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Methods for quantifying T cell receptor binding affinities and thermodynamics

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Abstract

$\alpha\beta$ T cell receptors (TCRs) recognize peptide antigens bound and presented by class I or class II major histocompatibility complex (MHC) proteins. Recognition of a peptide/MHC complex is required for initiation and propagation of a cellular immune response, as well as the development and maintenance of the T cell repertoire. Here we discuss methods to quantify the affinities and thermodynamics of interactions between soluble ectodomains of TCRs and their peptide/MHC ligands, focusing on titration calorimetry, surface plasmon resonance, and fluorescence anisotropy. As TCRs typically bind ligand with weak-to-moderate affinities, we focus the discussion on means to enhance the accuracy and precision of low affinity measurements. In addition to further elucidating the biology of the T cell mediated immune response, more reliable low affinity measurements will aid with more probing studies with mutants or altered peptides that can help illuminate the physical underpinnings of how TCRs achieve their remarkable recognition properties.

1. Introduction

$\alpha\beta$ T cell receptors (TCRs) are clonotypic membrane proteins on the surface of T lymphocytes responsible for recognizing peptide antigens bound and “presented” by class I or class II major histocompatibility complex (MHC) proteins. TCR recognition of a peptide/MHC complex is necessary for the initiation and propagation of a cellular immune response, as well as the development and maintenance of the T cell repertoire. TCR recognition of peptide/MHC is also involved in pathological conditions such as autoimmunity and transplant rejection. Given the central role these interactions play in health and disease, there has been intense interest in the physical mechanisms underlying TCR recognition of peptide/MHC as well as the physical correlates with immunological function.

TCRs are similar in some respects to antibodies, consisting of four immunoglobulin domains and an antigen binding site with multiple CDR (complementarity determining region) loops. However, TCRs and antibodies differ strikingly in the nature of the antigen that is recognized. Whereas antibodies can be elicited to molecules of nearly unlimited structural or chemical diversity, TCRs recognize a composite surface consisting of the antigenic peptide in an extended form flanked by the α helices that form the walls of the MHC peptide-binding groove (Figure 1). The peptide typically contributes approximately 30% of the recognized solvent accessible surface area (Rudolph et al., 2006), meaning that the MHC contributes significantly to the interface. This combined recognition of non-self (the peptide)

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in the context of self (the MHC) is a fundamental facet of cellular immunity. TCRs are also cross-reactive, capable of binding and initiating responses to multiple peptide/MHC antigens (Wucherpfennig et al., 2007). The properties of dual recognition of a composite surface together with extensive cross-reactivity have further stimulated interest in the physical underpinnings of TCR recognition of ligand.

Here, we discuss approaches that are useful in characterizing the affinities and thermodynamics for interactions between soluble ectodomains of TCRs and their peptide/MHC ligands, focusing primarily on isothermal titration calorimetry, surface plasmon resonance, and fluorescence anisotropy, and highlighting advantages, disadvantages, and potential pitfalls of each. As TCR-peptide/MHC interactions are typically of low-to-moderate affinity (K_D values for soluble constructs are typically in the single-to-double digit micromolar range) (Davis et al., 1998), we also discuss approaches beneficial in obtaining more accurate binding data useful in the analysis of mutants for alanine scanning studies or more complex experiments such as double-mutant cycles. The latter approach might be expected to shed light on the distribution of binding energy within TCR-peptide/MHC interfaces, addressing questions such as the “basal” level of affinity TCRs maintain towards MHC and the extent to which various loops are directed energetically towards the peptide vs. the MHC α -helices (Collins and Riddle, 2008; Garcia et al., 2009).

2. Isothermal titration calorimetry of TCR-peptide/MHC interactions

2.1. Introduction to titration calorimetry

Isothermal titration calorimetry (ITC) is an ideal method for characterizing receptor-ligand interactions, as it does not require the addition of a potentially interfering label nor does it require attachment of a binding partner to a surface. Further, as the signal reports directly on the binding enthalpy change (ΔH°) as well as the equilibrium constant and thus the free energy change (ΔG°), it is possible to obtain a nearly complete thermodynamic profile (ΔG° , ΔH° , and ΔS°) from a single experiment (ITC has recently been reviewed several times, most recently by (Freyer et al., 2008). A fourth thermodynamic parameter, the binding heat capacity change (ΔC_p) is available through a linear fit to enthalpy changes measured as a function of temperature. Knowledge of binding thermodynamics is becoming increasingly desirable when examining receptor-ligand interactions, as it can aid in deconvoluting the forces driving binding. Thermodynamic information is particularly useful when interpreting the physical consequences of mutations or in efforts to guide the design of interactions with stronger affinity. Interactions between TCRs and peptide/MHC complexes are no exception to these questions, and since the first report in 1999 (Willcox et al., 1999), ITC has been used several times to probe TCR-peptide/MHC interactions (Armstrong and Baker, 2007; Davis-Harrison et al., 2005; Jones et al., 2008; Krogsgaard et al., 2003; Miller et al., 2007), providing information about the forces driving individual interactions as well as general information about the role of flexibility in receptor specificity and cross-reactivity.

2.2. Concentration requirements and data quality

Although calorimetry remains one of the foremost techniques for characterizing macromolecular interactions, a downside of the technique is its relative insensitivity, requiring high concentrations and large volumes of protein. Although this has been mitigated somewhat with the introduction of new instrumentation with greater sensitivity and smaller cell volumes, it is still a problem for TCRs and peptide/MHC complexes given that these proteins require considerable effort to produce recombinantly. Although other expression systems are occasionally used, most recombinant TCR and class I peptide/MHC is produced by refolding from bacterially expressed inclusion bodies (Garboczi et al., 1992; Garboczi et al., 1996b). Refolding yields can vary dramatically, particularly with TCRs, for

which no single stabilizing strategy has proven consistently successful. The situation is further compounded by the weak-to-moderate affinity of most TCR-peptide/MHC interactions.

Importantly, in the absence of experimental constraints, obtaining a full set of accurate binding data from an ITC experiment normally requires the concentrations to be matched to both the affinity and the enthalpy change. The issue of the enthalpy change is obvious, as the smaller in magnitude this becomes, the weaker the ITC signal will be and correspondingly, the more difficult binding will be to detect. Weak enthalpies can be overcome by injecting more protein. Yet this brings up the more complex issue of concentrations and affinities: measuring both accurate affinities and enthalpy changes by ITC requires an optimal degree of curvature to the data that also allows estimation of pre- and post-saturation baselines. Practically, this is expressed in the concept of the c value: the product of the binding constant ($1/K_D$) and the concentration of the protein in the cell is recommended to lie in the range of 1–1000 in order to obtain an accurate fit to the data (Wiseman et al., 1989), although in practice a range of 10 to 500 is more realistic given the noise and experimental error present in most experiments. For TCR-peptide/MHC interactions, with a typical affinity (K_D) of 20 μ M, the concentration of protein in the cell should thus lie somewhere in the range of 200 μ M to 10 mM. With the need for much higher concentrations in the syringe, the practical challenges with calorimetry are understandable.

The challenge most likely to be encountered in ITC of TCRs and peptide/MHC interactions is very low c (<1), stemming from the weak binding affinities and low availability of protein. In low c experiments, the enthalpy change is usually poorly defined, leading to accuracy errors in ΔH° (and thus ΔS°) and precision errors in K_D . Fixing the stoichiometry (n value) can to some extent mitigate this problem (Turnbull and Daranas, 2003). However, this is likely to be a poor solution when working with TCRs or MHC proteins. Although only one peptide/MHC binds to a TCR, in practice the stoichiometry is often used as a correction factor accounting for inaccuracies in protein concentration. As the activity of refolded TCR or peptide/MHC is rarely 100%, fitting for stoichiometry is necessary to account for the level of inactive protein. In our experience, whether TCRs or peptide/MHC complexes are in the calorimeter cell, despite exhaustive purification and using fresh protein, the fitted stoichiometry parameter is often in the range of 0.8 to 0.9, but occasionally much lower.

In some cases, accurate equilibrium constants can be determined with ITC performed at very low c values, even if enthalpy changes cannot (Tellinghuisen, 2008; Turnbull and Daranas, 2003). Affinities determined this way still provide the opportunity to determine enthalpy changes (and thus entropy and heat capacity changes) via van't Hoff analysis. Yet such experiments require reaching full saturation in a binding experiment. If a low c experiment is being performed due to a low affinity interaction, the protein requirements for achieving saturation may be prohibitive.

2.3. Linkage effects in calorimetric experiments

Although the high sample requirements can make calorimetry with T cell receptors difficult, this challenge clearly can be overcome, and as noted earlier new instrumentation with greater sensitivity and lower sample needs is becoming increasingly available. Furthermore, there is interest in engineering high affinity TCR variants (Li et al., 2005; Shusta et al., 2000), and an ideal use of calorimetry is to examine the thermodynamic basis for improvements in binding affinity. For those interactions that can be characterized by ITC, what other opportunities and challenges can ITC provide?

A key issue when interpreting thermodynamic data for protein binding reactions is the influence of other equilibria that are linked to binding. Calorimetry, with its ability to measure a nearly complete suite of thermodynamic parameters in a single experiment, is especially useful for examining linked equilibria (Baker and Murphy, 1996; Fisher and Singh, 1995; Horn et al., 2002). The most commonly encountered form of linked equilibria is linkage of binding to changes in protonation, which occurs when the pK_a of an ionizable group changes upon binding. This can occur, for instance, when a charged group is placed in a less polar environment or becomes involved in a hydrogen bond or other electrostatic interaction (Fitch et al., 2002). When the pK_a shifts, protons are released to or taken up from solution. As binding reactions are invariably carried out in a pH-buffered solution, release of a proton into or removal of a proton from solution is countered by absorption or release of a proton from the buffer. Importantly, most biological buffers have very large enthalpies of proton absorption/release (HEPES, for example, has an ionization enthalpy of 5 kcal/mol). Thus, even a fractional pK_a change occurring upon binding will have enthalpic consequences, contributing significantly to the ΔH° that is measured in an ITC experiment. As the proton exchange does not influence the affinity, there will be a compensatory change in ΔS° (Baker and Murphy, 1996). In some cases, linkage to protonation can dramatically influence the binding heat capacity change (Guinto and Di Cera, 1996).

If the reasons for performing a calorimetric experiment are to obtain thermodynamic data for comparison with structural information, or if different interactions are to be compared (for example, two different TCR-peptide/MHC interactions), the potential influence of linked protonation should be examined for the comparisons to be most meaningful. An example of linked protonation occurring in TCR-peptide/MHC interactions is provided by recognition of the Tax₁₁₋₁₉/HLA-A2 ligand by the A6 TCR (Armstrong and Baker, 2007), where a pK_a change from 7.5 to 6.9 occurring upon binding imparts such an influence that the binding ΔH° and ΔS° varies by as much as 4-fold in different buffers (Figure 2).

The diagnostic for the influence of linked protonation is easy, if expensive from a protein requirement standpoint: perform multiple titrations at the same pH in buffers with different ionization enthalpies (for example, HEPES, phosphate, and imidazole). Ionization enthalpies are known for all common biological buffers (Christensen et al., 1976; Fukada and Takahashi, 1998). A plot of the measured ΔH° of binding vs. the ionization enthalpy of the buffer will reveal the extent of linkage present; this plot will be linear according to the following equation:

$$\Delta H_{obs}^\circ = \Delta H_o^\circ + nH^+ \Delta H_{buff}^{ion} \quad (\text{Eq. 1})$$

where ΔH_{obs}° is the measured binding enthalpy, nH^+ is the number of protons released at that pH, and ΔH_{buff}^{ion} is the ionization enthalpy of the buffer. ΔH_o° is the intercept of the line, and can be interpreted as the protein binding enthalpy removed from the influence of buffer effects. If the slope of this analysis is zero within error, then no proton linkage is present and no further decisions are necessary. However, a non-zero slope, as shown in Figure 2 for the binding of A6 to Tax₁₁₋₁₉/HLA-A2, requires further consideration, particularly when

interpreting the intercept (ΔH_o°). This value, the binding enthalpy no longer influenced by the buffer, still contains a contribution from the magnitude of the pK_a shift and its enthalpic component (Baker and Murphy, 1996). If the goal of the ITC experiments are to compare binding thermodynamics with those estimated from structure using methods that do not account for the energetics of the pK_a shift, such as commonly applied empirical surface area-based algorithms (Baker and Murphy, 1998; Spolar and Record, 1994), then further measurements also varying pH are necessary to extract the “intrinsic” binding

thermodynamics (Armstrong and Baker, 2007; Baker and Murphy, 1997; Barbieri and Pilch, 2006). However, for comparison of different interactions, values of ΔH_o° (and entropy changes determined from them) are often sufficient, provided the comparisons are performed at the same pH.

Calorimetry can also be used to characterize other forms of equilibria linked to binding, such as ion binding or release, or even conformational changes. To characterize these, the linked equilibria must have significant enthalpic consequences, as well as be present at sufficient levels to tease out during data analysis. Although such effects have yet to be explored calorimetrically in the study of TCR-peptide/MHC interactions, they remain a promising avenue of investigation, particularly with new, higher sensitivity instrumentation (Armstrong et al., 2008).

2.4. Other practical concerns for titration calorimetry

Beyond the issues outlined above, what other concerns are manifest in calorimetry on TCR-peptide/MHC interactions? One issue frequently encountered is the need to perform blank (baseline) titrations of titrant into sample buffer. These are usually described as being necessary to counter the heats of dilution and mixing that always exist when a titrant is titrated into a calorimeter cell, regardless of whether binding occurs. Yet performing blank titrations requires twice the titrant, further increasing the protein cost of an ITC experiment. One way this can be avoided is if the experiment clearly exceeds saturation, the post-saturation heats can be used to determine the dilution/mixing heat. Yet as noted earlier, exceeding saturation may not always be possible in TCR binding experiments.

A more practical method for accounting for the heat of dilution/mixing is to simply include it as a baseline offset during curve fitting. Baseline offsets are included in many other analysis techniques, and there is no fundamental reason they cannot be included in the analysis of ITC data. Indeed, this is routinely done in our laboratory (e.g., Armstrong and Baker, 2007; Davis-Harrison et al., 2005). The approach requires a simple modification to the fitting function, adding an adjustable baseline parameter to the penultimate equation describing the heat that is released at each injection. Unfortunately this is not easily achievable with the software distributed with current commercial calorimetric instrumentation, requiring the user to use other software tools for data analysis. However, if the integrated heats are available from the instrument software, writing a fitting function in any number of data analysis packages is straightforward and a good exercise for investigators wishing to gain insight into the equations describing calorimetric data and the process of nonlinear least squares analysis. Note that adding a baseline offset does add another adjustable parameter to the fit. In our experience, at very low c this can negatively impact the fitting such that fits cannot converge. However, for data with a modicum of pre- and post-saturation baselines, it provides no disadvantages to the quality of the fit. In a detailed, global analysis of multiple ITC experiments, inclusion of a baseline offset did not result in suboptimal parameter correlation or negatively impact the precision of the other fitted parameters (Armstrong and Baker, 2007).

3. Surface plasmon resonance studies of TCR-peptide/MHC interactions

3.1. Introduction to surface plasmon resonance

TCR-peptide/MHC interactions are notable in that, beginning with some of the first studies in 1994, they helped popularize the use of surface plasmon resonance (SPR) spectroscopy in characterizing macromolecular interactions (Corr et al., 1994; Matsui et al., 1994). The sample requirements for SPR are much lower than that of ITC, and the technique is more amenable for measuring weak-to-moderate affinities. Since the late 1990s, numerous studies

have used SPR to characterize TCR-peptide/MHC interactions (we regret not having sufficient space to reference all published studies using SPR with TCRs; the number of publications as of this writing exceeds 200).

As a technique for measuring biomolecular interactions, SPR is now well established. Briefly, a binding partner is tethered (either covalently via cross-linking or non-covalently via an affinity tag) to a sensor surface. A second binding partner is flowed over the surface, and the signal increases as mass accumulates on the sensor surface due to binding. The technique can be used to obtain binding kinetics and affinities, and via van't Hoff analysis, underlying binding thermodynamics (ΔH° , ΔS° , and ΔC_p).

Since its introduction, many investigators have discussed SPR experimental design, data acquisition, and data analysis. The technique's versatility and ease of use naturally lends itself to wide applicability, but this same versatility and ease has led to concerns about the way in which SPR is sometimes applied. Commonly discussed issues include immobilization chemistry, flow rates, blank corrections, replicate injections, model choice for analysis, and curve fitting strategies. These concerns have been reviewed several times (e.g., Myszka, 1999; Rich and Myszka, 2008). This literature is worth consulting to ensure the acquisition of high quality data and its proper analysis.

In addition to such concerns, TCRs and peptide/MHC complexes provide some additional challenges. One unique aspect is the noncovalent nature of the peptide/MHC complex. At the concentrations used for calorimetry, peptide dissociation is usually not an issue, as for most peptides the equilibrium will be shifted far towards the complexed state. For example, the Tax₁₁₋₁₉ peptide binds HLA-A2 with an affinity near 20 nM, well below the peptide/MHC concentrations needed to characterize TCR binding (Binz et al., 2003). However, in SPR, if very low peptide/MHC concentrations are used, or if the peptide/MHC is tethered to the surface, peptide dissociation could be problematic. In our laboratory, we typically couple TCRs to the sensor surface. Peptide/MHC complexes are stabilized by maintaining the sample storage chamber at low temperature, which reduces the peptide-MHC dissociation rate and thus limits accumulation of any peptide-free MHC. However, measurements with peptides that bind weakly to MHC molecules may necessitate additional safeguards, such as ensuring all samples are diluted with buffer containing a constant concentration of excess peptide (Jones et al., 2008). If the peptide/MHC complex is tethered to the surface, use of model that accounts for a decaying surface may be needed (Joss et al., 1998).

3.2. Use of surface plasmon resonance to measure low affinity TCR-peptide/MHC interactions

Although SPR may be more amenable for low affinity interactions than ITC, accurate measurements of low affinity interactions will usually be difficult when protein is limiting due to the inability to generate a full titration curve. In some cases, if the low affinity is due to a slow association rate with a reasonably long dissociation rate, then measuring affinity via kinetic methods may circumvent this problem (for example, the recognition of the Tax₁₁₋₁₉-IBA ligand by the A6 TCR occurs with an affinity near 160 μ M (Gagnon et al., 2006), a measurement that was obtained via kinetic rather than equilibrium methods). Yet often, low affinities arise from very rapid dissociation rates, which can preclude the use of kinetics in determining affinities by SPR.

However, SPR provides a means for greatly increasing the accuracy of binding constants that is particularly useful for low affinity TCR-peptide/MHC interactions. The primary problem in low affinity titration curves lies in knowing where saturation is. Very simply, if the K_D is the free ligand concentration where 50% binding occurs, how can one determine

the concentration that gives 50% bound if the concentration that gives 100% bound cannot be determined or reliably estimated?

The general hyperbolic equation that is fit to in a 1:1 equilibrium binding experiment is:

$$RU = RU_{\max} \frac{K[L]}{1 + K[L]} \quad (\text{Eq. 2})$$

where RU is the instrument response, $RU_{\max}$ is the activity of the sensor surface, K is the binding equilibrium constant (equal to $1/K_D$), and $[L]$ is the concentration of injected ligand. Typically, if 100% saturation is not reached, $RU_{\max}$ is estimated during the fitting process from the curvature of the binding response. However, how much saturation is required to accurately determine $RU_{\max}$ (and thus K_D) will be determined by many variables, including the number of data points, instrument noise, dilution errors, etc. Clearly, the greater the degree of saturation the more reliable the fit will be. Yet if a high degree of saturation cannot be reached, what options are available to ensure an accurate measurement?

One method available in SPR is to independently determine the activity of the sensor surface and fix this value in subsequent analyses. For example, if a peptide/MHC complex is on the sensor surface and a high affinity TCR variant is available, $RU_{\max}$ can be determined with the high affinity receptor before or after experiments are performed with the weaker binding wild-type molecule. Alternatively, if peptide/MHC or TCR variants are of interest, $RU_{\max}$ can be determined independently with the wild-type molecule.

The advantages of this approach are demonstrated in Figure 3, which highlights results from the analysis of 100 simulated, noisy datasets that reach only 33% saturation. The data are for an interaction proceeding with a 1 mM K_D and for a sensor surface with a $RU_{\max}$ of 1000. By most standards, given noise and experimental error, reaching only 33% saturation in a binding experiment would lead to suspicions about the accuracy of the fitted K_D . This is easily demonstrated, as highlighted in Figure 3B. A 1 mM K_D corresponds to a dissociation ΔG° of 4092 cal/mol at 25 °C. Analysis of the 100 datasets by the traditional means in which both $RU_{\max}$ and K_D are fitted parameters leads to an average of ΔG° 4098 ± 613 cal/mol. While at first glance the agreement with the actual value (4092 cal/mol) is impressive, this agreement only demonstrates the power of repeating experiments – one is likely not to repeat a binding experiment 100 times, and the standard deviation of 613 cal/mol indicates that any one ΔG° measurement is likely to be inaccurate. The situation is much worse if one examines the precision in the experiments: the average fitting error is 1208 cal/mol, or 30% of the actual binding free energy. This result indicates that any particular fit, even if it converged on an affinity close to the actual value, will have a large uncertainty. Moreover, of the 100 datasets simulated for Figure 3, 14 could not be fit due to near-perfect parameter correlation. Clearly the odds of obtaining an accurate and precise affinity from one of these datasets are low if one is fitting for both surface activity and K_D .

Yet when the same data are fit with the $RU_{\max}$ fixed at 1000, the situation is vastly improved. The average ΔG° is again very close to the real value, as expected (4099 cal/mol vs. 4092 cal/mol). However, the standard deviation in this value is only 90 cal/mol, a nearly 7-fold improvement in accuracy over the case when $RU_{\max}$ is floated. Furthermore, the average error in any one experiment is only 81 cal/mol, a 15-fold improvement in precision. Every data set could be fit, and for those datasets which could not be fit when $RU_{\max}$ was floated, the average ΔG° is still almost exactly correct: 4084 ± 95 cal/mol, with an average error of 82 cal/mol. For comparison, knowing the $RU_{\max}$ in advance when only 33% saturation is achieved results in the same level of accuracy and precision as would be achieved in a traditional analysis in which 90% saturation is reached. However, with a 1 mM affinity, reaching 33% saturation requires injecting a sample at a concentration of 500 μM,

difficult but achievable for TCRs or peptide/MHC complexes. Reaching 90% saturation, on the other hand, would require injecting a 9 mM sample, well above what is reasonably achievable for these molecules.

Obviously though, any independent measurement of $RU_{\max}$ includes error: when fitting 100 simulated noisy datasets that reach 90% saturation, $RU_{\max}$ was determined with an average error of 4%. What is the effect of error in a pre-determined $RU_{\max}$ in this analysis? Surprisingly, the effect is small. In Figure 3C, errors in $RU_{\max}$ were introduced into the analysis of the 33% saturation data. Every 1% error in $RU_{\max}$ led to an error in ΔG° of approximately 0.2%. Thus, even a very large error of 20% in $RU_{\max}$ leads to a tolerable error in ΔG° of only 4%.

What else is needed to accurately fix the maximum response in an SPR experiment? Given that the signal is proportional to the amount of active material on the sensor surface, the more the better. This advice is counter to common recommendations to limit the amount of material on a surface. However, low surface activities are usually recommended for kinetic experiments in order to reduce mass transport and rebinding effects, neither of which are issues when performing equilibrium experiments. Note that for these reasons flow rates can be reduced to a minimum when performing equilibrium studies with SPR, reducing the sample requirements for the injected ligand.

While of great utility, this method of enhancing accuracy and precision of binding affinities in SPR is not amenable in all cases. If a TCR or peptide/MHC surface decays significantly over the course of an experiment (due, perhaps to peptide dissociation from MHC or the detrimental effects of any necessary regeneration steps performed between injection cycles), $RU_{\max}$ will not be constant. More practically, if one is studying an interaction without the availability of a high affinity variant or not studying mutants of what is otherwise a reasonably affinity interaction, fixing $RU_{\max}$ is simply not an option. Yet in the study of T cell receptor recognition, many lines of investigation have turned towards examining the effects of mutations in the TCR or MHC or substitutions in the peptide, and the approach outlined above will be of considerable utility in such cases. One promising avenue of investigation is double-mutant cycle experiments, which involve measurements with two single amino acid mutants and one double mutant (Schreiber and Fersht, 1995). Depending upon the strength and type of interaction between the two sites that are mutated, affinities could drop substantially in these experiments. Yet double mutant cycle experiments have potential to address outstanding questions in TCR-peptide/MHC interactions, such as the “basal” level of affinity TCRs maintain towards MHC and the extent to which various loops are directed energetically towards the peptide vs. the MHC α -helices (Collins and Riddle, 2008; Garcia et al., 2009). The method outlined above may thus prove particularly useful in addressing immunologically relevant questions about how TCRs engage peptide/MHC.

3.3. Underlying binding thermodynamics from SPR experiments

Binding thermodynamics beyond ΔG° are available from SPR via van't Hoff-style analyses. Because interactions for protein-protein interactions are usually associated with large negative heat capacity changes (Stites, 1997), a direct fit to the temperature dependence of the free energy change is likely to be more preferable than a traditional natural log of K vs. inverse temperature van't Hoff analysis (provided the traditional analysis incorporates a ΔC_p the approaches will be numerically similar, but fitting to ΔG° vs. temperature will more effectively illustrate the heat capacity change). This approach has been used numerous times with T cell receptors, yielding data about the relationships between entropy, heat capacity, and conformational changes that may be occurring upon receptor binding (reviewed by Armstrong et al., 2008). It is important though to appreciate that the accuracy of the ΔH° , ΔS° , and ΔC_p values determined in this manner depends on the accuracy of the individual

ΔG° measurements. Ideally, realistic errors in ΔG° should be incorporated into the fit, which can easily be performed in most nonlinear curve fitting packages. In this regard, the approach outlined above for increasing the accuracy for low affinity measurements by SPR may be useful to determine underlying binding thermodynamics by SPR. Finally, note that the effects of linked equilibria on measured binding thermodynamics that were outlined for titration calorimetry will also be manifest in thermodynamics determined by van't Hoff methods (Horn et al., 2002; Horn et al., 2001). Although a linkage analysis by van't Hoff methods may be impractical, in its absence caution may be warranted when interpreting binding thermodynamics for very different protein-protein interfaces.

4. Fluorescence anisotropy as a tool for characterizing TCR-peptide/MHC interactions

4.1. Introduction to fluorescence anisotropy

Fluorescence has not routinely been used to characterize TCR-peptide/MHC interactions, primarily because there is little or no change in intrinsic fluorescence upon binding and the introduction of a probe within the protein-protein interface would likely interfere with recognition. FRET has recently been used in kinetic studies, with a donor on the MHC protein and an acceptor on the TCR, bypassing some of the limitations inherent to surface plasmon resonance (Gakamsky et al., 2007). Yet beyond these studies, the vast majority of quantitative studies on TCRs and peptide/MHC interactions has been performed with SPR, and to a lesser extent, ITC.

Fluorescence anisotropy (equivalent to fluorescence polarization via a simple mathematical relationship and recently reviewed by Jameson and Mocz, 2005) reports on molecular motion that occurs over the lifetime of a fluorescent probe. A mode of motion occurring in every molecule in solution is molecular tumbling, the rate of which to a first approximation is proportional to a molecule's size: the larger the size, the slower the tumbling, and the less tumbling contributes to the loss of anisotropy over the course of the fluorescence lifetime. For measuring binding, one simply measures the anisotropy of a fluorescent molecule in the presence of increasing concentrations of a binding partner. As the fluorescent molecule forms a larger complex, the slower tumbling of the complex is reflected as an increase in the anisotropy. Fluorescence anisotropy has a long history in the analysis of macromolecular interactions, and has seen particular use in investigating interactions between proteins and small molecules or nucleic acids (Heyduk et al., 1996; Sportsman, 2003), including the interactions of peptides with MHC proteins (Baxter et al., 2004; Binz et al., 2003; Buchli et al., 2006; Chen and Bouvier, 2007; Dedier et al., 2001). Fluorescence anisotropy is less commonly used to monitor protein-protein interactions, largely due to the availability and success of other techniques, as well as limitations placed by the relationship between the size and tumbling rate of the fluorescent molecule and the magnitude of the anisotropy change that occurs upon binding.

Yet fluorescence anisotropy deserves special mention here due to its potential for efficiently characterizing low affinity interactions, and TCR-peptide/MHC interactions in particular. This is because the advantages provided by SPR described in Section 3.2 also apply to fluorescence anisotropy, but as discussed below, because the maximum shift in anisotropy is an intrinsic property of a molecular complex rather than a property unique to each experiment, fluorescence anisotropy provides further advantages over SPR.

For a 1:1 binding interaction monitored by changes in fluorescence anisotropy, one way to represent the binding response is via a traditional hyperbolic binding curve of the form:

$$\Delta A = \Delta A_{\max} \frac{K[L]}{1 + K[L]} \quad (\text{Eq. 3})$$

where ΔA is the measured change in anisotropy that occurs upon binding, $[L]$ is the concentration of free ligand, and $\Delta A_{\max}$ is the maximum change in anisotropy, equal to the anisotropy of the complex minus the anisotropy of the free labeled ligand ($A_{\text{complex}} - A_{\text{ligand}}$). The anisotropy of the free ligand is easily measured. The anisotropy of the complex can be determined by either fitting for it in a binding experiment that achieves complete (or near-complete) saturation. Once the anisotropy of the complex is measured though, this value can be fixed for the analysis of lower affinity interactions involving similar proteins, such as MHC proteins presenting different peptides, or MHC or TCR mutants. All the advantages described in Section 3.2 apply to such an experimental approach, the advantage over SPR being that the anisotropy of the complex is unchanging from experiment to experiment (provided the mutations or different peptides do not fundamentally alter the shape and thus rotational properties of the ternary complex). In SPR, on the other hand, the maximum surface capacity changes with the preparation of each new surface and can degrade over the course of an experiment. As in SPR, the anisotropy of the complex can be measured through the use of high affinity TCR variants or determined with a wild-type TCR that binds with reasonably tight affinity.

Obviously, one of the proteins must be fluorescently labeled in order to use fluorescence anisotropy. The site of labeling must be distal enough from the binding interface so as not to influence binding, and if a peptide/MHC complex is labeled, not interfere with the peptide-MHC interaction. The site should not possess a high level of intrinsic mobility (e.g., a disordered loop), as this could result in such rapid depolarization of fluorescence that there is insufficient signal to report on changes in molecular tumbling (although this can be offset with the use of a longer lifetime fluorescent probe). A final requirement is tolerance to a cysteine mutation, as in most cases a cysteine reactive probe will be needed for site-specific labeling. An ideal position for labeling is thus an amino acid in a rigid unit of secondary structure with a solvent exposed side chain, preferably polar, as most commonly used fluorescence probes are highly polar themselves.

4.2. An example TCR-peptide/MHC interaction characterized by fluorescence anisotropy

To demonstrate the utility of using fluorescence anisotropy to monitor TCR binding to a peptide/MHC complex, we produced variants of the HLA-A2 class I MHC complex separately labeled with fluorescein at position 145 and position 195 of the HLA-A2 heavy chain. Position 145 is near the edge of the $\alpha 2$ helix in the HLA-A2 peptide binding domain, and position 195 is in a loop in the $\alpha 3$ domain distal to the peptide binding domain. The position 145 label required mutating a histidine to cysteine, whereas the position 195 label required mutating a serine. The side chains of both positions extend away from the protein surface and in available crystallographic structures do not appear to interact with other protein atoms. Despite being in the peptide binding domain, position 145 is fully solvent exposed and atoms of the imidazole ring are least 11 Å away from any TCR atoms in known ternary complexes with HLA-A2 (Buslepp et al., 2003; Chen et al., 2005; Ding et al., 1998; Gagnon et al., 2006; Garboczi et al., 1996a; Ishizuka et al., 2008; Miller et al., 2007; Stewart-Jones et al., 2003). The primary reason for choosing these two positions is that 195 appears to be highly flexible as it is in an exposed loop that is occasionally disordered in structures with HLA-A2, and position 145 is in a relatively rigid unit of secondary structure. These two positions should thus highlight the extent intrinsic flexibility has on signal and data quality: compared to the more rigid location at position 145, the more flexible location at position 195 should result in a lower overall change in anisotropy, as the greater

flexibility will diminish the contribution of molecular tumbling to the depolarization of fluorescence, degrading the signal that should be produced upon TCR binding.

The two mutant HLA-A2 complexes were expressed and refolded in the presence of the HTLV-1 Tax₁₁₋₁₉ peptide and purified according to standard procedures (Davis-Harrison et al., 2005; Garboczi et al., 1992), and labeled with a cysteine-reactive fluorescein derivative (fluorescein-5-maleimide). Extensive post-labeling dialysis and chromatographic purification was performed to ensure the samples were free of unreacted label, as the presence of free label, with its very low anisotropy would negatively influence the assay. Control labeling reactions performed with wild-type Tax₁₁₋₁₉/HLA-A2 indicated that this procedure resulted in no non-specific labeling and no residual free label.

Aliquots (120 μ L) of 100 nM fluorescently-labeled Tax₁₁₋₁₉/HLA-A2 were then incubated with increasing amounts of purified A6 TCR (assembled with the aid of a leucine zipper, see (Ding et al., 1999)), and the anisotropy measured using a Beacon 2000 fluorescence spectrometer. Final volumes for each sample were 200 μ L. As shown in Figure 4, for both samples, increasing the TCR concentration resulted in an increase in anisotropy. However, as anticipated, the more flexible position 195-labeled sample generated substantially poorer data than the position 145-labeled sample, with greater scatter and an overall change in anisotropy only 1/3 that of the position 145-labeled sample. Indeed, the starting anisotropy for the position 195-labeled sample was much less than that of the position 145-labeled sample (0.05 for position 195, 0.12 for position 145), reflecting the greater flexibility of the loop at position 195 compared to the more rigid helix at position 145. As the overall change in anisotropy reflects the dynamic range available to the assay, measurements with HLA-A2 labeled at position 145 will be substantially more reliable than those with protein labeled at position 195. On this note, a longer lifetime fluorescent probe is likely to provide even greater dynamic range, as the molecules will experience greater tumbling over the lifetime of the probe.

Fitting the position 145-labeled data to a single-site binding model yielded an affinity of 0.4 μ M, identical within error to the 0.3 μ M affinity determined by ITC (Armstrong and Baker, 2007). Note that the values of 0.3 – 0.4 μ M are slightly tighter than the 1–2 μ M affinity measured by SPR (Davis-Harrison et al., 2005). Yet unlike SPR the ITC and fluorescence anisotropy measurements are pure solution measurements and slight variations between SPR and pure solution measurements are not unusual. These results demonstrate the potential for using fluorescence anisotropy in monitoring TCR-peptide/MHC interactions. The small sample volumes, low protein requirements, and ability to fix the maximum shift in anisotropy make fluorescence anisotropy particularly attractive for assaying mutants, perhaps even more so than surface plasmon resonance. The technique is also easily adaptable for measuring binding thermodynamics via van't Hoff analysis: sealing the samples and simply measuring them at multiple temperatures will obviate the need to create new samples for each temperature point, as is necessary with SPR. For such an experiment, fluorimeters that can measure anisotropy in plate format will be particularly useful. Note that as with SPR, incubating the samples with excess peptide may be needed to ensure peptide/MHC stability over the course of the measurements. A final caution is that as changes in the anisotropy signal are closely linked to the size of the fluorescently labeled molecule and its binding partner, different TCR constructs may yield data of differing quality. As noted above, the A6 TCR construct used in Figure 4 was assembled with the aid of a leucine zipper (Ding et al., 1999; O'Shea et al., 1993). It is becoming more common to stabilize soluble TCRs with an engineered disulfide bond linking the α and β chain (Laugel et al., 2005); the resulting molecules are smaller than the equivalent zippered constructs and will likely yield smaller anisotropy changes than those shown in Figure 4. This could potentially be offset by the use of a longer lifetime fluorophore as discussed above.

5. Concluding remarks

Measurements of the interactions between T cell receptors and their peptide/MHC ligands continue to provide valuable insight into the biology of the cellular immune system. Although challenges in producing the proteins recombinantly and their weak-to-moderate affinities can make detailed physical studies difficult, the extraordinary molecular recognition properties displayed by TCRs, such as dual recognition of self and non-self and the simultaneous display of both cross-reactivity and specificity, make such studies well worth the effort. Comparisons of binding thermodynamics with structural and functional properties are likely to shed considerable light on how these properties are achieved, and new instrumentation will facilitate these experiments. Surface plasmon resonance has played and will continue to play a dominant role in characterizing TCR-peptide/MHC interactions, and its usefulness in characterizing low affinity interactions can be extended with simple experimental approaches. The approaches described here are particularly attractive for the study of mutants, and make informative double-mutant cycle experiments an attractive line of experimentation. Less commonly used techniques such as fluorescence anisotropy may be of even greater utility for such experiments.

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Figure 1.

Structural overview of a complex between a T cell receptor and a peptide/MHC molecule. The receptor is positioned at the top in dark grey. The peptide/MHC complex is underneath in light grey, with the peptide in black rendered in stick format. The structure is that of the B7 TCR bound to the Tax₁₁₋₁₉ peptide presented by the class I MHC HLA-A2 (Ding et al., 1998).

Figure 2.

Effects of linked protonation on the binding ΔH° (squares, solid line) and ΔS° (circles, dashed line) for recognition of Tax₁₁₋₁₉/HLA-A2 by the A6 TCR. The linkage results from a pK_a shift from 7.5 to 6.9 that occurs upon binding. The slope of the binding ΔH° vs. buffer ionization enthalpy yields the number of protons released at the experimental pH (6.4), and the intercept yields the buffer-independent binding enthalpy change at the experimental pH. The various buffers used are listed across the top according to ionization enthalpy. Data are from Armstrong and Baker, 2007.

Figure 3.

Fixing the activity of a SPR sensor surface dramatically enhances the ability to recover affinities from noisy, incomplete binding data. Binding data to 33% saturation were simulated for an interaction proceeding with a 1 mM K_D (ΔG° of 4092 cal/mol at 25 °C) and an RU_{max} of 1000, and 100 noisy data sets were generated by adding Gaussian-distributed random noise with a Gaussian width of 40 RU. The 100 noisy datasets were then fit with either RU_{max} either fixed or floated as a fitting parameter. **A**) Representative results from four of the noisy datasets. Solid lines represent the fits with both RU_{max} and K_D floated, dashed lines represent theoretically correct, perfect curves, and dotted lines represent fits with RU_{max} fixed at 1000. With RU_{max} floated, the ΔG° values recovered from the fits vary in their accuracy and have substantial errors in precision. The sample on the top right could not be fit due to extensive parameter correlation between RU_{max} and K_D . With RU_{max} fixed at 1000, however, the ΔG° values are much more accurate and the standard errors are substantially lower. **B**) Summary statistics from the analysis of the 100 datasets. Floating RU_{max} results in a large standard deviation in the recovered ΔG° values, and 14% of the datasets could not be fit. The average error in the recovered ΔG° values, 1208 cal/mol, is 30% of the actual ΔG° . Fixing RU_{max} brings the standard deviation in the recovered ΔG° down 7-fold, and results in a 15-fold improvement in the average error. As shown in the third entry in the table, fitting the noisy datasets that only go to 33% saturation with RU_{max} fixed is equivalent to traditional analyses (both RU_{max} and K_D floated) of datasets that reach 90% saturation. **C**) Errors in RU_{max} have a small effect on the error in the recovered ΔG° , with an approximately 0.2% error in ΔG° for every 1% error in RU_{max} .

Figure 4.

Binding of the A6 TCR to the Tax₁₁₋₁₉/HLA-A2 complex monitored by fluorescence anisotropy. Data are shown for Tax₁₁₋₁₉/HLA-A2 labeled at position 145 (squares, solid line) and position 195 (circles, dashed line). The starting anisotropy, dynamic range, and overall data quality data with the position 145-labeled sample are substantially greater than with the position 195-labeled sample, reflecting the influence of site-specific flexibility on the anisotropy data. Fitting the 145-labeled sample to a single site binding model yielded a K_D of $0.37 \pm 0.04 \mu\text{M}$, or a ΔG° of $8.77 \pm 0.06 \text{ kcal/mol}$, in close agreement with the value previously determined by ITC (Armstrong and Baker, 2007).