JPP JOURNAL OF PROTEINS AND PROTEOMICS
8(4), 2017, pp. 205-217

Review Article

THERMODYNAMICS OF PROTEIN-LIGAND INTERACTIONS AND THEIR ANALYSIS

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Abstract: Physiological processes are controlled mainly by intermolecular recognition mechanisms which involve protein–protein and protein–ligand interactions with a high specificity and affinity to form a specific complex. Proteins being an important class of macromolecules in biological systems, it is important to understand their actions through binding to other molecules of proteins or ligands. In fact, the binding of low molecular weight ligands to proteins plays a significant role in regulating biological processes such as cellular metabolism and signal transmission. Therefore knowledge of the protein–ligand interactions and the knowledge of the mechanisms involved in the protein-ligand recognition and binding are key in understanding biology at molecular level which will facilitate the discovery, design, and development of drugs. In this review, the mechanisms involved in protein–ligand binding, the binding kinetics, thermodynamic concepts and binding driving forces are discussed. Thermodynamic mechanisms involved in a few important protein-ligand binding are described. Various spectroscopic, non-spectroscopic and computational method for analysis of protein–ligand binding are also discussed.

Keywords: Protein–ligand interaction; Thermodynamics; Isothermal titration calorimetry; Kinetics.

1. Introduction

In each cell of an organism a series of reactions occur, which may be covalent and non-covalent. These reactions are co-ordinated and regulated both spatially and temporally. Each reaction is specific in its purpose and occurs due to finely-tuned inter- and intra-molecular recognition mechanisms. These reactions make a part of a complex network of interdependent multi-component reactions in inter linked compartments of the cell.

Proteins are amongst the many complex molecules that participate in such reactions. Proteins play a vital and essential role in cellular and in multicellular organisms or in extracellular activity. Due to a number of biological functions along with the molecular basis of biophysical properties of proteins, they have become of multidisciplinary interest which is usually biological such as biochemistry, pharmacology, physiology, immunology, etc. and physical such as chemistry, physics, mathematics and computing sciences. Molecular recognition means the process in which biological macromolecules interact with each other or with a variety of small molecules by non-covalent interactions to form a specific complex. Molecular recognition is an element of a functionally significant mechanism which involves the essential elements of life such as self-replication, metabolism, and information processing. For example, DNA replication taking place before cell division is accomplished by a series of complex enzyme-catalyzed reactions which depend on the recognition and binding between helicase and DNA

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Received: September 14, 2017
Accepted: October 23, 2017
Published: October 25, 2017

double helix, DNA polymerase and single strand DNA, and ligase and discontinuous DNA segments. Likewise, the extremely efficient and specific molecular recognition and binding acts as a prerequisite for enzyme-catalyzed reactions plays a key role in operating and controlling a metabolic network inolving thousands of chemical reactions occurring sidewise (Philo, 1999; Hensley, 1996). Cellular signal cascades also take place through a sequence of recognition, binding, and dissociation steps (Harding, 1993). As protein-ligand interactions play an important role in cellular metabolism, the detailed understanding of such interactions, microscopic as well as macroscopic level, is needed. For example antigen-antibody interactions and proteins which act as receptors; the ability of small and large molecules to recognize and bind to specific sites on large membrane bound receptors determines the neurotransmission. Similarly enzyme-ligand interactions form a large group of important complexes. Other important interactions studied at a molecular level, comprise ligand-structural proteins, protein-DNA, protein-saccharide, protein-protein, and protein- peptide interactions.

The term ligand means any molecule that can interact with any other molecule in this case it is proteins. Ligands can interact in a reversible, non-covalent way with a protein and hence regulate its biological role in a controllable manner.

To fully understand protein-ligand interactions the biophysical properties of both the protein and the ligand under study should be examined. The knowledge of the structure/conformation of the protein and the ligand in the unbound form at the atomic level is required for studying protein-ligand interaction.

1.1. Thermodynamics of protein–ligand interactions

The various energy changes taking place during protein ligand interactions depend upon many system-specific properties such as protonation states, binding of metal cations, conformational entropy changes from one ligand to another and so on. There is significant contribution of specific interactions to the binding energy involved in the formation of complex between protein and ligand. Various interactions involved are electrostatic interactions, van der Waals interactions, hydrogen bonding, hydrophobic interactions etc. The electrostatic interactions arising from charge-dipole interactions between ionised amino acid side chains and the dipole of the ligand or water molecule make significant contribution to the enthalpy change (ΔH) associated with the binding process. The magnitude of dipole moments of the polar side chains of amino acids of protein has been found to affect largely the interaction with ligands and hence heat changes, though van der Waals interactions are much weaker (0.1- 4 kJ/mol) as compared to covalent bonds or electrostatic interactions, Yet the large number of these interactions which take place upon molecular recognition processes make considerable contribution to the total free binding energy. Van der Waals interactions are generally taken as sum of pair wise inter atomic interactions (Wang *et al.*, 2004). The hydrogen bond may be considered as an electrostatic dipole-dipole interaction as it is a non-covalent, attractive interaction that exists between a hydrogen atom which is covalently bonded to an electronegative atom (donor) and another electronegative atom (acceptor), such as oxygen or nitrogen. Since proteins have a large number of hydrogen bond donors and acceptors in their backbone as well as in their side chains and the environment such as aqueous solvent, protein-protein network, lipid bilayers in which proteins are engrossed, too have many proton donors and acceptors so hydrogen bonding takes place between ligand and protein, within the protein itself and within the surrounding medium. Hydrogen bonds are weaker than ionic or covalent bonds. In biological systems, the hydrogen bond strength lies in the range of 5-30 kJ/mol (Emsley, 1980) and due to their weakness, hydrogen bonds can be formed and cleaved rapidly during binding processes, conformational changes, or protein folding. They play a vital role in molecular recognition and binding processes thus adjust the properties of the macromolecular system. The interactions between the hydrophobic side chains of proteins and ligands contribute considerably to the binding free energy. The hydrophobic groups repel water and other polar groups and cause attraction among the apolar groups of ligand and protein. Also non-polar and aromatic rings of tryptophan, tyrosine and phenylalanine participate in "stacking" interactions with aromatic parts of ligand due to hydrophobic interactions (Bissantz *et al.*, 2010, Perozzo *et al.*, 2004). Hydrophobic interactions are suitable for very diverse sets of ligands (Boehm and Klebe, 1996) and for protein-protein interactions (Vallone *et al.*, 1998).

Solvent effects also contribute significantly to binding energy. Generally ligand-protein interactions are mediated by water molecules concealed in the binding site and forming many hydrogen bonds with both the binding partners (Poornima and Dean, 1995). On ligand binding these bound water molecules are released to the bulk. These kinds of displacements may affect the thermodynamic energy changes of the binding between protein and ligand in a striking manner. The release of a water molecule from a rigid environment is considered to be entropically favourable. The upper limit of the entropy gained has been observed to be 2kcal/mol for transferring a water molecule from a protein to bulk solvent at room temperature (Dunitz, 1995) which may be compensated by loss of enthalpy.

The process of complex formation between a small ligand molecule and a protein involves a complicated equilibrium process in which the ligand and the protein in the solvated state exist as an equilibrium mixture of a number of conformers.

1.2. Kinetics of protein–ligand binding

Protein–ligand binding kinetics gives the rate at which these two components bind to each other. When a protein molecule let P and a ligand molecule L having mutual affinity are mixed in a solution, the interaction between them can be represented as:

$$P + L \rightleftarrows PL \quad (1)$$

This reaction is described by an association rate constant (k), a dissociation rate constant (k'), where PL stands for the protein–ligand complex, k and k' are the kinetic rate constants that describe the rate of the forward reaction which involves the association of protein and ligand and rate of backward reaction involving dissociation reaction, respectively. The units of k and k' are M s^{-1} and s^{-1}, respectively. At equilibrium, the rate of forward binding reaction $P + L \rightarrow PL$ becomes equal to the rate of backward unbinding reaction $PL \rightarrow P + L$ and

the equilibrium constant $K_b = k/k' = [PL] \ / \ [P][L]$ (2)

$$K_d = k'/k = [P][L] \ / \ [PL] \quad (3)$$

where the square brackets represent the molar concentration at equilibrium. The K_b (in unit of M^{-1}) binding constant, where Kd (in unit of M) is called dissociation constant. Hence, the higher the binding rate and lower the dissociation rate, the higher the binding affinity.

1.3. Enthalpic and entropic components of free binding energy

In a protein–ligand–solvent system, there are very intricate interactions and heat exchange among these substances. This heat transfer is related to various energy changes which abide the laws of thermodynamics. The driving forces for the association between protein and ligands are a combined outcome of a variety of interactions and energy exchanges among the protein, ligand, water, and buffer ions. Gibbs free energy, which is a measure of the capacity of a system to do maximum work at a constant temperature and pressure, is one of the most important of the driving forces (Gilson and Zhou, 2007; Gibbs, 1873). At equilibrium, the binding constant K_b is related to the standard Gibbs free-energy change ($\Delta G°$) of the reaction under conditions of constant pressure by equation

$$\Delta G° = - RT \ln K_b \quad (4)$$

where R is the gas constant (8.314472 J mol^{-1} K^{-1}) and T is the temperature (in Kelvin). As in case of any spontaneous process, protein–ligand binding takes place only when the Gibbs free energy change ($\Delta G°$) of the system is negative at constant pressure and temperature. The extent of protein–ligand association is determined by the magnitude of the negative $\Delta G°$, which also determines the stability of protein–ligand complex and in turn the binding affinity of a ligand to a protein. The more negative the value of ΔGo, the more favourable the reaction (Xie *et al.*, 2014) The change in free energy itself is related to standard enthalpy change ($\Delta H°$ i.e. the heat given out on making of bonds or heat taken up on breaking of bonds) and standard entropy change ($\Delta S°$), which represents changes of the degree of freedom within the system upon ligand binding, by following relationship.

$$\Delta G° = \Delta H° - T \Delta S° \quad (5)$$

ΔH is negative in the exothermic and positive in the endothermic processes, respectively. For a binding process, ΔH is referred to as the binding enthalpy. It gives the energy change when the ligand binds to the protein by non covalent interactions such as Van der Waals attractions, hydrogen bonds, ion pairs and other polar and non polar interactions at the binding interface. However,

the heat effect of a binding reaction involves the entire system, including contributions from the solvent also such as interactions between the protein and solvent and between the ligand and solvent, (Cooper and Johnson, 1994; Perozzo *et al.*, 2004). Actually, the enthalpy change upon binding is a net result of forming and breaking many individual interactions, including the disruption of the hydrogen bonds and Van der Waals interactions existing between the protein and solvent and between the ligand and solvent, the formation of the non covalent interactions between the protein and ligand, and the solvent interaction near the complex surfaces (Perozzo *et al.*, 2004; Liu *et al.*, 2012). Entropy is a measure of the disorder or randomness in atoms and molecules in a system. Entropy change (ΔS) is a thermodynamic property of a system having positive and negative signs which indicate the overall increase and decrease in the degree of the freedom of the system, respectively. The total entropy change associated with binding, may be called as the binding entropy (ΔS) is consists of three entropic terms (Chaires, 2007) i.e. (i) ΔSsolv. which represents the solvent entropy change arising mainly due to solvent release upon binding and usually makes a favourable contribution to the binding entropy because of its large positive value; (ii) ΔSconf. which represents the changes in the conformational degree of freedom of both the protein and ligand upon binding, which may make favourable or unfavourable contribution to the total binding entropy as the degree of freedom of the complex may increase or decrease as compared to those of free protein and ligand before binding (MacRaild, 2007; Bronowska 2011); (iii) ΔSr/t represents the decrease of translational and rotational degrees of freedom of the protein and ligand upon complex formation, hence contributes unfavourably to the binding entropy. For binding to occur (Liu *et al.*, 2012) the binding reactions would have to overcome the negative ΔSr/t upon binding (Amzel, 1997; Amzel, 2000) through either large solvent entropy gain i.e. positive ΔSsolv or favourable protein–ligand interactions i.e. negative binding ΔH. The protein–ligand binding processes are driven by the decrease in total Gibbs free energy of the system. So the ideal optimization strategy should be to maximize the favourable enthalpy change or entropy change contribution while to minimize the unfavourable enthalpy change or entropy change.

The heat capacity change ΔCp upon complex formation is also characteristic for a particular bimolecular interaction and it is related to the temperature and the enthalpy change by the relationship $\Delta Cp = d\,\Delta H / dT$. The Gibbs free energy is temperature dependent. In a thermodynamic analysis the aim is to determine $-\Delta G$, $-\Delta H$, $-\Delta S$, and their temperature dependence by Cp, as these four parameters offer a complete description of the energies governing molecular interactions.

$$\Delta G\,(T) = \Delta H(T_0) - T\,\Delta S(T_0) + \Delta Cp\,[T - T_0 - T \ln T / T_0] \quad (6)$$

T_0 is an appropriate reference temperature.

2. Analysis of protein-ligand interactions

The analysis of protein–ligand interactions is important to understand the regulation of biological functions and to design novel bioactive molecules that will regulate protein function or inhibit protein–protein interactions (Keiser *et al.*, 2010; Wells and McClendon, 2007). Various biophysical techniques have been developed to illustrate protein–ligand complexes such as spectroscopic techniques like fluorescence polarization assay and fluorescence resonance energy transfer and non-spectral techniques like isothermal titration calorimetry, surface plasmon resonance, differential scanning calorimetry, enzyme-linked immunosorbent assay, microscale thermophoresis and electrospray ionization mass spectrometry (Arkin and Wells, 2004; Renaud and Delsuc, 2009; Niesen, 2007; Jonker, 2011). With surface plasmon resonance and microscale thermophoresis techniques we can measure kinetic parameters in protein–ligand interactions, while isothermal titration calorimetry can be used to measures the thermodynamic parameters of protein–ligand interactions in solution (Trott and Olson, 2010; Huang, 2004).

2.1. Spectroscopic techniques

The Spectroscopic techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, Laue X-ray diffraction, small-angle X-ray scattering, and cryo-electron microscopy are used for resolving atomic structures (Joachim, 2009; Kay, 2011).

2.1.1. X-ray-diffraction

X-ray-diffraction data provides information about intermolecular non covalent interactions i.e. the

enthalpy contribution and about some spatial disorderness about the average structure as reflected by the B-factors i.e. entropy contribution (Bronowska, 2011; Henzler-Wildman and Kern, 2007); Laue X-ray diffraction has been used to measure the structures and kinetics simultaneously and has the advantage of delivering the timescale of local motions (Bourgeois and Royant, 2005); small-angle X-ray scattering and cryo-electron microscopy have been used to determine directly the structural assembly with moderately low resolution under the experimental conditions, but they cannot describe the timescales of conformational transition (Henzler-Wildman and Kern, 2007). Single-molecule fluorescence spectroscopy (Weiss, 2000) and time-resolved hydrogen-deuterium exchange mass spectrometry (Graf *et al*., 2009) are some other experimental techniques that can be used to study the protein dynamics involved in binding.

2.1.2. Mass spectroscopy (MS)

It permits a conversion of solution phase into the gas phase of the analyte in mass spectrometer gently so that even weakly bound non covalent complexes can be detected intact and their mass can be analysed. MS methods such as ESI-Ms have been used for determining the association constants of non-covalent interactions. The main advantage of MS methods is that signals due to protein, ligand, and protein-ligand complex can be detected separately. Also higher order structure formation such as dimerization can also be detected by these methods.

2.1.3. Fluorescence spectroscopy

This technique is very sensitive and compatible for measurement of protein-ligand interactions and structures. However, it can be used to determine only the structure of the fluorescent probe and its immediate environment and the interpretation of the data obtained is often difficult. However, fluorescence spectroscopy can be used widely in studying the physico-chemical properties of proteins, protein-ligand interactions and protein dynamics as almost all proteins have been found to contain naturally fluorescent amino acid residues such as tyrosine and tryptophan. Also, a large number of fluorescent dyes have been obtained that can be applied to particularly probe the function and structure of macromolecules. Fluorescence spectroscopy is the most appropriate method for studying properties such as stability, thermodynamics, kinetics and ligand binding when only limited quantities of proteins are available because of its wonderful sensitivity.

2.1.4. Circular dichroism (CD)

This is a commonly available and widely used technique for determination of secondary structure of protein. It has the advantage that comparatively smaller quantities of material are required and results can be obtained in shorter time than in the case of NMR and X-ray. Circular dichroism technique have also been used to study the effects of environmental conditions of pH, temperature etc., on the protein conformation, in the presence and absence of a ligand. CD used in the stop-flow manner is mainly helpful for studying protein-ligand interactions in real time. In CD studies, the solution components should be UV transparent preferably in order to minimize the signal to noise ratio as a result it limits the use of some buffers, salts and solvents. However, due to theoretical problems, the technique cannot be used to determine the tertiary structure of protein.

2.1.5. Fourier transform infrared spectroscopy (FTIR)

This technique is especially helpful for determination of secondary structure of proteins as in CD techniques. Nevertheless there is quite flexibility in FTIR from the point of view of the nature of samples which can be studied for example tissue slices, cells, solid state samples (powdered or freeze dried), crystals, thin films, aqueous proteins and protein-ligand samples. FTIR have also been used for examining the structure of proteins in the presence and absence of ligands and for solution of protein D_2O. H_2O can be problematic to be used. It is also useful in investigating the effect of changes in conditions of solution on proteins and protein-ligand interactions. FTIR can also be applied for studying the presence and absence of hydrogen bonds but FTIR is limited to the use of short path length cells.

2.1.6. Raman spectroscopy

Raman spectroscopy has been used for many years for the investigation of structural, enzymatic proteins and protein-ligand interactions. It is a very good method for studying the variable conditions in the solution state. The resonance Raman has modified specificity and sensitivity relative to off-resonance Raman and electronic states can also be

obtained from resonance Raman spectra. It can be used for different sample states as in FTIR, without any limit to cell path length.

2.1.7. NMR spectroscopy

Among the spectroscopic methods, NMR spectroscopy is a well-known technique for studying intermolecular interactions. Protein–ligand interactions with a large range of affinities (10.9–10.3 M) are measured using NMR spectroscopy (Pellecchia *et al.*, 2008; Peng *et al.*, 2004; Goldflam *et al.*, 2012). The main power of NMR-based screening is the high sensitivity of chemical shifts. Fragments that bind to the protein with even millimolar affinity can be detected. The analysis of protein–ligand complexes is done using the protein-observed and ligand-observed NMR experiments where the NMR parameters of the protein and the ligand, are respectively compared in their free and bound states (Pellecchia *et al.*, 2008; Peng *et al.*, 2004; Goldflam *et al.*, 2012). The chemical shift is the most typical and the easiest to follow parameter in protein-observed methods. The ligand binding site is found by identifying the chemical shift perturbations of the protein resonances observed on ligand addition. So we can distinguish specific binding from non-specific binding. The resolution of the structure needs molecular dynamics calculations using experimental NMR restraints resulting from chemical shifts, nuclear Overhauser effects (NOEs), scalar couplings, paramagnetic interactions or dipolar couplings (Key, 2011; Billeter *et al.*, 2008; Cavanagh, 2007). The protein-observed methods require highly stable and soluble protein with low molecular masses (less than 30 kDa) and long experimental time which are the major drawbacks of this method. NMR parameters such as longitudinal, transverse and cross-relaxation rates highly depend on the molecular rotational correlation time that is correlated with the molecular weight.

On the other hand ligand-observed NMR experiments are based on the modification of such size-sensitive NMR parameters for the ligand in the presence of a protein receptor (Peng *et al.*, 2004; Meyer and Peters, 2003). Unlike protein-observed experiments, ligand-observed experiments are more sensitive to protein with larger molecular masses and need lesser amount of protein without any isotope labelling. Ligand-based methods can provide relevant structural information on the protein–ligand complexes along with the measurement of protein–ligand affinities. So the ligand-observed NMR experiments have greater applicability. The three methods which depend on intermolecular magnetization transfer: transferred-NOE spectroscopy (NOESY), saturation transfer difference (STD) spectroscopy and water–ligand observed via gradient spectroscopy (Water LOGSY) experiments are mainly used. Applications of the methods are particularly due to their roles in the fragment-based drug design (FBDD) approach (Erlanson, 2012). Fragment-like molecules are very small compounds having molecular mass below 300 Da, with poor complexity; as a result they bind to proteins with feeble affinities, but show high ligand efficiency. NMR spectroscopy is a great method in FBDD, and with ligand-observed methods we can, not only identify ligands but also get information about the affinities, specificity and 3D structures of protein–ligand complexes. NMR methods have the advantage of characterizing the protein–ligand interactions over a wide range of timescales from picoseconds to seconds (Mittermaier and Kay, 2006) and, therefore, are powerful for determining entropic contribution to the binding free energy (Meyer and Peters, 2003) Also the NMR experiments are conducted in solution conditions which are nearer to the biological conditions. They provide information on dynamics such as relaxation, rotational and translational, vibrational motion of atoms measurement and identify individual side chain motions. Secondary structure of protein can be obtained from limited experimental data. The main disadvantages of NMR methods include the requirement of concentrated solutions, which may cause a risk of aggregation also it is limited to determination of relatively small proteins.

2.2. Non- Spectral Techniques

2.2.1. Isothermal Titration Calorimetry (ITC)

The information about the full thermodynamic profiles comprising the entropy, enthalpy and the binding free energy could not be obtained from the structural and dynamic data alone, even when subjected to the most advanced computational methods and so binding affinity may not accurately predicted (Chaires, 2008). But the calorimetric techniques such as the isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) give the quantitative thermodynamic data which can be used to study the stability of the protein-ligand complex and to

explain the driving forces for binding. In DSC technique we can measure the enthalpy and the heat capacity of thermal denaturation with the help of which the stability of protein–ligand complexes can be determined (Sturtevant, 1987; Celej, 2006). The only technique which can directly measure the heat exchange during complex formation at a constant temperature is ITC which has become the golden standard for the determination of the driving forces for the binding process (Perozzo, 2004; Bronowska, 2011).

The simplicity and accuracy of ITC methods for thermodynamic description, combined with modifications in instrumentation (Wiseman, 1989) have resulted in their increasing use in the investigation of protein–ligand interactions (Ababou and Ladbury, 2007) and in drug discovery (Holdgate and Ward, 2005) over the last decade. The only technique by which ΔG°, ΔH° and TΔS° can be correctly determined from a single experiment is ITC. A usual ITC experiment is consist of three steps: (a) a ligand is titrated into a solution containing the protein) of interest; (b) the heat released or absorbed in the binding process is measured; and (c) the primary ITC data obtained is processed and fitted to get the values for binding constant (Kb), Gibbs free energy of binding (ΔG), binding enthalpy (ΔH) and entropy (ΔS), and the stoichiometry (n) of the binding event (Pierce, 1999). In addition, the heat capacity change (ΔCp) of a binding reaction can be obtained if ITC experiments are done at different temperatures. In ITC instruments the sample cell containing the protein and the reference cell filled with buffer or water are maintained at the same temperature by making use of a power compensation design. During the experiment using a titration system the ligand is delivered to the sample cell in exactly known fractions as a result heat is either released or absorbed depending on the nature of the reaction and, therefore causes a temperature difference between the reference and sample cell. This difference in temperature is compensated for by regulating the feedback power applied to the cell heater i.e. in exothermic reactions decrease and in case of endothermic reactions increase the power to the sample cell. On the whole measurements are simply the time-dependent input of the power needed to maintain equal temperatures of the sample and the reference cells at each titration. The results of a large number of reported isothermal titration calorimetry (ITC) studies of protein–ligand complexes for which structural information is also available have been collected so as to investigate the potential relationships between structure and thermodynamics of protein–ligand interactions. In an ITC experiment, the incremental heats of reaction are measured when one component is titrated into the other, then ΔH° and K_b (ΔG°) are obtained by nonlinear fitting of the resulting titration curve [9h]. Using Eq. (5), the entropy change coupled with interaction can be obtained. The ΔCp, which measures the change in heat with temperature at a constant pressure, can be obtained by determining ΔH values at a range of temperatures with ITC which is followed by calculating the slope of the temperature-dependent ΔH curve with linear waning analysis. ΔCp provides a connection between thermodynamic parameters and the structural information of proteins due to the strong relationship between ΔCp and the surface area buried on forming a complex (Perozzo, 2004; Prabhu and Sharp, 2005). On complex formation, the water of hydration is released leading to a change in heat capacity and the magnitude of ΔCp is proportional to the amount of surface area concerned. The dehydration of both the protein and ligand molecules upon binding can make positive or negative contribution to ΔCp, depending on the burial of the apolar or polar surface areas leading to negative ΔCp or positive ΔCp respectively (Perozzo, 2004; Syme *et al.*, 2007). ITC can determine the binding stoichiometry (n) from the molar ratio of the protein and the ligand at the equivalence point, if the concentrations of both are known. In data fitting, the parameter n can be fixed as the number of binding sites per protein molecule. The heat exchange observed by the ITC experiment is the net heat effect in the sample cell upon addition of the ligand, including the heat released or absorbed due to binding reactions, the heat effects due to dilution of the ligand and protein, mixing of the two solutions with different compositions, temperature differences between the sample cell and the syringe, and so on. So, in order to obtain the heat of complex formation, the control experiments have to be performed to determine these non-specific heat effects. The major advantages of ITC include non-destructive nature of the technique as the thermodynamic parameters of the interaction can be measured in solution without immobilization, modification, or labelling of the binding protein and ligand; it does not impose any molecular weight restrictions (Bouchemal,

2008) high precision with error in the range of 5% in the determination of binding constant (Krell, 2008) the ability to measure high affinity interactions (Freyer and Lewis, 2008; Doyle et.al., 1995). However, there are still certain disadvantages to ITC. Since heat is a universal signal and each process contributes to the measured global heat effect, the evaluation of the contribution from the binding is complicated. Despite the high sensitivity, challenges still exist for extracting heat effects of complex formation when the binding exhibits rather small binding enthalpy (resulting in relatively low signal to noise) and when the binding processes are very slow (leading to the neglect of kinetically low processes). The disadvantages of ITC include: the need of a large amount of sample as a result this technique cannot be applied to certain proteins which are difficult to prepare in large amounts. Traditional ITC method is laborious, time-consuming, and has low yield so it is not much suitable for biotechnological and pharmaceutical applications which demand low labour and high yield (Chaires, 2008; Rajarathnam and Rösgen, 2016; Ghai *et al.*, 2012) . However the development of the modern ITC instruments by MicroCal (Worcestershire, UK) and Calorimetry Sciences Corporation (Lindon, UT, USA), has changed the situation. These robotic automated instruments recognize cell loading and data collection from large numbers of samples in an almost self-sufficient way which has made a revolution in the manner that ITC can be engaged in high yield research. Also, the developments in the form of array-based nanocalorimeters permit simultaneous enthalpy measurements for a right high-throughput screening (HTS).

2.2.2. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) employs an optical method used to measure a change in refractive index of the medium in close vicinity of a metal surface (usually thin gold layer) which can be used to observe the binding of analyte molecules (ligand) to receptor molecules (protein) immobilised on the metal surface. In a conventional SPR instrument, there is a sensor chip that consists of a glass plate on which a thin layer of metallic gold (usually 50 nm thick) is coated on which biomolecular interaction occurs. The protein to be studied is immobilised on this gold surface. When an incident beam of light strikes the surface at a particular angle, the SPR phenomenon results in a graded reduction in intensity of the reflected light depending on the thickness of a molecular layer at the metal surface hence the adsorption of molecules on the metal surface and their ultimate interactions with particular ligands can be measured accurately. SPR has been developed and performed mainly using Biacore™ technology (Rich and Myszka, 2001; Real-Fernández *et al.*, 2015). The Biacore instrument contains a sensor surface which is a thin film of gold on a glass support and forms the floor of a flow cell through which an aqueous solution flows continuously. The protein molecules (receptor) are immobilized on the sensor surface, and the ligand (analyte molecule) is injected into the aqueous solution to find the binding reaction. As ligands bind to immobilized receptor protein molecules, an increase in the refractive index which is expressed in response units, RU, is observed. When all binding sites of the receptor molecules are occupied i.e. after a desired association time, a solution without ligand is injected through the flow cell so as to dissociate the protein–ligand complex. As the ligand dissociates from the immobilized protein, we will observe a decrease in refractive index. From the time vs RU curves the kinetic association rate constant k and the dissociation rate constant k' can be calculated. Using equation (3), the binding constant K_b can be obtained. The capacity of SPR to measure the real-time binding data makes it well suited to analyses of the binding constant K_b. The technique is applied to the measurement in real-time of the kinetics of ligand–receptor interactions. This technique has many advantages over conventional methods used for affinity measurements (Dunitz, 2009) such as it requires a much smaller amounts of protein sample (Van der Merwe, 2001), does not require any labelling with a tracer and is very fast. A major drawback of the technique is the requirement for the regeneration of the sensor chip for measurements with various concentrations. SPR has the ability to measure higher binding affinities as compared to ITC i.e. in the ranges of 10´6–10 μM (Joshi and Isha, 2006). SPR can be used to estimate binding enthalpy via van't Hoff analysis due to its highly reproducible affinity measurements, in combination with exact temperature control (Willcox *et al.*, 1999). Though conventional SPR technique is not much suitable to high-throughput assays (Van der Merwe, 2001), but recent developments in SPR instrumentation, in the design of sensor chip, and strategies for sample preparation have shown that SPR has great potential for HTS

screening of membrane protein ligands (Patching, 2014, Maynard *et al.*, 2009). However, it should be noted that the protein immobilization affects the conformational and translational/rotational entropies, and hence, the association rate (Kastritis and Bonvin, 2012).

2.2.3. Fluorescence Polarization (FP)

Fluorescence-based techniques applied for measuring intermolecular interactions include fluorescence anisotropy (Owicki, 2000), fluorescence correlation spectroscopy (Lieto, 2016), time resolved fluorescence (Handl and Gillies, 2005), FP (Rossi and Taylor, 2011) etc. The fluorescence polarization among these has the capability to determine the kinetics and thermodynamics of protein–ligand binding. Fluorescence polarization (FP) is a non-disruptive method for measuring the association of a fluorescent ligand with a larger molecule such as protein. Using this technique we can determine the standard Gibbs free energy change (ΔG°), standard enthalpy change (ΔH°) and standard entropy change (ΔS°) of ligand binding to the protein. This method is suitable to any ligand-binding site for which an appropriate fluorescent ligand is available. FP can be used to measure low-affinity interactions in real-time without using any radioactive materials. The fluorescence has a broad spectrum of wavelengths so multiple colours can be used for detecting the binding of the fluorescent-labelled ligand to a target protein. The principle of FP is based upon the fact that an initially polarized fluorescence emission becomes depolarized over a period of time, and the depolarisation takes place faster in the unbound than in the bound state of ligands to the protein molecules (Jameson and Croney, 2003; Lea and Simeonov, 2011). FP can use the competitive binding analyses, in which the fluorescent-labelled ligand molecules are bound to the protein and, consequently are displaced by the unlabelled competing ligands to measure the binding affinities of labelled as well as unlabelled ligands. FP has linear proportion to the percentage of bound/ unbound species, which can be used to determine the $IC_{1/2}$ i.e. inhibitor concentration or the concentration of the unlabelled ligands required to displace half of the labelled ligand. Consequently, the Ki/Kd (where Ki is the inhibition constant of the unlabelled ligand) can be calculated with the help of the Cheng–Prusoff equation (Cheng and Prusoff, 1973; Munson and Rodbard, 1988). Binding enthalpy can be estimated via van't Hoff analysis using K_d values measured at different temperatures (Rossi and Taylor, 2011). FP technique involves the use of single fluorescent label approach and does not involve the filtration or separation steps and as a result it needs comparatively lesser number of reagents, lesser quantity of protein, and fairly inexpensive equipment than do SPR and ITC. In addition FP is a simple technique and FP detection does not destroy samples. As a result the technique becomes very suitable for application to HTS of large numbers of unlabelled ligands (Rossi and Taylor, 2011). FP is a ratiometric method so the response determined by FP does not gives a direct measure of the binding but proportional to the binding (Kastritis and Bonvin, 2012; Wilkinson, 2004) which makes the measured affinity values to be related with the experimental conditions used (Kastritis, 2011). In addition, the use of the fluorescent-labelled ligand may influence the binding behaviour. FP is applicable only to soluble proteins and ligands and only for fluorescent ligands which are significantly smaller than the protein to which they bind.

3. Computational Methods

A number of computational methods for identifying and characterizing binding sites have been developed which can be classified into three categories: (a) geometric algorithms to find concave invaginations in the protein; (b) methods based on energy considerations; and (c) mapping/docking algorithms which use a number of small molecules and functional groups as probes and thus give direct input for fragment-based design. The geometric approaches involve the coating of the protein surface with a layer of spherical probes and then filtering out those that are not adequately buried (An and Abagyan, 2006). These methods are usually capable of recognizing and listing the various pockets without accounting for any measure of druggability. Though simple energy-based techniques are as fast as the geometric methods, but they are more sensitive and specific. Recently published two algorithms of this kind are Pocket Finder (An and Abagyan, 2006) and Q-Site Finder (Laurie, 2005) based on calculating the van der Waals interaction energy of the protein for aliphatic carbon probes on a grid, and retaining the probes with favourable interactions. PocketFinder algorithm (An and Abagyan, 2006) estimates the envelope of such probes and the envelopes are sorted out by their volumes, whereas Q-SiteFinder

(Laurie, 2005) ranks the clusters of probes with favourable interactions on the basis of their total interaction energies. The ligand binding sites have a tendency to be among the highest ranked pockets. It has been noted that in most of complexes studied, the 93% of real binding sites are among the top two largest predicted binding sites (An and Abagyan, 2006). However, the observations obtained from these methods do not provide structural information for fragment-based design. The various methods that involve mapping/docking and scoring with a number of molecular probes begins with the revered GRID algorithm (Goodford, 1985) which calculates a potential on a grid superimposed on a protein, and detects the locations which are favourable binding the various fragments. Multiple Copy Simultaneous Search (MCSS) is another accepted method which uses many ligand copies concurrently, each one of which is transparent to the other but subject to the full force of the receptor (Miranker and Karplus, 1991). As emphasized by (Mattos and Ringe, 1996). The major problem with studies by GRID and MCSS is that they lead to many energy minima on the surface of the protein, and it becomes hard to determine which of the minima are actually applicable. As these two methods do not account for salvation, the re-ranking the MCSS results with potentials that include terms demonstrating solvation results in significant improvement in its performance (Zoete, 2005; Schechner, 2004).

4. Conclusion

The global driving force for the binding reaction is the decrease in total Gibbs free energy of the protein-ligand-solvent system, which depends on the enthalpic and entropic contributions. Although the entropy–enthalpy compensation has an adverse effect on a large free energy change, The increase in solvent entropy due to solute desolvation and the enthalpy decrease due to the favourable interactions between the binding partners tend to overcompensate for the unfavourable contributions from the enthalpy increase due to the energy required for desolvation and the entropy decrease due to conformational entropy decrease and the loss of the rotational and translational entropy of the ligand, thus net effect is lowering of the free energy of the system and leading to a funnel-like binding similar to the protein folding funnel

The analysis of protein–ligand complexes using NMR fragment based screening gives important information for chemical biology research and for drug design thus helps to evaluate new protein targets. As analysis of protein–ligand interactions is now considered as a dominant method to achieve insight into protein functions and structures therefore the use of NMR experiments is likely to rise in the future.

Among experimental methods used for investigating the protein-ligand binding, the ITC due to its capacity to give a total thermodynamic profile of the system under study, has been considered a gold standard in determining the binding driving forces for protein-ligand binding and the stability of the protein-ligand complex. SPR can be used to estimate the kinetic rate constants directly. FP can determine the equilibrium dissociation constant by competition binding analyses. If measurements are performed at different temperatures these two methods can be often used to measure the binding affinity and to determine the binding enthalpy. The computational methods are important as these are relatively less laborious, more economic, and faster than experimental methods and can also assist the interpretation of the existing experimental data and help in the design of new experiments. The main challenges in the calculation of free energy are to increase the speed of the methods and to improve the accuracy and reliability of calculated results. The development and use of more accurate force fields, better sampling techniques, more precise solvent models, and combining the existing methods by using their strong points could be the main guidelines.

Acknowledgement

The information has been congregated from reported scientific research and books to update the inclusive overview of the subject for students. I wish to express my grateful thanks to all the scientists and researchers who have put a lot of effort for various inventions in this field and have very kindly shared their research information, which was of invaluable help in preparing this review article. I am grateful to the authorities of my institute for providing the necessary facilities for the preparation of the paper. I express my sense of gratitude to all sources which have been used directly or indirect1y during the completion of this work.

Abbreviations

DNA, Deoxyribonucleic acid; P, Protein; L, Ligand; MS, Mass Spectroscopy; CD, Circular Dichroism; FTIR, Fourier Transform Infra Red spectroscopy; NMR, Nuclear Magnetic

Resonance; NOESY, Nuclear Overhauser Effect Spectroscopy; STD, Saturation Transfer Difference; LOGSY, Ligand Observed via Gradient Spectroscopy; FBOD, Fragment Based Drug Design; ITC, Isothermal Titration Calorimetry; SPR, Surface Plasmon Resonance; RU, Response Units; HTS, High Throughput Screening; FP, Fluorescence Polarization; MCSS, Multiple Copy Simultaneous Search.

Conflict of interest

The author declares no conflict of interest.

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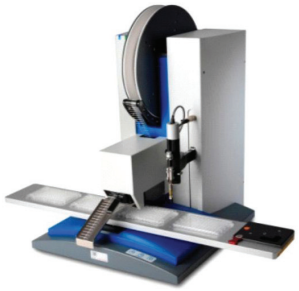

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