The binding of one protein to another provokes a variety of biophysical changes that can then be used as a measure of the binding reaction. Optical spectroscopy, particularly fluorescence, is the most flexible technique, but surface plasmon resonance biosensors, microcalorimetry and mass spectroscopy have recently shown significant development.

Introduction

Unsuccessful discussions are often accused of ‘generating more heat than light’. Presumably, light is associated with illumination and clarity whereas heat is linked to conflict and confusion. Happily, among the biophysical methods of measuring protein–protein binding, it appears that both heat and light are equally informative. We have tried to cover recent developments in the fields of calorimetry, optical spectroscopy, mass spectroscopy (MS) and surface plasmon resonance (SPR) but in such a wide area we acknowledge that many valuable contributions will not receive their correct recognition. The use of analytical ultracentrifugation, which has recently been given new impetus by much improved technology, is covered in papers from a recent meeting [1,2].

Those who study protein–protein interactions hope to resolve the high resolution structure of their chosen complex, and more and more of these are being published. Nevertheless, before and after this goal is reached, methods for determining quantitative data relating to these complexes are important. In the same way that we can resolve a protein structure without understanding why it folds that way, we can also obtain high resolution structures of complexes without comprehending the forces that stabilise them. Biophysical data must be employed to achieve the ultimate objective: an ab initio method of predicting the binding interface. In selecting articles, we have tried to concentrate on those that rely primarily upon optical and calorimetric biophysical methods rather than structural analysis.

SPR or resonant mirror techniques

These techniques can be used at two levels of analysis. Firstly (and this is where they really score), in providing simple yes/no answers to the question does A bind to B. Secondly, they can, with care, provide accurate binding affinity data. Both methods are sensitive to the binding of mass to an activated optical chip surface [3*]. The real difference as far as the experimenter is concerned derives not from the alternative optical systems used but from the sample chamber. SPR devices from BIAXare (Uppsala) use fluidic technology to deliver a constant flow of buffer that delivers or removes sample from the sensor surface. The Resonant Mirror device from Affinity Sensors (Cambridge, UK) uses a stirred cuvette above the chip to which sample is added or removed by robotic or manual pipettes. Although the latter allows for better measurements at equilibrium, both systems aim to increase the speed of analysis by analysing the apparent kinetics of binding. It was shown [4] that the initial rate analysis can provide the most rapid, and indeed, more accurate data rather than nonlinear regression analysis of the entire binding profile. Results from the latter method often deviate from the pseudo first order kinetics that are assumed for simple 1:1 receptor–ligand interactions and, unfortunately, these deviations have been interpreted as arising from a complex protein–protein interaction, but evidence is mounting that the majority of cases arise from the technology itself. It has been noted by O’Shannessy and Winzor [5] that “the combination of a highly automated data acquisition system and an inbuilt computer analysis of those collected data has been taken as grounds for absolving the experimenter of any interpretative responsibility”. Problems may arise from mass transport effects on the ligand binding kinetics [6,7] and these effects can be predicted by computer simulation [8].

The use of global fit routines, which have gained prominence recently, may not remove these effects but the use of well-designed control experiments can resolve the mass transport problem [9]. For the nonspecialist wishing to obtain more than qualitative binding data, a set of basic tests have been developed that should highlight inconsistencies in the data [10**]. This and other work [11] shows that equilibrium data obtained from a series of ligand concentrations fitting a Langmuir isotherm is the surest measure of binding affinity. Dissociation rate constants are slowed by the inefficient removal of ligand from the surface and rebinding to the locally high concentration of fixed binding sites in the surface layer may be partly responsible. ‘Competition’ studies show that...
the addition of free binding sites to the mobile phase can largely eliminate rebinding effects [12,13].

Inaccuracies may arise from the effects of flow rate, heterogeneity of the immobilised or soluble component, and the parking problem (where binding sites are not independent because of proximity within the dextran surface). This can be helped by more specific binding, for example, if a single cysteine residue is present, biotinylation [7] or disulphide formation has been successful [14]. Alternatively, histidine tags may be useful [15,16] although less strongly bound. Self-assembly of monolayers of an antigenic peptide and a thio-alcohol spacer directly onto gold surfaces has been reported [12]. Stoichiometry can be measured because the signal is proportional to the mass of receptor and ligand bound [17] and this can help to ensure the validity of results. It should also be noted that the expensive carboxymethyl cellulose chips can be regenerated [18].

**Isothermal titration calorimetry**

ITC relies upon the accurate measurement of heat changes caused by the interaction of molecules in solution and possesses the advantage of not requiring labelling or immobilisation of the components [19]. The raw data (Figure 1) consists of peaks of heat output generated by successive injections of ligand and, when integrated, these provide the total heat output per injection. This provides the binding isotherm. Analysis requires the use of an appropriate binding model [20] but since this is an equilibrium method using a range of ligand concentrations, the initial data interpretation is less controversial than with surface plasmon resonance. Nevertheless, the thermodynamic consequences of the resulting data are still contentious [21]. The final calculable information consists of association constants, stoichiometry, and changes in enthalpy ($\Delta H$), Gibbs free energy ($\Delta G$), entropy ($\Delta S$) and (if experiments are performed over a range of temperatures) heat capacity ($\Delta C_p$). The recent literature provides good examples of how these may be analysed [20,22,23,24]. It has also proved possible to obtain such data from BIAcore measurements over a range of temperatures [25]. The results were different from previously obtained fluorescent and ITC data [26] but more comparisons are needed before the true relationship between the two sets of data is clear.

Because ITC requires higher concentrations of protein than most other methods, many of the proteins are recombinant and thus a large proportion of mutagenesis studies are reported. The well-characterised barnase–barstar complex was analysed using mutagenesis of interacting residue pairs to provide a comprehensive treatment of the binding interface [27]. This procedure allows the overall $\Delta H$ and $\Delta G$ changes to be calculated for interacting residue pairs and it is found that, in most cases, $\Delta H$ is greater than $\Delta G$. This provides a clear example of entropy–enthalpy compensation [23,28–31] in which protein and solvent entropic changes partly balance the direct effects of the mutation on the enthalpy. In the growth hormone–receptor interface, many residues can be replaced by alanine without any alteration in binding affinity and it was shown that with three buried residues this resulted from almost exact entropy–enthalpy compensation [23,24]. Hence although a contact surface may be large, the $\Delta G$ may be determined by a few critical contacts. This was also observed in the combined folding and binding of mutated coiled coils [22] and confirms the belief that changes in $\Delta G$ ($\Delta \Delta G$) are the preferred thermodynamic description of mutational effects.

Changes in heat capacity ($\Delta C_p$), on the other hand, appear more difficult to interpret in protein–protein interactions.

![Figure 1](image-url)

An example of the raw data obtained from an ITC experiment. Successive injections of ligand over an 80 min period show a decreasing heat output that correlates with the increasing saturation of the binding sites present on the receptor within the cuvette. When the integrated heats of binding are plotted against injection number a binding isotherm is produced. Injection number is proportional to both ligand concentration and the ratio of ligand to receptor. As a result, curve fitting based upon a one or more site model provides the affinity ($K_d$) and $\Delta H$ directly. Other parameters are calculated using the relationship $\Delta G=RT \ln K_d=\Delta H−T\Delta S$. 
than in protein folding [23••,24,27••] where predictions of Cp based upon the burial of nonpolar surface area can be quite successful. In the example of growth hormone binding to its receptor, the simple replacement of a phenylalanine (buried at the interface) by alanine has an unexpectedly large effect upon the ΔCp of binding [23••]. Because ΔCp is usually calculated from plots of ΔH versus temperature, it is important to ensure that no thermal denaturation occurs [23••,24], but even then unpredictable ΔCp values can still be obtained. The burial of charged groups is favoured entropically by their dehydration if they have good enthalpic interactions with other charges on the interface [24]; however, when binding results in a change in the pKₐ of an ionisable group, it shows pH dependency or proton linkage and this may account for some discrepancies between calculated and measured ΔCp [32,33•]. This occurs in the case of ovomucoid third domain binding to elastase where good agreement is shown between ΔCp based upon changes in polar and apolar accessible area and Cp based upon measured values [32,33•]. Testing the accuracy of predicted ΔCp will be important if we are to successfully predict binding surfaces and affinities. Use of model compounds can help to probe the binding site as shown for pp60c-src Src homology 2 domains [34] and the SHC phosphotyrosine interaction domain [35]. Calorimetric studies of proton linkage can also provide the bound and unbound values of pKₐ for the ionisable groups involved [31].

In the case of homo-oligomers, dilution calorimetry can be employed in which the concentrated protein is injected into the titration microcalorimeter. Here, the injection heat also gets smaller as the experiment progresses because the dissociation is inhibited by the increasing protein concentration in the cuvette [20••,36,37•]. By this means, the dissociation of insulin oligomers by the solubilising agent cyclodextrin has been measured [36].

**Optical spectroscopy**

Fluorescence is by far the most important optical spectroscopic method for measuring protein–protein interactions because fluorophores are highly sensitive probes of environmental changes [38•,39]. They can also be used to measure changes in mobility associated with binding [40,41]. Furthermore, the technology exists to measure minute quantities of fluorescent material. Within the review period, several developments have shown the direction in which this established technology may develop. The biosynthetic incorporation of fluorescent amino acids at defined sites in the tachykinin neurokinin-2 receptor by the use of stop codon suppression has been used to follow peptide ligand binding by fluorescence resonance energy transfer (FRET) [42•]. This avoids the need for in vitro labelling and provides data directly from. Xenopus oocyte membranes. The alternative way to avoid in vitro labelling is to use green fluorescent protein (GFP) in gene fusions. One indication of things to come is the use of FRET between modified GFP domains to detect calcium ion induced structural changes; the same methods being equally applicable to the measurement of protein–protein interactions within the cell [43••]. Another in vivo FRET assay has shown the dissociation of the cholera toxin A subunit from the pentameric B ring structure within the Golgi compartment of Vero cells [44•]. This elegant use of fluorescence shows that in situ determination of protein–protein interactions has arrived.

Circular dichroism (CD) has much more limited applications but has shown itself to be applicable where binding is accompanied by secondary structural change [45]. Difference spectroscopy can be used to view the ligand in the bound state [31] whilst correlations with observed solution structure of active and inactive peptide models can indicate the conformation of the binding interface [46,47]. Class I MHC molecules are stabilised to different extents by bound peptides and this is measurable by changes in the melting temperature (Tₘ) as measured by CD. When the log (Kₐ) for a range of peptides was plotted against Tₘ, a linear relationship was discovered showing that CD can be used to directly determine Kₐ [48•]. Independently of their CD study, Gao et al. [49] used an ingenious histidine-tag dilution assay [50] to demonstrate that the transcripsional form of the Vesicular stomatitis virus phosphoprotein is a trimer.

**Mass spectroscopy**

Under defined conditions, protein complexes may be observed to remain intact during electrospray or matrix-assisted laser desorption ionisation-time of flight MS (MALDI-TOF-MS) [51–54]. Consequently, these methods may be used to probe protein–protein interactions. One particular strength is the ability to identify peptide ligands by using an affinity selection procedure prior to analysis by MS. In this way, combinatorial libraries [51] or peptide digests [54] can be analysed for specific binding sequences because the accurate masses obtained allow for a detailed if not completely unambiguous identification of the ligand. MALDI-TOF-MS has been used to confirm the proposed noncovalent heptameric structure of the aerolysin homooligomer [55] and, using photolabelled peptides, identify the substance-P binding site in the neurokinin-1 (NK-1) receptor [56].

**Conclusions**

It is clear that each method described can only address a subset of the range of interactions being studied but, if correctly interpreted, all the methods provide accurate binding data. In the future, improving ITC instrumentation will increase the possible applications of calorimetry. Fluorescence methods will continue to provide data at the ‘outer limits’ and open up the cytoplasm as a medium in which to study protein–protein interactions.
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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


A more detailed treatment of recent developments in SPR data analysis than is possible in this broader review.


13. Nieba L, Krebber A, PlÈ uckthun A: A more detailed treatment of recent developments in SPR data analysis than is possible in this broader review.


A clear example of the difference between contact residues that alter the free energy and those that don’t. The role of entropy–enthalpy compensation is well explained.


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A ﬁne example of the use of kinetics obtained from ﬂuorescence binding data to distinguish between different models of protein–protein interaction. Here, the GTPase-activating protein (GAP) and the neurofibrillarytosis type-1 (NF1) protein appear to participate directly in GTP cleavage by Ras.

39. Li W, Dennis CA, Moore GR, James R, Kleanthous C: Structure-function analysis of TAF130: identiﬁcation and characterization of a GTPase-activating protein (GAP) and the neurofibrillarytosis type-1 (NF1) protein appear to participate directly in GTP cleavage by Ras.


The only way to use ﬂuorescence in cells is to use probes whose wave-lengths are beyond range of the cellular background. Here, the promising results from stop codon suppression show how unnatural amino acids can be incorporated to give useful probes of protein–peptide binding.


A clear indication of how GFP engineering will open up new possibilities for measurements of interactions in vivo.


This paper gives precise data on the intracellular site of the dissociation of the A and B subunits. Confocal microscopy is used with FRET to study the fate of an internalised protein complex.


This paper is a good example of how a particular property of a protein–protein interaction can be exploited to provide a novel means to measure afﬁnity.


