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Review

Analysing structure-function relationships with biosensors

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Abstract. Elucidating the nature of the relationship between the structure and function of biomolecules remains one of the major challenges in biology. Biomolecules are dynamic entities that possess a variety of structures, and their functions at the molecular, cellular and organismic levels are quite different. Since there is no single causal link between structure and function, the search should be for correlations rather than causal relations. Biosensor instruments based on surface plasmon resonance are widely used for establishing correlations between the chemical structure of binding sites and their binding activity. Mutagenesis studies have shown that only a small percentage of the residues located in a binding site contribute to the binding energy. Since substitutions in residues located far away from the binding site are able to affect binding activity, this greatly complicates the rational design of proteins endowed with improved functions. However, biosensors can be used to determine and predict the influence of the chemical environment and of the structure of a ligand on binding kinetics.

Key words. Biosensor; binding kinetics; protein structure; protein function; quantitative structure-activity relationship.

Introduction

To understand what impact biosensors have had on the understanding of structure-function relationships in proteins, it may be helpful first to examine what is meant by a structure-function relationship. This somewhat mysterious relationship is continuously being referred to in the biochemical and biological literature without any explanation being given about its nature. Despite the many ambiguities that surround the concept of function, elucidating structure-function relationships is often presented as the ultimate problem that needs to be solved if molecular biology is to succeed in unifying chemistry and biology in a grand synthesis.

The deceptive simplicity of concepts like structure and function may lead one to believe that nothing would be gained by analysing their meaning. My first aim, therefore, wil be to show that such an analysis is in fact essential if some common misconceptions are to be avoided. At a time when the rise of functional genomics is giving credibility to the claim that it will soon be relatively easy to relate the sequence of genes to the function of gene products [1], it may be valuable to remind oneself that the connection between the structure and function of biomolecules is not at all straightforward and that its elucidation remains one of the major challenges in biology.

What is a structure?

Woodger [2] defined structure as the result of selective attention to the visual experience of an object at a specific time. The structure of an organ in a living organism, for example, corresponds to a visual fixation of biological material at one particular time. Biological macromole-

cules are usually also depicted as static structures, but their small size mostly does not allow them to be observed directly and their representation is therefore based on a theoretical modelling of chemical structure. This type of static representation which excludes the dimension of time tends to conceal the fact that pictures of biomolecules correspond to visual time-slices of dynamic systems. One consequence of thinking in terms of static structures is that it influences the way we imagine biomolecules interact. Static images reinforce the appeal of lock- and -key models for describing and explaining the process of molecular recognition and they make it more difficult to visualize the role that mutual adaptation of two proteins plays in facilitating their interaction [3].

There is also a tendency to view the secondary and tertiary structures of a protein as unique conformations determined solely by a particular nucleotide sequence. Such a view makes the protein-folding problem even less tractable than would otherwise be the case, since it ignores the role played by the physico-chemical environment in controlling the folding process. A particularly good example of the influence of chemical space on the conformation of a molecule is provided by cyclosporin which was found to have completely different conformations in different cell compartments [4, 5].

The quaternary structure of virus particles also tends to be viewed as a static configuration and this has greatly hampered our understanding of the biological properties of viruses. Moderate changes in pH and temperature have been shown to lead to a rearrangement of parts of the protein subunits that form the viral capsid. As a result, regions of the protein that are internal in the X-ray structure become transiently exposed to proteases, cellular receptors and antibodies [6–9]. Only when these dynamic features of virus particles are taken into account, does it become possible to explain some of the observed biochemical, immunological and biological properties of viruses.

Even the sequence of the RNA genome of a virus species should not be considered to have a single primary structure since the error-prone replication process ensures that the genomes in individual virus particles always contain many mutations. It is thus more appropriate to view the genome of an RNA virus as a master sequence corresponding to the most fit genome sequence in a given environment together with innumerable competing mutant sequences [10] .

Since the analysis of biological systems must always include the dimensions of time, history and evolution, biological structure needs to be thought of as a dynamic rather than a static property, biomolecules possessing a variety of structures rather than a single structure. The term structure is thus best declined in the plural rather than the singular and it should be understood as a collective notion.

What is a function?

According to Kitcher [11], the function of an entity is what it is designed to do. Customarily, only two sources of design are considered: the intentions of a cognitive agent, which is relevant when an artifact has been designed, and the process of natural selection which favours the retention of a useful function during evolution. The relationship between design and the operation of natural selection can be clarified as follows [11]:

a) The function of X is what X is designed to do.
b) What X is designed to do is that for which X was selected.

According to this view, the performance of a function must confer some good to the biological system as a whole, for example by contributing to its health, performance, survival or reproduction. When functions are defined as arising from natural selection and adaptation, some authors prefer to speak of the biological role or biological value of the corresponding feature of the system rather than of its function. Such authors refrain from giving the term function any teleological connotation of purpose, intention and biological value, and consider that the function of a system is simply what it does, i.e. its functioning or activity [12]. According to this definition, the function of a protein corresponds to what it does and how it acts, as well as to when and where it acts [13]. In this restrictive sense of function, the only activity that is considered is binding activity, and function then becomes synonymous with binding activity. The meaning of function is then restricted to the level of the protein molecule itself, and functions that would become meaningful only at the level of the cell or of the organism as a whole are ignored [14]. Since proteins are able to interact with many other molecules in different cellular compartments, thereby contributing to the complex and integrated activities of the cell, the functions of a protein at the cellular level are very difficult to unravel. Thousands of interactions become possible in a given context of protein expression and regulation and in the absence of information on the precise chemical environment in the cell at a given time, any disentangling and prediction of cellular function become hazardous. At higher levels such as metabolic pathways, physiological subsystems and organ function, the activity of complete genomes must be analysed to determine how phenotypic properties at the level of the organism arise from the cooperative action of many gene products [14]. Higher-order functions of a protein can only be analysed in the biological context of the entire organism. The development of proteomics and bioinformatics may in due course give us the information necessary to understand and predict at least some protein functions at the cellular and phenotypic level.

At the present time, protein function is analysed mainly at the molecular level. This involves studying binding interactions between the protein and various ligands. To predict functional activity, one needs information on the tertiary structure of the protein, on the location of binding sites and on the occurrence of conformational changes and post-translational modifications. When only models or low-resolution structural data are available, it is usually possible on the basis of our knowledge of proteins of known function to predict that a new protein is an enzyme or a cell surface receptor. From medium-resolution data, one can further deduce, for example, that the protein is a protease, but high-resolution data would be required to identify the specific nature of a particular ligand. Although recognizing the location of a binding cavity on the protein surface is fairly easy, this does not suffice to establish which ligand will be able to bind specifically to a particular set of atoms within the binding pocket. Our inability to predict binding activity from structural information is partly due to the insufficient resolution of available structural data but is further compounded by the impossibility of accounting for solvation and induced-fit effects. As a result, our ability to predict protein function from structure is at present severely limited [15].

The structure and binding activity of proteins are widely believed to be connected by a causal relationship epitomized by the dictum: 'Structure determines function'. The mistaken belief that structure causes activity arises from a failure to appreciate that causality is a relation existing between successive events and not between material objects nor between a structure and an event. A cause is an event that is a necessary and sufficient condition for the occurrence of a later event, called the effect. A biological event such as a binding reaction can thus not be caused by something that is not an event, for example the structure of a molecule. The absence of a unique, causal link between structure and activity is related to the fact that a single protein structure can have a multiplicity of activities while the same activity can be generated by a variety of structures.

Deducing the activity of a protein from its structure is also impossible because any activity dependis on the context, i.e. the physico-chemical and cellular environment, as well as on the existence of a relational nexus between the protein and a particular ligand. All binding sites are relational entities defined by their partners and not by intrinsic structural features identifiable and existing independently of this relationship [16] . The structure of a binding site, as opposed to the structure of a molecule, cannot be described without considering the binding partner.

In view of the absence of a simple causal link between the structure and activity of a biomolecule, the analysis of so-called structure-function relationships consists mainly in uncovering correlations between the atomic structure of binding sites and biological activity. Instead of searching for a single, non-existing causal link between structure and function, the aim should be to investigate the multiplicity of factors that can influence binding activity.

Searching for structure-activity correlations with biosensors

The biological activity of any substance always involves a process of molecular recognition which in turn depends on an initial, specific binding step between two partners. It follows that the primary criterion for assessing any biological activity is the ability of a substance to bind specifically in a binding assay. In recent years, the measurement of the binding characteristics of biomolecules has been greatly simplified by the development of biosensor instruments based on the optical phenomenon of surface plasmon resonance (SPR). These instruments make it possible to visualize the binding process on a computer screen as a function of time, in terms of change in mass concentration occurring on a sensor chip surface. One of the interacting partners is immobilized on the surface of the chip and the binding of the other is followed by the increase in refractivc index caused by the mass of bound species. None of the reactants needs to be labelled, which avoids the artefactual changes in binding properties that often occur when molecules are labelled. A major advantage of the technique is that interactions are measured in real time, allowing kinetic rate constants and equilibrium affinity constants to be determined [17–19].

The most widely used biosensor instrument is the BIACORE developed by Biacore AB (Uppsala, Sweden). This instrument has been used in about 90% of the studies published so far [20, 21]. The BIACORE consists of an optical detector system, exchangeable sensor chips, a processing unit and a personal computer for control and evaluation. The processing unit contains the SPR monitor, an integrated microfluidic cartridge and an autosampler for dispensing samples automatically [22]. The sensor chip is a glass slide coated on one side with a gold film covered with a dextran layer extending about 100 nm from the surface. The dextran is usually carboxymethylated, allowing the immobilisation of compounds containing primary amines. Various other immobilisation strategies are also available [23]. The system measures the binding between a ligand immobilised on the chip and an analyte introduced in a flow passing over the surface. The microflow cell has a volume of 0.06 µl and with flow rates ranging between 1 and 100 µl/min, a typical interaction can be analysed in less than 10 min. Four independent flow cells are present on each sensor chip. Changes in SPR signal corresponding to the mass of bound molecules are monitored continuously and are presented on the computer screen as a plot of resonance units (RU) versus time, known as a sensorgram. In the case of proteins, a signal

of 1000 RU corresponds to a surface concentration change of 1 ng/mm^2. After each analysis, the sensor surface can be regenerated by introducing a small volume of a suitable dissociating agent which removes the analyte from the immobilized ligand. As many as 100 analytical cycles can be performed on the same ligand surface.

Although biosensor instruments are simple to operate, high-quality data can only be obtained if experiments are designed correctly and various system-dependent artefacts arc avoided. Important aspects that must be taken into account include mass transport effects [24, 25], aggregation, heterogeneity induced by surface immobilisation of the ligand, avidity and matrix effects as well as non-specific binding [26]. Binding data should be analysed using global curve fitting and numerical integration and a theoretical best-fit curve to the primary data should be presented to show that the correct model has been used to interpret the binding interaction [18, 19, 27]. The range of kinetic constants that can be measured with precision with BIACORE is $10^3 - 10^7$ M^{-1} s^{-1} for the on-rate (k_a) and 10^{-5} to 10^{-1} s^{-1} for the off-rate (k_d) [28].

The ease with which quantitative binding data can be obtained with biosensors has made this technology the method of choice for measuring the affinity of a biomolecular interaction in an attempt to establish correlations between the structure and activity of proteins. The part of the protein structure that is most relevant is of course the particular binding site for the ligand of interest and the general aim is thus to uncover correlations between the atomic structure of the binding site and its binding or functional activity. Biosensors have also been found to be powerful tools for identifying binding partners in crude mixtures by so-called ligand fishing.

A structural binding site corresponds to the set of surface atoms in the protein that are in van der Waals contact with the ligand. In the case of protein antigens, for example, an area of about 700–900 Å^2 of the protein surface, comprising between 15 and 22 amino acid residues, is usually found to be in contact with about the same number of residues of the antibody-binding site. However, it should be stressed that the units of recognition are not whole residues but individual atoms. The binding forces that contribute to the energy of interaction are hydrogen bonds, salt bridges and van der Waals bonds. An electrostatic interaction and a hydrogen bond contribute about 5 and 0.5–1.5 kcal of binding energy, respectively. Since a tenfold increase in binding affinity corresponds to a free energy change at 25 °C of only 1.4 kcal/mol (5.8 kJ/mol) and since the majority of affinity constants of proteins lie in the range $K = 10^5 - 10^9$ M^{-1} (corresponding to free energy changes of 7.0-12.6 kcal/mol), it follows that the binding energy and affinity of an interaction can be significantly affected by a very small structural alterations that change only a few of the existing bonds between the two partners.

The most detailed information available on the structure of binding sites is obtained by X-ray crystallography of protein-ligand complexes. However, these studies are usually done at insufficient resolution so that they do not identify all the features that may be important in binding, for example the number and position of water molecules present at the interface. It is at present not possible to calculate in a reliable manner the energetic contribution to the binding affinity of all the atoms in the binding site, and thus to translate structural information into binding energy [29].

To evaluate the contribution of individual residues to the energy of interaction, the most common approach is to modify the proteins by site-directed mutagenesis or alanine scanning mutagenesis and to assess the effect of mutations on the binding affinity [30]. Such studies invariably indicate that only a few of the residues located in the binding site defined on a structural basis contribute to the binding energy and affect the affinity parameters in a significant manner. Side-chain substitutions in a few critical residues can result in a drop in affinity constant of 2–3 logs whereas substitutions elsewhere in the contact region lead to little or no change in affinity. The general conclusion, therefore, is that the so-called 'functional' binding site is always smaller than the 'structural' binding site [31]. Instead of assessing the effect of mutating entire residues, it is also possible, using BIACORE, to study the effect on binding affinity of discrete atomic substitutions in a residue. Such a study was done with cyclosporin analogues presenting very limited chemical modifications and the results showed that the removal of a single CH_3 or OH group could drastically affect the binding of a cyclosporin antibody [32]. These findings demonstrate that binding reactions should always be analysed in terms of single atomic interactions and not in terms of whole residues. Speaking of contact residues is actually misleading since all or even most of the atoms of a given residue are unlikely to participate in the binding interaction.

Substitutions of residues that are not in contact at the interface between two proteins are also frequently found to affect the binding affinity [33]. Substitutions of whole residues in a protein seem to produce small structural shifts that propagate far beyond the mutated region [34, 35]. Small perturbations are often not detected at the current resolution of structural data although they are able to significantly affect binding affinity [36].

When the binding properties of 15 engineered Fabs, directed to an epitope of tobacco mosaic virus protein, which included four single, two double and three triple Fab mutants, were compared with BIACORE, multiple substitutions were found to have non-additive (cooperative) effects on the dissociation kinetics [37]. Substitutions in residues located far away from each other and away from the Fab-combining site were able to affect the binding in a significant manner.

One of the aims of unravelling how single residues in a protein contribute to the binding affinity for a specific ligand is to use such knowledge for the rational design of proteins endowed with improved functions [15]. However, the effects of multiple mutations on protein function seem to be very difficult to predict even when high-resolution structural data are available. As a result, the rational design of improved proteins by site-directed mutagenesis has met with little success [38]. Multiple mutations can lead to improved phenotypic characters by new combinations of properties that are not manifested in the parents and which therefore cannot be part of a rational design strategy. Beneficial changes in the active site of a protein can also be brought about by the accumulation of mutations away from the site, and such effects are also not predictable [39, 40]. Such findings have led some authors to conclude that the engineering of improved protein function need not be based on structural information but is more likely to succeed by the combination of interactive rounds of random mutation, recombination and selection [38, 41].

Attempts to establish correlations between the structure and binding activity of proteins have a long history [42, 43]. The analysis of quantitative structure-activity relationships (QSARs) has mostly been based on scales of the electronic, hydrophobic and steric properties of amino acid residues designed for multivariate analysis [44, 45]. Recently, this approach was used to analyse the interaction between a peptide epitope of tobacco mosaic virus protein and a monoclonal antibody, in an attempt to develop QSARs that would allow the prediction of the kinetic parameters of the interaction [46]. Using BIACORE, the kinetics of the interaction were characterized with 18 analogues of the peptide, using 20 buffers of various pH values and containing various chemical additives. For multivariate peptide design, three amino acid positions were selected because their modification was known to moderately affect binding without abolishing it entirely [47]. The results showed that when a proper experimental design was used, it was possible to determine the influence of the chemical environment and of the chemical nature of the peptide on the off-rate kinetics and thus to derive mathematical models for predicting binding [46]. By measuring the kinetic rate parameters in buffers containing various additives, information could also be obtained on which non-covalent forces are involved in the interaction since different forces are targeted by various additives [48]. If further optimised, this approach may become useful in drug development as well as for defining the molecular properties required for obtaining a certain activity in a particular cellular environment. Drug development requires knowing which chemical forces contribute to the desired activity as well as to the unwanted side-effects. As stated by Kauvar and Villar [49]: 'A drug is not a unique key that can open only one lock. Rather, all drugs show varying degrees of interaction with a plethora of proteins. A successful drug is a compound for which proteins at the top of its preference list contribute to the desired therapeutic effect while those that are detrimental are at the bottom of the list'. Unfortunately, our ability to predict the many unwanted side-effects of drugs is very limited and there is little prospect that by using structure-based drug design, we will be able to do away with the trial and error screening of biologically active compounds [15].

In view of the complexity of biological systems [50], trying to develop successful drugs only on the basis of binding characteristics measured outside the context of an integrated organism is counterproductive. It may be appropriate to emphasize once more that our understanding of protein function at the cellular and organismic levels is extremely sketchy and that the search for correlations between structure and function discussed here pertains only to binding activity.

In the past, binding activity was assessed mainly in terms of equilibrium affinity constants, but since the advent of biosensors, a more meaningful correlation between structure and binding properties has been found in terms of dissociation rate constants.

Since most association rate constants (k_a) of protein-protein interactions tend to be approximately 10^5–10^6 M^{-1} s^{-1}, differences in observed binding properties are mainly the result of differences in off-rates. In the case of antibodies, a dissociation rate constant (k_d) slower than 10^{-3}–10^{-4} s^{-1} (corresponding to a half-life of the complex of 10–100 min) would offer no advantage and would not be selected during the immune response since the signal transduction that occurs after binding and which is required for activating B cells takes place within a few minutes [51]. This is the reason why the affinity ceiling observed with antibodies is around $K_a = 10^{10}$ M^{-1} ($k_a/k_d = 10^6$ M^{-1} $s^{-1}/10^{-4}$ s^{-1}) [52, 53].

An important functional property of certain antibodies is their ability to neutralize the infectivity of pathogens. In the case of viruses, the capacity of viral antibodies to neutralize virus infectivity tends to be correlated with antibody off-rates rather than with their equilibrium constants [54]. Since the biological function of an interaction is greatly dependent on the presence of favourable kinetics, the biosensor technology that measures on- and off-rales with considerable ease is likely to become an indispensable tool for studying structure-function relationships.

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