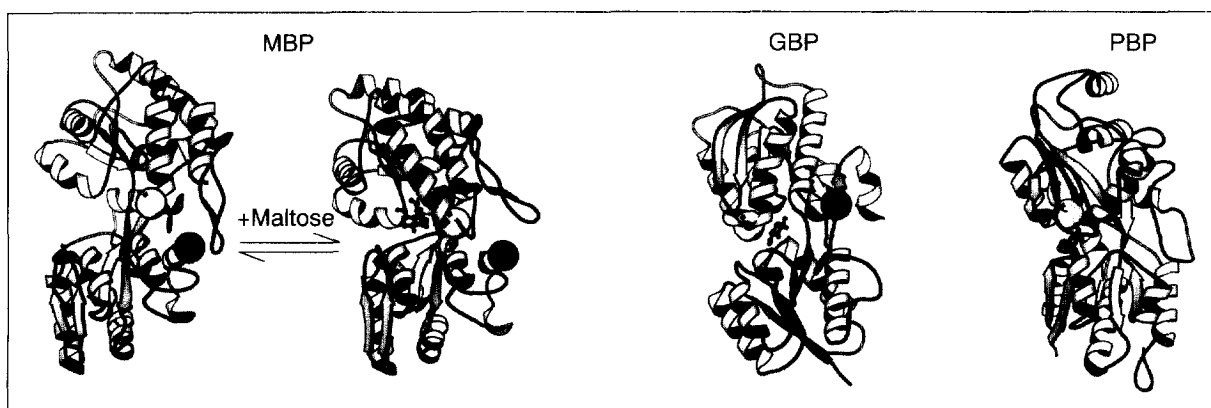


Figure 1

Fluorescent-signal transduction in engineered *Escherichia coli* periplasmic binding proteins (MBP, maltose binding protein^{7,9,27}; GBP, glucose/galactose binding protein¹⁰; PBP, phosphate binding protein⁸). Each protein consists of two domains separated by a flexible hinge region. The structures of MBP with and without bound maltose reveal extensive rearrangement of the two domains upon ligand binding. This can be detected in each of these proteins by placing microenvironmentally sensitive fluorophores either in the ligand-binding pocket (yellow) or in allosterically coupled sites (red). Structures were generated using MolScript⁴⁰ from coordinates obtained from the Brookhaven Protein Data Bank (accession numbers: apo-MBP, 1omp; maltose-MBP, 1mdq; GBP, 1glg; PBP, 1qul).

distant from the binding site and sense ligand binding indirectly via an allosteric-coupling mechanism based on detection of the domain movements^{9,10}. For this to work, the allosteric sites must be located in regions that undergo a local conformational change in concert with the interdomain bending motion. In the case of MBP, the positions of such sites could be deduced by comparison of the experimentally determined structures of both protein conformations⁹. By structural homology, this analysis could be extended to GBP, which has not been studied in such structural detail¹⁰. The allosteric sensing mechanism has the advantage that there is no direct interaction between the fluorophore and the ligand, and, consequently, the binding constant is essentially unaffected. Furthermore, in the case of MBP, it could be shown that the binding site could be manipulated without destroying the allosteric linkage with the reporter group. In this way, the response of the maltose sensor could be 'tuned' for various concentration ranges, creating a sensor that accurately measures maltose over a six-orders-of-magnitude concentration range (0.1 μM –20 mM).

Metalloproteins

The detection of trace metals has numerous applications in basic biological research, as well as in environmental and clinical chemistry. Metalloproteins can display remarkably selective binding and high affinity. Three natural metal-incorporating motifs or metalloproteins have been engineered to incorporate fluorescent signal-transduction mechanisms: zinc fingers^{2,3,23}, carbonic anhydrase^{3–5,28–30} and calmodulin (see below).

Zinc fingers (ZFs) are small (25–30 residue) protein domains that selectively bind zinc with dissociation constants in the nM to pM range. The peptides are unfolded in the absence of zinc, and this zinc-dependent folding transition has been used to measure binding by observing changes in the fluorescence of fluorophores incorporated by total chemical synthesis of the peptide (Fig. 2). Folding can be followed either by using FRET between a donor-acceptor pair placed at the N- and C-termini of the peptide, respectively², or by the incorporation of a single microenvironmentally sensitive fluorophore in the hydrophobic core of the folded peptide³. The primary coordination sphere of the bound zinc is formed by two histidine and two cysteine residues, which means that ZFs are very susceptible to the formation of unwanted disulfide bonds through oxidation by molecular oxygen or redox-active transition metals such as copper or iron. Signal transduction does not involve direct interaction of the fluorophore with the coordinated zinc, and so the fluorophore and binding site can be manipulated and optimized independently in a modular manner²³. Thus, replacement of one of the cysteines with histidine, aspartate or glutamate could be explored as a method of identifying a peptide with a reduced sensitivity to oxidation.

Carbonic anhydrase (CA) is a metalloenzyme that has a tightly bound zinc ion located at its active site, coordinated by three histidines and a bound water. A zinc-biosensor system has been developed based on changes in the fluorescence properties of dansylamide³¹, a CA inhibitor that binds only in the presence of zinc. This basic scheme (Fig. 3) can be further improved by optimizing the dansylamide reporter group²⁸ or by setting

up FRET between the dansylamide and fluorophores at nearby locations on the protein surface, which can be introduced by general covalent modification of surface lysines³² or site-specifically by engineered cysteines⁵. Energy-transfer effects between the attached fluorophores and the electronic absorption spectra of bound transition metals can also be exploited⁴. Although this CA biosensor is not fully integrated (the signal-transduction element diffuses freely), it is an excellent model system, because the properties of the metal-coordination sphere are well understood and have been manipulated in a variety of interesting ways by genetic engineering³³.

The affinity of CA for zinc can be manipulated by mutagenesis. Structure-based design has been used to extend the primary coordination by introducing a fourth ligand, which results in an enzyme with femtomolar affinity ($K_d = 0.02 \text{ pM}$)³⁰. At the other extreme, changing one of the coordinating histidines to an

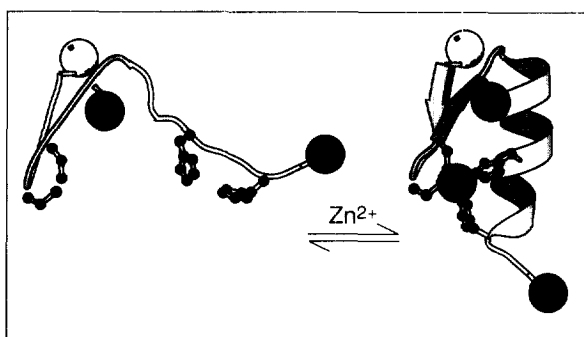


Figure 2

A zinc sensor based on the zinc finger (ZF) peptide. Zinc (purple) is bound by a His₂Cys₂ primary coordination sphere in the folded ZF. The ZF undergoes a folding transition upon metal binding, which can be monitored either by fluorescence-resonance-energy transfer between donor (yellow) and acceptor (red) fluorophores located at the N- and C-termini of the peptide² or by changes in the micro-environment of a fluorophore located in the hydrophobic core³ (green). Structures were generated using MolScript⁴⁰ from coordinates obtained from the Brookhaven Protein Data Bank (accession number 1zaa).

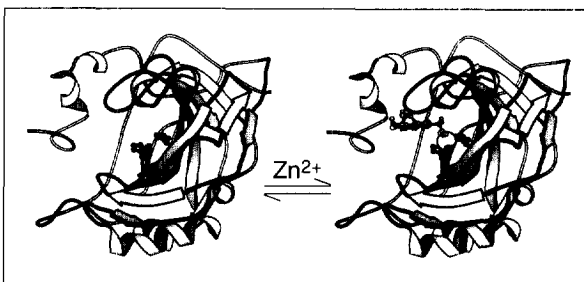


Figure 3

A metal sensor based on carbonic anhydrase (CA). Zinc (yellow) is bound by three histidines in the CA active site. The binding event is transduced by changes in the fluorescence properties of dansylamide (blue; the structure of this complex was not determined experimentally but generated by manual docking, guided by visual inspection), an inhibitor that binds only in the presence of zinc³¹. Structures were generated using MolScript⁴⁰ from coordinates obtained from the Brookhaven Protein Data Bank (accession number 1uga).

aspartate³⁴ created a protein with a $K_d = 70$ nM. Mutations in this secondary coordination resulted in variants with binding constants spanning the region between these two extremes³³. Ultimately, an array of CA variants could be constructed to measure zinc over a large range of concentrations.

The release of zinc from the enzyme is very slow (10^{-7} s⁻¹), ruling out the use of wild-type CA in real-time sensing applications. Remarkably, a single point mutation in the second coordination sphere, which converts one of the histidines in the primary coordination sphere from a hydrogen-bond donor to an acceptor³⁵, increases the rate of metal release to approximately 1 s⁻¹.

It is also possible to isolate binding mutants using genetic-screening approaches. A small combinatorial library has been constructed from three hydrophobic residues that flank two of the three histidines in the primary coordination sphere and are thought to affect the rigidity of this region. A zinc-benzenesulfonamide affinity resin was used to select metal-binding variants from this library²⁹. The resulting variants exhibited large changes in their zinc-binding constants and metal-release rates.

Adapting proteins with intrinsic reporter groups by engineering binding sites

Enzyme activity

Even though enzyme activity-based assays are, by definition, not reagentless, they offer the possibility of signal amplification by integrating over time. An appealing way to capitalize on a readily measured enzymic assay system is to link the enzyme activity to an allosteric effector site, the specificity of which can be manipulated in a modular fashion. The engineering of *E. coli* alkaline phosphatase has provided such an opportunity^{17,36}. Short (13–15 amino acids), linear, heterologous epitopes derived from the human-immunodeficiency-virus (type I) gp120 or hepatitis-C-viral proteins could be inserted at a distance of 35–40 Å from the active site (Fig. 4). Binding of monoclonal antibodies (mAbs) resulted in significant inhibition of enzyme activity with a K_i corresponding to the approximate affinity of the mAb for the epitope. Remarkably, the introduction of an additional single point mutation, known to increase the general flexibility of the active site, resulted in the activation of enzyme activity upon mAb binding. This suggests that the modulation of the activity by the bound mAb occurs via an allosteric mechanism involving changes in conformational states, rather than through steric hindrance. The observed modulation (three- to fourfold) and probable independence of the inserted amino acid sequence suggest that this system may be generally applicable for the detection of antibodies or protein receptors.

Green fluorescent protein: an intrinsic optical reporter

GFP contains a highly fluorescent fluorophore that is formed by the autocatalytic cyclization of a tripeptide contained within its own polypeptide sequence. This genetically encoded chromophore is the perfect candidate for constructing a biosensor system with an intrinsic fluorescent transducer function, provided that an appropriate binding site can be introduced. Two recent reports^{15,16} describe such a system.

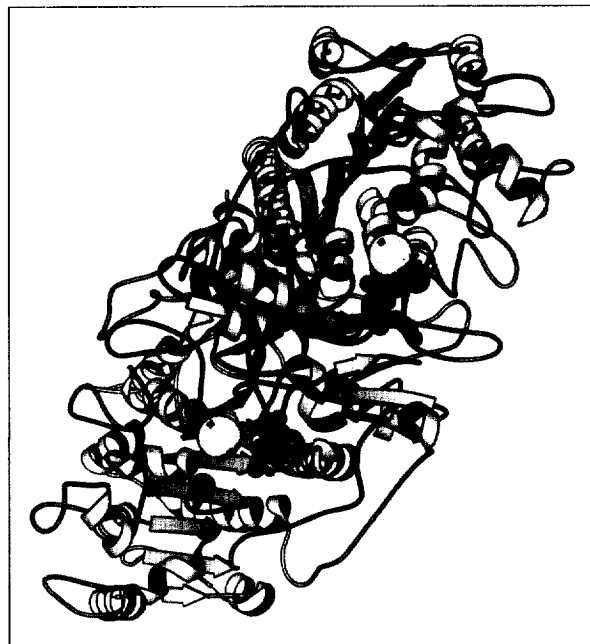


Figure 4

Allosteric antibody-binding sites in the alkaline-phosphatase dimer^{17,36} (AP). Viral epitopes can be inserted on the protein surface between residues 407 and 408 (red), located near the active site (yellow). Binding of antibodies to these epitopes partially inhibits AP activity. If additional single point mutations are constructed at positions 101 (blue) or 153 (purple), the bound antibody activates, instead of inhibiting, the enzyme. Structures were generated using MolScript⁴⁰ from coordinates obtained from the Brookhaven Protein Data Bank (accession number 1alk).

Calcium sensors have been constructed by creating a gene fusion in which two GFP variants are joined, either by a calmodulin-binding peptide (CaMBP) alone¹⁵, or by combining calmodulin (CaM) and CaMBP in one construct¹⁶ (Fig. 5). One of the GFP variants emits blue light that can excite the other GFP by FRET. Thus, the fluorescence of the system is modulated by the separation distance of the two proteins. This is dependent on the conformation of the intervening segment, which is, in turn, dependent on the Ca²⁺ concentration. In the absence of calcium, CaM assumes an extended structure and does not bind to CaMBP, which is in a random-coil conformation. Upon Ca²⁺ binding, CaM folds into a compact structure and binds CaMBP, which then becomes α helical. In the CaMBP fusion construct, the system is highly fluorescent in the absence of Ca²⁺ and loses fluorescence on Ca²⁺ binding, resulting in a sixfold change in the ratio of emissions at 440 nm to 505 nm. It is thought that the unstructured CaMBP allows the two GFPs to form antiparallel dimers that exhibit efficient FRET, and that Ca²⁺-dependent binding of CaM disrupts this dimer, increasing the distance between the fluorophores and resulting in destruction of the FRET. The CaM::CaMBP fusion appears to have a different structural mechanism, because the addition of Ca²⁺ decreases emission at 445 nm and increases emission at 510 nm, indicating improved FRET, albeit a much smaller effect than that observed in the CaMBP construct (70% change in ratio). This is interpreted as resulting from the formation of a compact structure by

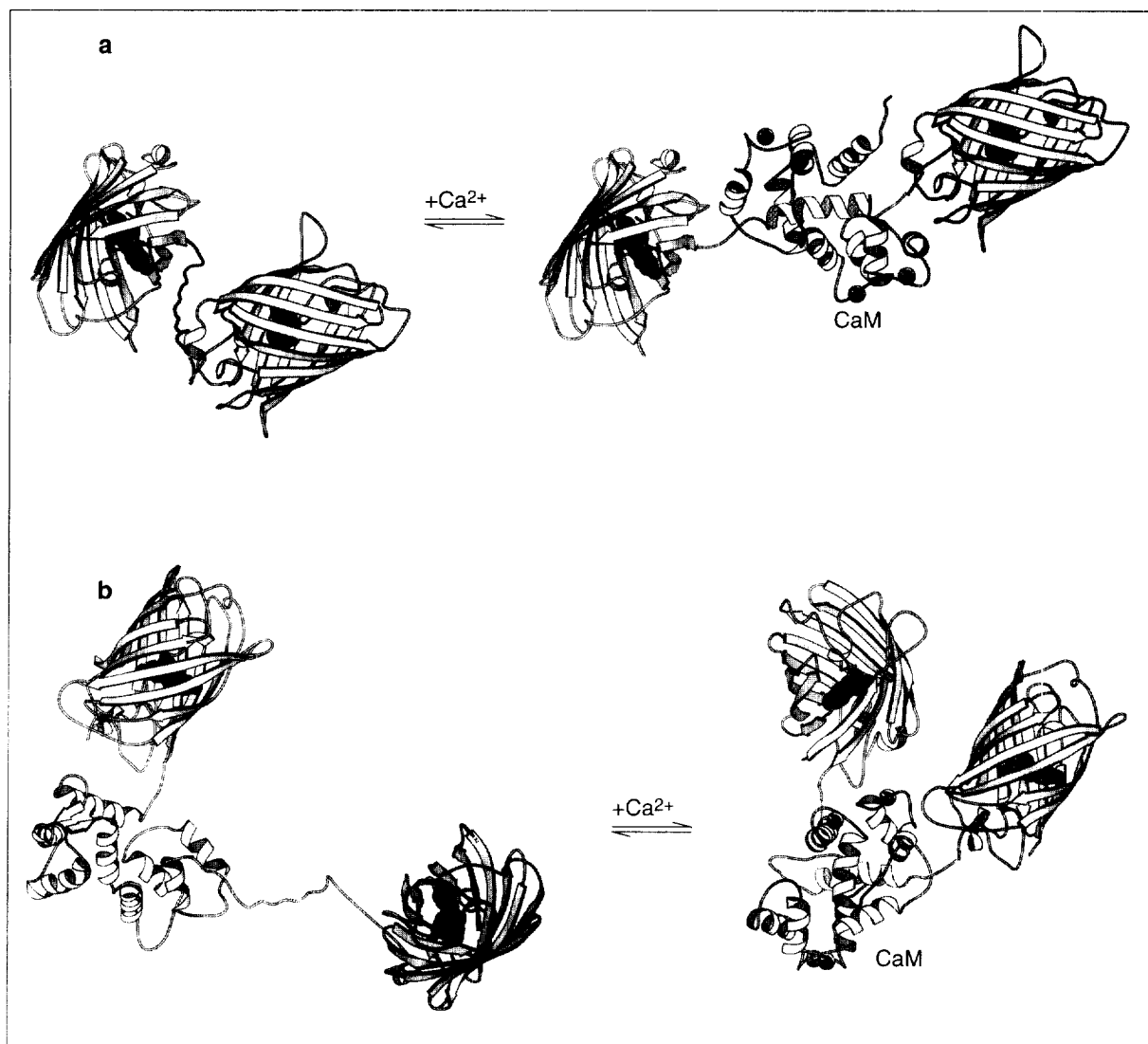


Figure 5

Calcium sensors based on the green fluorescent protein (GFP). **(a)** The blue-emitting GFP is separated from the green-emitting GFP by a calmodulin-binding peptide¹⁵ (red). In the absence of Ca^{2+} , the two GFPs form a dimer that allows efficient fluorescence-resonance-energy transfer (FRET) between the two intrinsic fluorophores in the protein (indicated in blue and green). Binding of Ca^{2+} (purple) stabilizes a conformation of calmodulin (CaM) that forms a complex with the calmodulin-binding peptide, thereby disrupting FRET between the GFPs. **(b)** The two GFPs are separated by a fusion that combines both calmodulin and calmodulin-binding peptide¹⁶. In the absence of Ca^{2+} , this segment assumes an extended conformation, and Ca^{2+} binding results in the formation of the compact form of calmodulin and binding of the peptide, bringing the two GFPs closer together. The change in FRET is not as pronounced in this system, indicating that the changes in the distribution of interchromophore distances are not very large. The structures shown here are modelled by combining the individual components. Structures were generated using MolScript⁴⁰ from coordinates obtained from the Brookhaven Protein Data Bank (accession numbers: GFP, 1ema; calmodulin-calmodulin-binding-peptide complex, 2bbm; apo-calmodulin, 1cfd).

the CaM::CaMBP segment, bringing the two GFPs closer to each other. Both systems have been used successfully for the measurement of intracellular calcium concentrations^{15,16}.

α -Haemolysin: an intrinsic electrochemical reporter system

Another intrinsic signal-transduction mechanism is provided by the measurement of electrical currents resulting from the opening and closing of protein pores in membranes. This is more complex than the use of an integrated fluorophore, because it involves the formation of a multicomponent macromolecular assembly and requires the fabrication of a robust lipid bilayer.

Staphylococcal α -haemolysin is an unusual membrane protein, in that it is particularly amenable to protein engineering and therefore well suited to use in sensor construction. Unlike most membrane proteins, it is relatively straightforward to manipulate biochemically, because it is expressed in a water-soluble form that is readily produced in an *E. coli* heterologous-expression system; this water-soluble form can then be inserted into certain membranes to form heptameric pores. It has been possible to engineer these pores genetically by introducing a number of triggers and switches that respond to different physical or chemical signals³⁷.

The channel in the pore is formed by a large β barrel. This structural motif is ideal for the introduction of

three histidine residues to form a primary coordination sphere for transition metals (in a similar way to CA). Based on this concept, a structure-based rational-design approach was used to construct such a metal-binding site at the bottom of the pore¹⁴. This construct is a sensitive metal sensor, because its single-channel currents are modulated by the addition of transition metals, each of which produces a characteristic distribution of channel opening and closing.

Tapping the greatest reservoir of natural binding diversity: engineered antibodies

Antibodies provide the most readily available source of diversity in binding sites. However, the development of a general method for incorporating signal-transduction functions into antibodies to construct homogeneous assay systems has been difficult. In some cases, it is possible to use chemical modification for the site-specific attachment of reporter groups¹². Recently, a very general approach has been developed in which antibodies are coupled to an electrochemical reporter function by macromolecular assembly¹³ to construct ion-channel switches (Fig. 6).

Gramicidin is a small helical peptide that spans half a lipid bilayer. An ion-conductive transmembrane channel is formed when two peptides located in the inner and outer membrane leaflet of the lipid bilayer align. Two types of gramicidin have been introduced into a membrane that is tethered to a gold surface by half-membrane-spanning lipids – (1) immobilized gramicidins attached to the gold surface and located in the inner leaflet, and (2) mobile biotinylated gramicidins in the outer leaflet. The assembly is completed with the introduction of two forms of biotinylated antibody – (1) mobile antibodies that are attached to the mobile gramicidin half-channels located in the outer leaflet, using a streptavidin adaptor molecule that binds both antibody and gramicidin molecules via their biotin groups, and (2) immobilized antibodies that recognize a different epitope on the surface of the macromolecular target antigen (analyte), and that are attached to a biotinylated membrane-spanning lipid that is itself tethered to the gold substratum. In the absence of antigen, the gramicidins in the outer leaflet move freely by diffusion and form intermittent ion-conductive channels by aligning with the tethered half-channels in the inner leaflet. The addition of antigen forms crosslinks

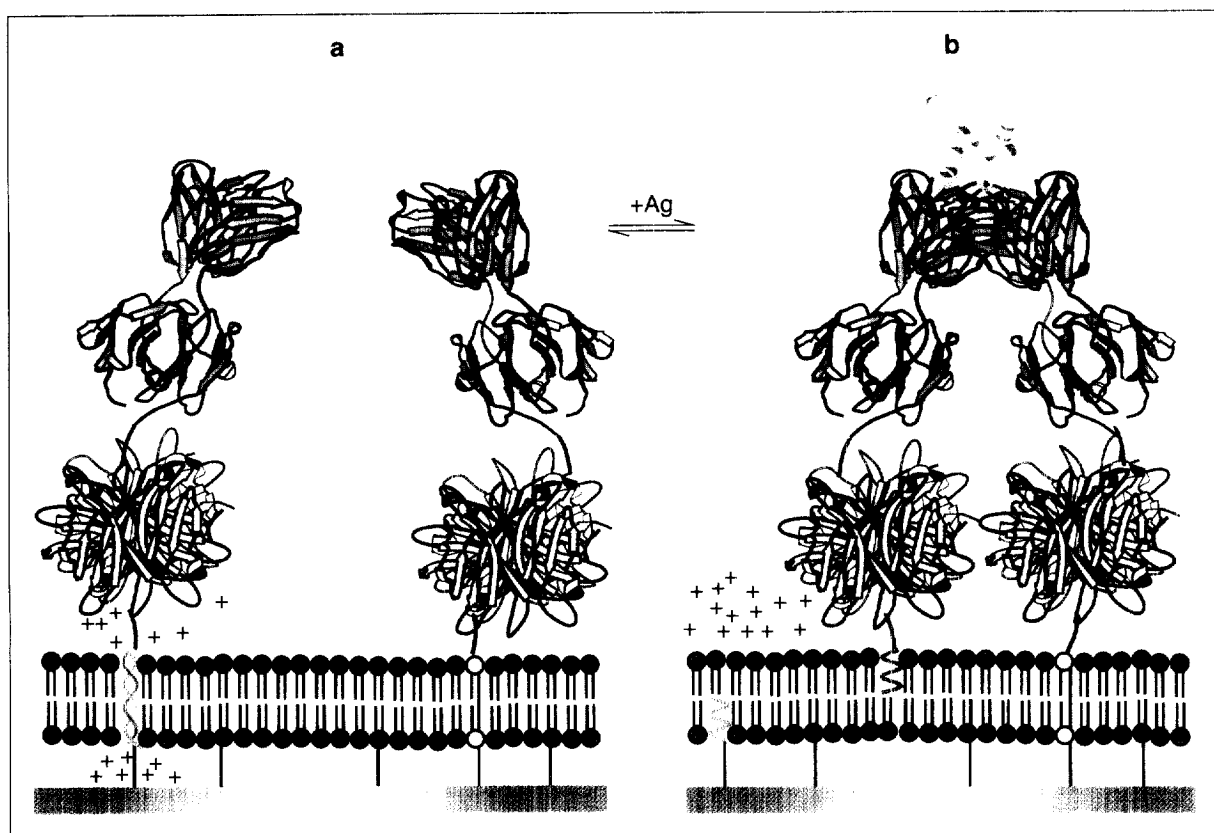


Figure 6

Antibody-controlled ion-channel switches¹³, constructed by the assembly of a tethered membrane, gramicidin, streptavidin, antibody and antigen onto a gold substrate (shaded yellow). The membrane is tethered by half-membrane-spanning lipids (solid-black head groups) that are immobilized on the gold surface by thiol linkages. The gramicidin channels are formed from two halves, one of which (pink) is tethered to the gold, and the other (purple) to a mobile antibody. Other antibodies are immobilized via a transmembrane lipid tethered to the gold substrate. Antibodies are coupled to their two attachment points using a biotin-streptavidin (red) adaptor. In the absence of antigen (**a**), the mobile gramicidin half-channel is free to form complete channels, but on binding (**b**) of antigen (Ag, blue), the antibodies are crosslinked, restricting the formation of gramicidin channels (note that, in order for this to work, the mobile and tethered antibodies need to recognize different epitopes). This figure was generated by combining the coordinates of the various macromolecular components from the Brookhaven Protein Data Bank using MolScript⁴⁰ (accession numbers: gramicidin half-channel, 1mag; full channel, 1gma; streptavidin tetramer, 1str; antibody-antigen (lysozyme) complex, 1fdl).

between the antibodies and restricts the movement of the gramicidins in the outer leaflet, reducing the number of open channels and resulting in a decrease of the transmembrane current that provides the detectable signal.

Perspective and future developments

As yet, there is no protein, engineered or natural, that fulfills all the desirable features for the construction of generic biosensors; an intrinsic signal-transduction function that is easily measured using robust (and miniaturizable) instrumentation; a readily accessible, highly diverse collection of binding sites; and a minimum number of molecular components to reduce complexity in construction. The difficulty lies in combining a good signal-transduction mechanism with diverse binding sites. This is illustrated by the two systems that represent the two extremes. On the one hand, there is the beautiful simplicity of the fully integrated optical signal-transduction mechanism of GFP^{15,16}, but this is not easily combined with an analyte-binding site. On the other hand, we can harness the vast natural molecular diversity of the immune system using ion-channel switches¹³, but at the cost of needing to construct an intricate molecular-assembly system. The way out of this quandary is to combine a simple signal-transduction mechanism with techniques for systematically manipulating the properties of binding sites. The studies on the periplasmic binding proteins⁹ and metalloproteins^{23,29,30,34} demonstrate the power of this approach. To date, there has been no report of a change in specificity of these engineered biosensor proteins, but it is to be hoped that this is just a matter of time. Powerful approaches for rebuilding binding sites or creating entirely new sites in proteins of known structure are under active development. These involve either empirical selection or screening schemes to isolate proteins with altered binding properties from large combinatorial libraries³⁸ or rational design approaches³⁹. Ideally, general strategies will be developed to construct new binding sites in systems with good intrinsic signal-transduction properties, such as GFP, as shown by the introduction of a metal-binding site into α -haemolysin¹⁴ and other (non-sensory) proteins³⁹ using rational, structure-based design.

The ultimate goal of all of these protein-engineering approaches is to keep the detection instrumentation as simple and generic as possible, and manipulate the biomolecules accordingly. Although these objectives have not yet been achieved, a new paradigm is emerging in biosensor technology: adapt the molecule to the instrument, rather than the instrument to the molecule.

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