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TCR Binding to Peptide-MHC Stabilizes a Flexible Recognition Interface

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Summary

The binding of TCRs to their peptide-MHC ligands is characterized by a low affinity, slow kinetics, and a high degree of cross-reactivity. Here, we report the results of a kinetic and thermodynamic analysis of two TCRs binding to their peptide-MHC ligands, which reveal two striking features. First, significant activation energy barriers must be overcome during both association and dissociation, suggesting that conformational adjustments are required. Second, the low affinity of binding is a consequence of highly unfavorable entropic effects, indicative of a substantial reduction in disorder upon binding. This is evidence that the TCR and/or peptide-MHC have flexible binding surfaces that are stabilized upon binding. Such conformational flexibility, which may also be a feature of primary antibodies, is likely to contribute to cross-reactivity in antigen recognition.

Introduction

The adaptive immune response is dependent on the ability of T and B cells to recognize antigens associated with any potential pathogen. This is achieved through the generation of a large repertoire of clonally expressed T and B cell antigen receptors. Because an individual has far fewer lymphocytes than there are potential antigens, it would be advantageous if each antigen receptor could cross-react with a large number of antigens (Hodgkin, 1998; Mason, 1998). The need for cross-reactivity is perhaps most evident in the case of the TCR (Mason, 1998). T cell antigen recognition is already limited to the subset of pathogen peptides that can be processed and presented on host MHC molecules. If, in addition, host TCR repertoires were unable to recognize a large proportion of these peptides, pathogen evasion of T cell antigen recognition would be easier. There is now a large body of evidence indicating that individual TCRs can cross-react with many, often minimally homologous, peptide antigens bound to the same MHC molecule (Bhardwaj et al., 1993; Reay et al., 1994; Evavold et al., 1995; Wucherpfennig and Strominger, 1995; Kaliyaperumal et al., 1996; Kersh and Allen, 1996; Mason, 1998). This raises the question as to how TCRs achieve this level of cross-reactivity.

As with conventional cell–cell recognition molecules (van der Merwe and Barclay, 1994; Davis et al., 1998b), TCR/peptide-MHC interactions have a low affinity (Kd 1–90 μM) (Davis et al., 1998a). However, in contrast to other cell–cell recognition molecules, in which the low affinity is a consequence of a fast dissociation rate constant (k_{off}) (van der Merwe and Barclay, 1994; Davis et al., 1998b), the low affinity of TCR/peptide-MHC interactions is also a consequence of slow association rate constants (k_{on}) (Davis et al., 1998a). The k_{on} values (10^2–10^4 $M^{-1} \times s^{-1}$) reported for TCR/peptide-MHC interactions are up to three orders of magnitude slower than those measured for other cell–cell recognition molecules (van der Merwe et al., 1994; Davis et al., 1998a, 1998b). Similarly, reported k_{off} values (10^{-2}–10^{-1} s^{-1}), although fast when compared with high-affinity protein–protein interactions ($<10^{-3}$ s^{-1}), are much slower than protein–protein interactions with equivalent affinities (>1 s^{-1}) (van der Merwe et al., 1994; Davis et al., 1998a, 1998b).

One possible explanation for the slow kinetics of TCR/peptide-MHC interactions is that binding is accompanied by conformational adjustments at the binding interface. A recent crystallographic study has revealed large conformational changes at a TCR/peptide-MHC interface upon binding (Garcia et al., 1998), but the significance of these changes has been unclear. It is possible that these adjustments reflect underlying flexibility of the TCR antigen-binding site, which could contribute to TCR cross-reactivity. However, crystallographic studies provide little information on conformational flexibility or the effect that this has upon binding ligand. In an attempt to address these questions, we have carried out a detailed kinetic and thermodynamic analysis of two distinct TCR/peptide-MHC interactions, using surface plasmon resonance (van der Merwe and Barclay, 1996) (SPR) and isothermal titration calorimetry (ITC) (Ladbury and Chowdhry, 1996). Our results support the idea that the antigen-binding site on the TCR is flexible and that the low affinity and slow kinetics of TCR/peptide-MHC interactions are a consequence of this conformational flexibility. Finally, we propose that conformational flexibility contributes to the cross-reactivity characteristic of TCR/peptide-MHC interactions.

Results and Discussion

T Cell Receptors Evoked by Viral Infection Bind Peptide-MHC with Low Affinity and Slow Kinetics

We studied two TCRs isolated from human and murine cytotoxic CD8+ T cells that had been evoked by influenza virus infection. Such cytotoxic T cell responses

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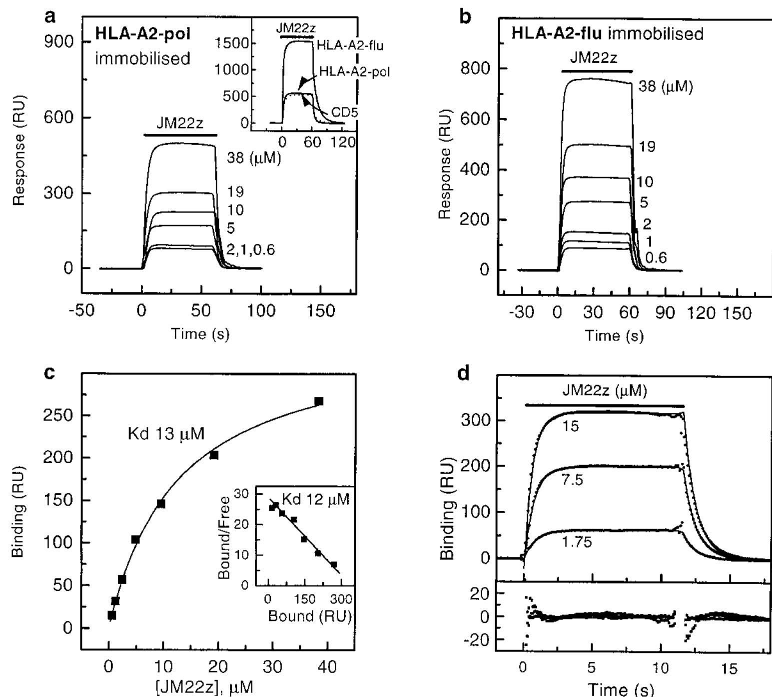

Figure 1. Affinity and Kinetics of JM22z Binding HLA-A2-flu

(A–C) Measurement of affinity by equilibrium binding analysis.

(A and B) JM22z was injected (bars) at the indicated concentrations at a flow rate of 5 $\mu L \times min^{-1}$ over surfaces to which HLA-A2-pol (1700 RU) or HLA-A2-flu (1900 RU) had been immobilized. (Inset) JM22z (28 μM) was injected at 5 $\mu L \times min^{-1}$ over surfaces with immobilized HLA-A2-flu (2800 RU), HLA-A2-pol (4200 RU), or CD5 (4300 RU).

(C) The difference between the equilibrium response with injection over HLA-A2-flu and HLA-A2-pol represents binding and is plotted for each JM22z concentration. Nonlinear curve-fitting of the 1:1 Langmuir binding (A + B↔AB) equation [$AB = B^*AB_{max}/(K_d + B)$] to the binding data yielded a K_d of 13 μM. (Inset). A Scatchard plot of the same data was linear, giving a K_d (slope of plot $= -1/K_d$) of 12 μM.

(D) Kinetic analysis. JM22z was injected at different concentrations at a high flow rate (100 $\mu L \times min^{-1}$) over HLA-A2-flu (2800 RU). The traces shown have had their corresponding background responses (obtained with injection over the HLA-A2-pol surface) subtracted. Numerically integrated rate equations ($dAB/dt = k_{on}{*}A{*}B - k_{off}{*}AB$, $dB/dt = k_{off}{*}AB - k_{on}{*}A{*}B$) derived from the 1:1 Langmuir binding model (A + B↔AB) were simultaneously fitted to the association and dissociation phases of all three injections (global fitting) using BIAevaluation (version 3) software (BIAcore AB). Residual errors from the fits are shown in the bottom panel.

play an important role in the control of acute and persistent viral infections (Doherty et al., 1997; McMichael and Phillips, 1997; Rickinson and Moss, 1997). The human TCR (JM22) studied is specific for an immunodominant epitope comprising residues 58 to 66 from the influenza A matrix protein (M58-66) presented by HLA-A2 (HLA-A2-flu) (Moss et al., 1991; Lehner et al., 1995). The murine TCR (F5) is specific for residues 366–374 of the influenza nucleoprotein presented by $H2\text{-}D^b$ ($H2\text{-}D^b$-NP) (Townsend et al., 1984, 1986; Palmer et al., 1989). Soluble forms of both receptors were expressed in *Escherichia coli* with C-terminal leucine zipper motifs (termed JM22z and F5z) and refolded in vitro (see Experimental Procedures).

Affinity and kinetic analysis was performed using SPR, which measures changes in refractive index near a sensor surface within a small flow cell. The low affinity of the interactions under study necessitated the injection of high concentrations of protein through the flow cell. Because this increases the bulk refractive index of the sample, a background response is observed, which is measured by injecting the sample over a control surface with an irrelevant protein immobilized (Figure 1A, inset). Injection of soluble JM22z over immobilized HLA-A2-flu gave a greater response than injection over either HLA-A2 presenting a peptide from HIV reverse transcriptase (HLA-A2-pol) or a control protein (CD5), indicating specific binding (Figure 1A, inset). Because there was no detectable binding of TCR to HLA-A2-pol at the concentration range used (Figure 1A), the latter was used to measure the background response in subsequent experiments. Affinity measurements were performed by measuring the equilibrium binding response (with background response subtracted) at a range of TCR concentrations (Figures 1A–1C). In this way, the dissociation constant (Kd) was determined to be ~15 μM at 37°C and ~6.6 μM at 25°C (Figure 1C; Table 1). A similar affinity (Kd ~5.6 μM at 25°C) was obtained when measured in the reverse orientation, with the JM22z TCR covalently coupled and the soluble, monovalent HLA-A2-flu in solution (Table 1). This is within the range of values previously reported for nonalloreactive mouse TCR/peptide-MHC interactions (Kd 1–90 μM at 25°C) (Davis et al., 1998a).

A number of pitfalls, including protein aggregation, mass-transport limitations, and rebinding, can lead to underestimation of the binding kinetics (van der Merwe and Barclay, 1994; Schuck, 1997; Davis et al., 1998b). We therefore sought first to confirm the slow kinetics of TCR/peptide-MHC interactions reported by others (Davis et al., 1998a), using an approach designed to avoid these pitfalls. Care was taken in the preparation of the proteins to avoid contamination with multivalent aggregates (see Experimental Procedures). Kinetic constants were derived by global analysis of the data (Karlsson and Falt, 1997), which involves simultaneous nonlinear curve fitting to the complete binding curves obtained with several concentrations of soluble ligand (Figure 1D).

Table 1. Summary of Affinity Constants

Temp (°C)	Immobilized Ligand	Soluble Ligand	Kd (μM)*
37	HLA-A2-flu	JM22z	15 ± 4 (n = 7)
25	HLA-A2-flu	JM22z	6.6 ± 2 (n = 14)
25	JM22z	HLA-A2-flu	5.6 ± 4 (n = 3)
25	$H2\text{-}D^b$-NP	F5z	11 (n = 1)

*Mean ± SD of n determinations.

Table 2. Summary of Kinetic Constants

Temp (°C)	Immobilized Ligand	Soluble Ligand	Immobilization Level (RU)	k_{on}($M^{-1}s^{-1}$)	k_{off} (s^{-1})	Kd_{calc} (μM)
37	HLA-A2-flu	JM22z	600	6.7×10^4	1	14
37	HLA-A2-flu	JM22z	1250	6.9×10^4	1.2	17
37	HLA-A2-flu	JM22z	2800	4.2×10^4	1.1	26
25	HLA-A2-flu	JM22z	800	3×10^4	0.12	4
25	JM22z	HLA-A2-	3240	4×10^4	0.16	4
25	H2-D^b-NP	F5z	1400	nd*	0.8	—

*Not determined.

Satisfactory global fits (Figure 1D) were obtained using the simple (1:1 or Langmuir) binding model (A + B↔AB), yielding a k_{on} of 4.2×10^4 $M^{-1}s^{-1}$ and a k_{off} of 1.1 s^{-1} at 37°C. The fact that the complete binding curves conform to the 1:1 binding model is strong evidence that multivalent aggregates are not contributing to binding. To eliminate mass-transport or rebinding artefacts, we performed the kinetic analysis at low levels of immobilized HLA-A2-flu (Table 2). Decreasing the level of immobilized HLA-A2-flu from 2800 to 1250 RU (response units) resulted in a small increase in the measured k_{on} and k_{off}, suggesting that mass-transport/rebinding artefacts were present at the higher immobilization level (Table 2). However, a further decrease in HLA-A2-flu from 1250 to 600 RU had no effect on the measured k_{on} and k_{off} (Table 2), indicating that mass transport/rebinding artefacts were absent at the two lower surface densities. Importantly, the Kd values calculated from the k_{on} and k_{off} (Kd_{calc} 14–17 μM) agreed very well with the Kd (~15 μM) determined by equilibrium binding (Tables 1 and 2). Finally, the same kinetic constants were obtained in both orientations, with either HLA-A2-flu or JM22z immobilized (Table 2). Taken together, these results provide strong evidence that the kinetic constants reported in this study are accurate and unaffected by artefacts that can occur in BIAcore experiments (van der Merwe and Barclay, 1996).

One poorly understood aspect of the T cell response is the extent to which the binding properties of the TCR determine which T cell(s) dominate(s) the response to an infection. Although there have been a number of studies of TCR/peptide-MHC interactions (Plaksin et al., 1997; Davis et al., 1998a), one novel aspect of this binding study is that we have analyzed TCRs from T cells evoked in response to an infection. Cytotoxic T cells with TCRs very similar or identical to JM22 appear to dominate the response in influenza A-infected HLA-A2 individuals (Moss et al., 1991; Lehner et al., 1995), indicating that this TCR/peptide-MHC interaction is likely to be functionally important. The binding kinetics reported here for the JM22z/HLA-A2-flu interaction are faster than previously reported for TCR/peptide-MHC interactions (Davis et al., 1998a). However, these previous studies were performed at unphysiological temperatures (≤25°C). When measured at 25°C, the kinetics of JM22z/HLA-A2-flu interaction were significantly slower than at 37°C (Table 2), with the k_{on} (3×10^4 $M^{-1}s^{-1}$) and k_{off} (0.12 s^{-1}) now at the high end of the range of values previously reported. Similar results were obtained with the mouse F5 TCR (see below). Taken together, these results confirm that, when measured at 25°C, the binding kinetics of TCR/peptide-MHC interactions are indeed much slower than other, equally weak, cell–cell recognition molecule interactions, but they highlight the fact that, at physiological temperatures, these differences are much reduced (van der Merwe and Barclay, 1994; Davis et al., 1998b). While the slow kinetics have important implications with regard to understanding the molecular basis of antigen recognition (see below), it is less clear that the difference between the kinetics of TCR/peptide-MHC and other cell–cell recognition molecules has any functional significance. This is because it is not known whether these differences will be evident when the molecules are in their natural environment, tethered to plasma membranes, where the binding kinetics are expected to be limited by diffusion with the plane of the membrane (Bell, 1978; van der Merwe and Barclay, 1994; Davis et al., 1998b).

The Striking Increase in Binding Kinetics with Temperature Indicates that Significant Energy Barriers Impede Association and Dissociation

The large difference in binding kinetics measured at 25°C versus 37°C led us to investigate in more detail the variation of kinetics with temperature. The kinetics slowed dramatically when the temperature was decreased from 37°C to 5°C (Figure 2A, left). In contrast, the kinetics of soluble CD8αα binding to the same peptide-MHC changed little over the same temperature range (Figure 2A, right). Dissociation of the TCR followed first-order kinetics at all temperatures, as shown by the linear logarithmic plots of the dissociation phases (Figure 2A, inset). The k_{off} slowed ~300-fold over this temperature range, from 1 s^{-1} to 0.003 s^{-1} (Figure 2A, inset). An Arrhenius plot ($\ln k_{off}$ versus 1/T) of the JM22z/HLA-A2-flu k_{off} was linear between 5°C and 37°C and yielded a remarkably high dissociation activation energy (E_a^{diss}) of ~31 kcal × M^{-1} (Figure 2B). An Arrhenius plot of the k_{on} was also linear between 5°C and 37°C and gave an association activation energy (E_a^{ass}) of ~11 kcal × M^{-1} (Figure 2C), which is much higher than expected from effects of temperature on water viscosity (4–5 kcal × mol^{-1}) (Day et al., 1963). The activation energy can be thought of as the thermal energy required in order to cross an energy barrier preventing association or dissociation (Figure 3C). The high E_a^{ass} and E_a^{diss} values measured for the JM22z/HLA-A2-flu interaction indicate that association and dissociation are impeded by a significant energy barrier (Figure 3C).

Several lines of evidence indicate that this striking temperature dependence of binding kinetics is not an artefact of SPR or the recombinant proteins themselves.

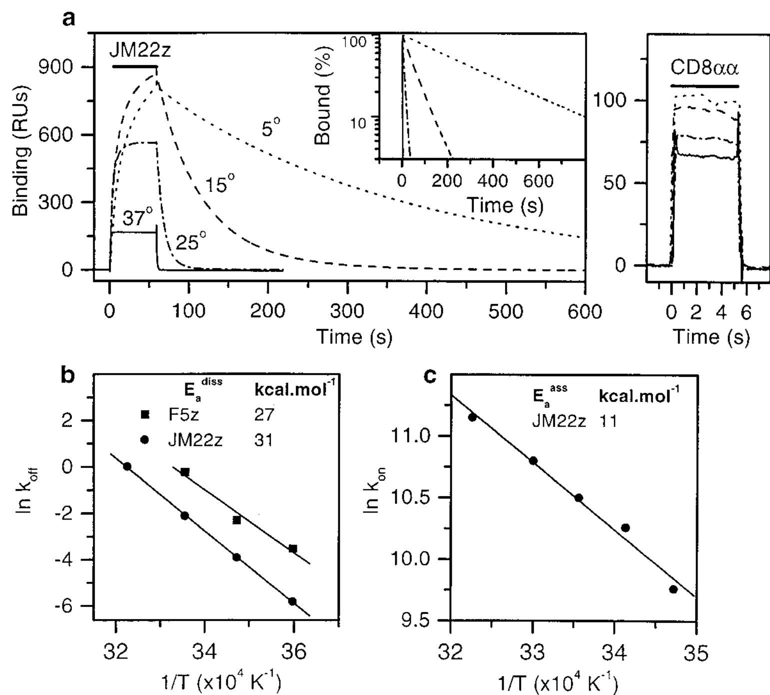

Figure 2. Temperature Dependence of Binding Kinetics

(A) (Left) JM22z (7.5 μM) was injected (bar) at 40 μl × min^{-1} over HLA-A2-flu (2960 RU) at the indicated temperatures. The responses observed with injection of the same samples over HLA-A2-pol (2200 RU) have been subtracted. (Inset) Semilogarithmic plots of the dissociation phases were linear, indicating that dissociation followed first-order kinetics at each temperature. (Right) Soluble CD8αα (42 μM) was injected (bar) at 100 μl × min^{-1} over HLA-A2-flu (2300 RU) at the indicated temperatures. The responses observed with injection of the same samples over a control surface (biotinylated OX68, 2900 RU) have been subtracted.

(B and C) Arrhenius plots of the variation of k_{off} and k_{on} values with temperature for the JM22z/HLA-A2-flu and F5z/H2-D^b-NP (k_{off} only) interactions. The slopes were determined by linear regression (correlation coefficients > 0.99), and the activation energies (E_a) of dissociation and association were calculated from the relationship slope = -E_a/R, where R is the gas constant. Very similar E_a values for association and dissociation of JM22z were obtained when measurements were performed at two different surface densities (2960 and 1250 RU) of HLA-A2-flu.

First, the kinetics of another ligand for HLA-A2-flu, namely CD8αα, varies little between 5°C and 35°C (Figure 2A, right). Second, we have studied the binding kinetics of several low-affinity cell-surface receptor/ligand pairs including CD80/CD28 (Kd 4 μM), CD2/CD48 (Kd ~60 μM), and the killer immunoglobulin receptor KIR2DL3, which binds HLA-Cw7 (Kd ~10 μM). In all cases, the k_{off} varied less than 14-fold between 5°C and 37°C (P. A. V., unpublished data), in contrast to more than 300-fold for the JM22z/TCR/HLA-A2-flu interaction. Third, very similar temperature-dependent kinetics (J. J. Boniface et al., unpublished data) have been measured independently for the 2B4 mouse TCR and its ligand moth cytochrome c-I-E^k, both of which were expressed as lipid-anchored molecules in eukaryotic cells (Lin et al., 1990; Wettstein et al., 1991). Finally, the faster dissociation rate at high temperatures was reversed when the temperature was decreased (data not shown), indicating that it was not a consequence of temperature-dependent denaturation or degradation of the interacting proteins.

The Low Affinity of These TCR/Peptide-MHC Interactions Is a Consequence of Highly Unfavorable Entropic Effects

The affinity of an interaction can be represented as its binding free energy or ΔG° (see Table 3). This binding energy is the sum of enthalpic (ΔH) and entropic (−TΔS) components, either of which can be favorable (i.e., act to increase the affinity) or unfavorable. Whereas ΔH (heat absorbed or released upon binding) can be measured experimentally, the entropy is calculated from ΔG° and ΔH (Table 3; Figure 4). We first estimated the enthalpy indirectly by van't Hoff analysis, which involves measuring the dependence of affinity on temperature (Figure 3B). The van't Hoff enthalpy (ΔH_{vH}) was determined to be ~−23 kcal × mol^{-1} at 15°C–30°C (Figure 3B). Because of the difficulties associated with van't Hoff analysis (Figure 3, legend) and because discrepancies have been noted between van't Hoff enthalpies and directly measured enthalpies (Naghibi et al., 1995), we also measured the enthalpy directly using a microcalorimeter (Figure 3B). The calorimetric enthalpy (ΔH_{cal} ~ −19.7 kcal × mol^{-1} at 25°C) agreed well with the van't Hoff enthalpy. From the affinity (ΔG° −7.1 kcal × mol^{-1} at 25°C, obtained by SPR) and enthalpy, the entropic term (TΔS) was calculated to be between −15.9 and −12.6 kcal × mol^{-1} at 25°C (Table 3). For proteins interacting in solution, the activation energy E_a is equivalent to the heat input required to overcome the energy barrier (activation enthalpy or ΔH‡). It follows that the difference between E_a^{ass} and E_a^{diss} should equal the heat input (ΔH) for the overall reaction (i.e., $\Delta H = E_a^{ass} - E_a^{diss}$, Figure 3C). For the JM22z/HLA-A2-flu interaction, there was good agreement between the ΔH calculated from the E_a^{ass} and E_a^{diss} determinations (−20 kcal × mol^{-1}) and the ΔH measured by van't Hoff analysis or calorimetry (Table 3). It is noteworthy that, because the ΔH and TΔS almost always become more negative as the temperature increases (Ladbury and Chowdhry, 1996; Stites, 1997), the thermodynamic effects (large favorable enthalpy, large unfavorable entropy) measured here at 25°C are likely to be more pronounced at 37°C.

These results indicate that the low affinity of the JM22z/HLA-A2-flu interaction is largely a consequence of highly unfavorable entropic changes upon binding (or increase in order). This is in striking contrast to typical protein–protein interactions (excluding antibodies, see below) in which the entropic changes are typically favorable and therefore contribute to the increased affinity

Table 3. Summary of Thermodynamic and Arrhenius Constants[1]

Interaction	ΔG^{o2}	$\Delta H_{vH}{}^{3}$	$\Delta H_{cal}{}^{3}$	$T\Delta S$	E_a^{ass}	E_a^{diss}
JM22z/HLA-A2-flu	−7.1	−23	−19.7	−15.9 to − 12.6	11	31
F5z/H2-D^b-NP	−6.7	−19	nd[4]	−12.3	8[5]	27

[1] All figures in kcal × M^{-1}. $\Delta G°$ (from Table 1) and ΔH_{cal} (from Figure 3A) measured at 25°C. ΔH_{vH} (from Figure 3B) was measured in the range 15°–30°C for JM22z and 5°C–25°C for F5z (Figure 3B). E_a^{diss} was measured in the range 5°C–37°C for JM22z and 5°C–25°C for F5z (Figure 2B). E_a^{ass} was measured in the range 15°C–37°C for JM22z (Figure 2C).
[2] $\Delta G° = R \times T \times \ln Kd$, where R is the Gas constant and Kd is the equilibrium dissociation constant expressed in units M.
[3] ΔH_{vH}, van't Hoff enthalpy; ΔH_{cal}, calorimetric enthalpy.
[4] Not determined.
[5] Calculated. $T\Delta S = \Delta H - \Delta G°$; $E_a^{ass} = \Delta H° + E_a^{diss}$

(Figure 4). In principle, this unfavorable entropy could arise either from changes in the interacting proteins themselves (e.g., conformational adjustments upon binding that result in a decrease in mobility or flexibility of the binding surfaces) or from changes in solvent (e.g., incorporation of ordered water molecules into the binding interface) (Ladbury, 1996). Solvent effects are an unlikely explanation since the binding of two molecules leads to the net expulsion of water molecules from the interacting surfaces (where they are ordered) into free

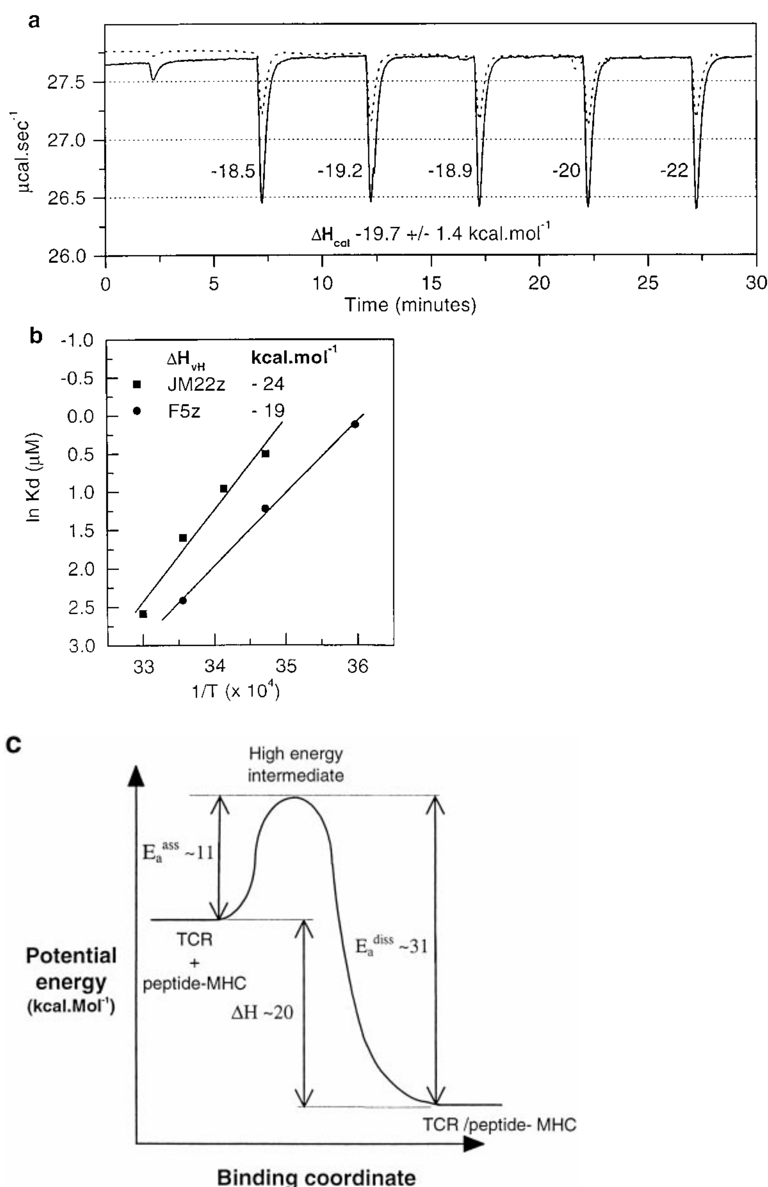

Figure 3. Thermodynamic Analysis of TCR Binding

(A) Measurement of enthalpy by calorimetry (ΔH_{cal}). The rate of heat release (at 25°C) is shown during injection (large deflections) of five 1 nmol aliquots of HLA-A2-flu complex into a cell containing either 119 µM JM22z (solid line) or buffer alone (dotted line). From these data, the ΔH_{cal} for each injection was calculated as described in the Experimental Procedures. The mean (±SD) ΔH_{cal} for five injections was −20 ± 1.3 kcal × mol^{-1}.

(B) Measurement of enthalpy by van't Hoff analysis (ΔH_{vH}). Affinity constants for the JM22z/HLA-A2-flu and F5z/H2-D^b-NP interactions were measured at several temperatures as described in Figures 1A–1C. For each receptor, the decrease in affinity with increase in temperature was fully reversible (data not shown). The slopes were determined by linear regression (correlation coefficients > 0.98) and used to calculate the indicated ΔH_{vH} values (slope = $-\Delta H_{vH}/R$). Because ΔH usually varies with temperature for protein–protein interactions, van't Hoff plots are not strictly linear. The ΔH_{vH} values shown are therefore approximations of the true ΔH_{vH} at the mid-point of the range of temperatures used for the linear fit (15°C–30°C and 5°C–25°C for the JM22z and F5z TCRs, respectively).

(C) A reaction profile illustrating the potential energy changes as JM22z binds to HLA-A2-flu. Data from Table 3.

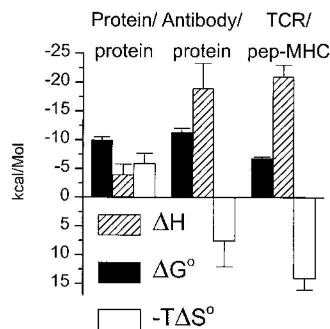

Figure 4. Thermodynamic Parameters for Protein–Protein Interactions

The binding energy (ΔG°) is the sum of the enthalpic (ΔH) and entropic (−TΔS°) components (ΔG° = ΔH − TΔS°). Binding is favored by more negative values (note the inverted y axis scale). Data for the TCR/peptide-MHC interactions (n = 2) are from Table 3. Data for multiple protein–protein (n = 30) and antibody/protein (n = 13) interactions are taken from Stites (1997). The bars show mean values and the error bars represent the SEM (n > 2) or range (n = 2).

solution (where they are disordered). Furthermore, unlike conformational adjustments, the trapping of ordered water molecules at the binding interface would not explain the slow, temperature-dependent kinetics of TCR/peptide-MHC binding. It seems more likely therefore that the unfavorable entropic changes arise from a substantial decrease in the conformational flexibility of the TCR and/or peptide-MHC upon binding, as has been observed with other protein–protein interactions (Stites, 1997). Consistent with this, the slow k_{on} and high E_a^{ass} suggest that conformational adjustments are required for binding.

The binding enthalpy is surprisingly high for such a low-affinity protein–protein interaction (Figure 4) and suggests a large net increase in the number of favorable noncovalent bonds (e.g., hydrogen bonds and van der Waals contacts) following binding. In principle, these new bonds could be between water and proteins (within or outside the binding interface) or directly between the interacting proteins themselves. However, the relatively slow k_{off} and very high E_a^{diss} imply that the TCR/peptide-MHC complex is stabilized by direct contacts, strongly suggesting that the favorable enthalpic effects arise from direct protein–protein contacts and/or protein/water/protein bridges. In support of this, the binding interfaces of two recently determined TCR/peptide-MHC complexes (Garboczi et al., 1996; Garcia et al., 1998) are actually larger (total buried surface area ~ 1880–2010 Å^2) than most well-characterized protein–protein interactions (1400–1600 Å^2), most of which have a substantially higher affinity (Janin, 1995; Jones and Thornton, 1996; Stites, 1997). Although the fit or complementarity between the TCR and peptide-MHC binding surfaces is quite poor (Garboczi et al., 1996; Garcia et al., 1998), available structural (Garboczi et al., 1996; Garcia et al., 1998) and mutagenesis (Manning et al., 1998) data suggest that there are as many energetically significant contacts at TCR/peptide-MHC interfaces as found in high-affinity protein–protein interactions (Janin, 1995; Jones and Thornton, 1996). Taken together with our thermodynamic and kinetic data, this suggests that the low affinity of the TCR/peptide-MHC interactions is a consequence not of insufficient contacts at the interface but rather of the entropic penalty associated with the conformational adjustments and reduction in flexibility required for binding.

A Mouse TCR/Peptide-MHC Interaction Has Similar Kinetic and Thermodynamic Properties

In order to address the possibility that the binding characteristics of the JM22z/HLA-A2-flu interaction are unique to this particular TCR, we extended this analysis to the mouse F5 TCR. The F5/H2-D^b-NP interaction had a somewhat lower affinity (Kd ~11 μM or ΔG° ~−6.7 kcal × mol^{-1} at 25°C) and faster k_{off} (0.8 s^{-1} at 25°C) than the JM22z/HLA-A2-flu interaction. More importantly, the kinetics and affinity were also highly temperature-dependent. Arrhenius analysis of dissociation yielded a very high activation energy (E_a ~27 kcal × mol^{-1}) (Figure 2B). The binding enthalpy determined by van't Hoff analysis was also high (ΔH° −19 kcal × mol^{-1} at 5°C–25°C) (Figure 3A) so that the calculated entropy was highly unfavorable (Table 3, TΔS° ~−12 kcal × mol^{-1}). The calculated E_a^{ass} of the F5 interaction was ~8 kcal × mol^{-1} (Table 3), indicating a significant energy barrier to association. Thus, the F5z and JM22z TCRs show very similar thermodynamic and kinetic properties. Taken together with similar results recently obtained with a mouse MHC class II–restricted TCR (J. J. Boniface et al., unpublished data) and the slow kinetics reported in all other studies of nonalloreactive TCRs (Davis et al., 1998a), this suggests that unfavorable entropic effects and slow, temperature-dependent kinetics may be a common feature of TCR/peptide-MHC interactions.

The Binding Properties of TCR/Peptide-MHC Interactions Suggest that a Reduction in Conformational Flexibility Accompanies Binding

The thermodynamic and kinetic data reported here support the following model for TCR/peptide-MHC binding (Figure 3C). The TCR and/or peptide-MHC possess a degree of conformational flexibility in their unbound states, with only a subset of conformations compatible with binding. Binding requires that collisions occur with both molecules in the correct conformation or with sufficient kinetic energy to drive the necessary conformational rearrangement. Thus, k_{on} values are slow and are strongly temperature-dependent. The bound complex is stabilized by a large number of contacts, but these are liable to be disrupted at higher temperatures as mobility at the TCR/peptide-MHC interface increases. Thus, dissociation (k_{off}) is comparatively slow for such a low-affinity interaction and increases dramatically as the temperature increases.

Our results do not show whether the conformational flexibility resides within the TCR, the peptide-MHC, or both. Two X-ray crystallography studies that compared these molecules in the bound and unbound states demonstrated conformational differences in the TCR complementarity-determining region (CDR) loops (Garcia et al., 1998) as well as in the peptide-MHC (Garboczi et al., 1996; Garcia et al., 1998). However, much larger

differences were observed in the TCR (Garcia et al., 1998). Structural studies of unliganded peptide-MHC molecules argue against substantial conformational flexibility of the peptide-MHC binding surface, although this is somewhat controversial (Madden, 1995).

Functional Implications of Conformational Flexibility

It has been argued that, in order for the repertoire of T cell specificities in a single animal to be reasonably complete, individual TCRs need to be able to recognize a large number ($\sim 10^6$) of the peptides that can bind the MHC molecule to which they are restricted (Mason, 1998). This is necessary because there are far more potential TCR ligands ($>10^{10}$ nonamer peptides can bind each MHC class I molecule) than there are distinct T cell specificities (Mason, 1998). Indeed, recent experimental studies indicate that individual TCRs can cross-react with a very large number ($\sim 10^6$) of peptides presented by the same MHC molecule (Bhardwaj et al., 1993; Reay et al., 1994; Evavold et al., 1995; Wucherpfennig and Strominger, 1995; Kaliyaperumal et al., 1996; Kersh and Allen, 1996; Mason, 1998). It should be stressed that, despite cross-reacting with such a large number of peptides, TCRs are still functionally specific because they will recognize only a small fraction ($<0.01\%$) of the peptides that can be presented by a particular MHC molecule (Mason, 1998).

TCR cross-reactivity may be enhanced by poor shape complementarity between the binding surfaces, which has been observed in high-resolution structures of TCR/peptide-MHC complexes (Garboczi et al., 1996; Garcia et al., 1998). This is facilitated by water, which can fill the gaps at molecular interfaces and function as molecular "glue" (Ladbury, 1996). Cross-reactivity may also be enhanced by conformational flexibility of the antigen-binding site, as previously suggested (Garcia et al., 1998). Evidence for the latter has been provided by mutagenesis studies that sought to identify MHC residues that contribute to TCR binding (Ehrich et al., 1993; Ono et al., 1998). Mutation of TCR contact residues in a peptide presented by MHC altered the pattern of MHC mutations that disrupted TCR binding (Ehrich et al., 1993; Ono et al., 1998). These results are readily explained if it is postulated that the TCRs undergo conformational adjustments to accommodate the peptide mutation and, in so doing, alter the TCR/MHC contacts elsewhere in the binding interface. In conclusion, the findings in this report and a similar study by Boniface et al. (unpublished data), taken together with structural data (Garcia et al., 1998), and the slow kinetics reported for other TCR/peptide-MHC interactions (Davis et al., 1998a), strongly suggest that conformational flexibility is a general feature of TCR/peptide-MHC interactions. It seems likely therefore that both conformational flexibility and poor shape complementarity contribute to TCR cross-reactivity.

Conformational Flexibility and the Evolution of Antigen Receptors

An important question is whether conformational flexibility of the TCR has evolved for a particular purpose (e.g., increased cross-reactivity) or whether it is instead a consequence of the mechanism by which a diverse TCR repertoire is generated. It seems unlikely that flexibility has "evolved" in the conventional sense because the TCR antigen binding site is never encoded within the germline and cannot be passed on to progeny. It is conceivable however that positive selection favors TCRs with "flexible" binding sites, perhaps because this enables them to engage a greater proportion of the self-peptide-MHC complexes. Another possibility is that conformational flexibility is a consequence of the structure of the TCR and the way it is generated. Antigen binding sites on antigen receptors are comprised of several peptide loops, which are assembled by imperfect splicing of many possible combinations of gene segments. We suggest that because such loops inherently lack secondary structure, and because of the stochastic nature of the process by which they are generated, it is highly likely that antigen binding sites will possess a degree of conformational flexibility.

This raises the question as to whether antibodies, which are structurally similar and generated by the same mechanism, also have flexible antigen binding sites. There is evidence for conformational flexibility in the antigen-binding sites of antibodies (Foote and Milstein, 1994; Wilson and Stanfield, 1994; Braden et al., 1996; Wedemayer et al., 1997b), although the conformational adjustments that accompany binding are usually smaller than is the case for the single TCR for which data are available (Garcia et al., 1998). A comparison of published thermodynamic data (Stites, 1997) reveals that antibody/protein interactions have much more unfavorable binding entropies and more favorable binding enthalpies than many protein–protein interactions (Figure 4). Thus, the thermodynamic properties of antibody/protein interactions fall into a class that is intermediate between protein–protein and TCR-peptide-MHC interactions (Figure 4). Furthermore, slow kinetics have been measured for a subset of antibody/protein interactions (Mason and Williams, 1986; Foote and Milstein, 1991; Braden et al., 1996). These data suggest that antibodies possess some conformational flexibility in their antigen binding sites, but perhaps not as much as TCRs. However, the large majority of the antibodies that have been studied are secondary antibodies, which will have been subjected to affinity maturation by somatic hypermutation. The significance of this is highlighted in a study by Wedemayer et al. (1997). Here, they showed that, whereas a primary antibody undergoes a large conformational change upon binding to its ligand, affinity maturation of this antibody stabilizes the antibody in the ligand-binding conformation so that it is "preconfigured" for binding. It seems likely that the resulting 3×10^4-fold increase in affinity in the secondary antibody is at least partly a consequence of a reduction in the entropic penalty of binding.

These considerations suggest that many TCRs and primary antibodies possess a degree of conformational flexibility in their antigen-binding site. This would have the advantage of increasing their cross-reactivity for antigen but would have the unfavorable thermodynamic and kinetic effects described here for the TCR. In the case of antibodies, somatic hypermutation provides an opportunity to modify these properties by introducing

mutations that stabilize the binding configuration, thereby reducing the entropic penalty of binding and increasing the k_{on} (Wedemayer et al., 1997a). In agreement with this, affinity maturation has been associated with increases in the k_{on} (Foote and Milstein, 1991). In contrast, the TCR, which is not thought to undergo affinity maturation, would be expected to retain the unfavorable entropy and slow kinetics described in this and other studies.

Experimental Procedures

Soluble MHC Class I Preparation

HLA-A2 heavy chain (residues 1–276) was expressed in *E. coli* and refolded as previously described (Garboczi et al., 1992) together with the Flu (GILGFVFTL) or Pol (ILKEPVHGV) peptides (Genosys) and β2-microglobulin. Biotinylated HLA-A2 was prepared by refolding with β2-microglobulin that had been chemically biotinylated using N-hydroxysuccinimido-biotin (Sigma). HLA-A2-flu eluted at ∼43 kDa on gel-filtration. This is similar to its molecular mass (∼45 kDa), indicating that it does not self-associate. The extinction coefficient of HLA-A2-flu was determined by amino acid analysis to be 66150 $M^{-1} \times cm^{-1}$. H2-D^b heavy chain (residues 1–280) was engineered with a 13-amino-acid carboxy-terminal BirA recognition sequence (Schatz, 1993) and expressed in *E. coli*. It was refolded as described (Garboczi et al., 1992) together with the influenza NP peptide (ASNENMDAM) and mouse β2-microglobulin. The complex was biotinylated as described (O'Callaghan et al., 1998) using BirA (a gift from Dr. C. A. O'Callaghan) and purified by gel-filtration.

Soluble TCR and CD8αα Preparation

JM22 utilizes gene segments AV10S2 and BV17S1, while F5 utilizes gene segments AV4 and BV11. Expression, refolding, and purification of JM22z and F5z are to be described in detail elsewhere (B. E. W. et al., unpublished data). In brief, TCR α and β chains carrying c-Jun (α) and c-Fos (β) heterodimerization motifs at their carboxyl termini were expressed separately in *E. coli*, solubilized from inclusion bodies, and refolded together. Refolded αβ heterodimers were purified by ion exchange chromatography and gel filtration. JM22z and F5z eluted at ∼70 kDa on gel-filtration. This agrees with their molecular mass (∼60 kDa), indicating that they do not self-associate. JM22z eluted at the same position even at the high concentrations (>20 μM) used in the BIAcore experiments. The extinction coefficient of JM22z was determined by amino acid analysis to be 105500 $M^{-1} \times cm^{-1}$. The extinction coefficient of F5z was calculated to be 79840 $M^{-1} \times cm^{-1}$. Soluble, monovalent CD8αα homodimers were produced as previously described (Wyer et al., 1999).

Surface Plasmon Resonance

SPR studies were performed using a BIAcore 2000 (BIAcore AB, St. Albans, UK) in HBS (BIAcore). HBS contains 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P20. Streptavidin (Sigma) and the JM22z TCR were covalently coupled to Research Grade CM5 sensor chips (BIAcore) via primary amines using the standard amine coupling kit (BIAcore). For coupling, streptavidin was injected at 0.5 mg/ml in 10 mM sodium acetate (pH 5.5). Coupling levels ranged from 6000 to 11000 RU. Biotinylated proteins were immobilized at the indicated levels by injection at 50 to 150 μg/ml for 0.5 to 10 min over streptavidin-coupled surfaces. For coupling, JM22z was injected at 14 μg/ml in 10 mM sodium acetate (pH 5). Coupling levels ranged from 3200 to 11400 RU. To avoid multivalent aggregates, soluble proteins used in SPR experiments were purified by gel-filtration on a Superdex S-200 column, stored at 4°C, and used within 48 hr with minimum amounts of concentrating.

Microcalorimetry

Direct enthalpy measurements (ΔH_{cal}) were performed at 25°C in HBS buffer using an MCS isothermal titration micocalorimeter (Microcal). After an initial 3 μL trial injection, five aliquots of HLA-A2-flu complex were injected (15 μL at 69 μM or 1 nmol) into a 1.334 mL cell containing either JM22z (119 μM) or, for measuring the heat of dilution, HBS buffer alone. Assuming a Kd of 6.6 μM, it was calculated that 94.5, 94.3, 94.1, 93.9, and 93.7% of injected HLA-A2-flu bound in the 1st to 5th injections, respectively. The molar heat release was determined by integrating each peak, subtracting of the heat of dilution, and adjusting for the proportion (see above) of injected HLA-A2-flu that bound.

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