THE CONTRIBUTION OF CONFORMATIONAL ADJUSTMENTS AND LONG-RANGE ELECTROSTATIC FORCES TO THE CD2/CD58 INTERACTION *

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Running title: Conformation and electrostatics in CD2 binding to CD58.

CD2 is a T cell surface molecule that enhances T and Natural Killer cell function by binding its ligands CD58 (humans) and CD48 (rodents) on antigen presenting or target cells. Here we show that the CD2/CD58 interaction is enthalpically-driven and accompanied by unfavourable entropic changes. Taken together with structural studies, this indicates that binding is accompanied by energetically-significant conformational adjustments. Despite having a highly charged binding interface neither the affinity nor rate constants of the CD2/CD58 interaction were affected by changes in ionic strength, indicating that long-range electrostatic forces make no net contribution to binding.

The CD2 family of cell-surface glycoproteins are structurally-related members of the immunoglobulin (Ig') superfamily (1,2). Expressed mainly on haematopoietic cells, they modulate immune responses by homotypic or heterotypic interactions with other members of the CD2 family. CD2 binds CD58 (in humans) or CD48 (in rodents), and these interactions enhance T cell recognition of antigen on antigen-presenting or target cells. This enhancement is thought to involve both adhesion and signalling mechanisms (1,3-7).

CD2 and its interaction with its ligands have been intensively studied and have emerged as an important paradigm for understanding the molecular basis of cell-cell recognition (1,8,9). CD2 and its ligands have structurally-related ectodomains comprised of two Ig domains, with the membrane-distal domains involved in ligand binding (1,8). The interaction of human CD2 with CD58 is characterised by a low affinity (Kd ~10 μM at 37°C), which is the result of a very fast dissociation rate constant (k_off > 4 s⁻¹) (10). Structural studies of the individual proteins and site-directed mutagenesis have located the binding sites on the equivalent GFCCC'β-sheets of CD2 and CD58, and revealed them to be highly charged (11-13). Solution of the crystal structure of the complex between the human CD2 and CD58 ligand binding domains has provided a detailed view of the binding interface (14). This is relatively small (buried surface area ~1160 Å²) and has poor surface-shape complementarity, consistent with the low affinity (14). Comparison of the structure of the complex with the structure of unbound CD2 (15-17) and CD58 (12,13) revealed significant differences, particularly in the case of CD2. The most prominent differences were in the C'C'' and FG loops of both molecules (14,16). In addition NMR analysis has shown that the CD58 binding site on unbound CD2 is highly flexible, with most of the movement occurring in the C'C'' and FG loops (16,17). Taken together, these data suggest CD2 binding to CD58 is accompanied by conformational adjustment and stabilization of a flexible interface. While these conformational changes provide an explanation for the low affinity of the CD2/CD58 interaction they appear inconsistent with its relatively fast k_off (10).
In order to further investigate these putative conformational changes and the discrepancy between the structural and kinetic data we undertook a detailed thermodynamic and kinetic analysis of the CD2/CD58 interaction. We show that the interaction is enthalpically-driven and accompanied by unfavourable entropic changes, consistent with stabilisation of a flexible binding interface. We also show that, despite having a highly charged binding interface, long-range electrostatic interactions have no net effect on the CD2/CD58 interaction.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Soluble forms of CD2 and CD58 were prepared and purified as previously described (18). These comprised the full ectodomains with C-terminal oligohistidine tags. The C-termini of the encoded CD2 and CD58 were SCPEKHHHHHHH and TCIPSSHHHHHHH respectively.

**Surface Plasmon Resonance**—These studies were performed on a BIAcore 2000 (BIAcore AB) (19). Unless otherwise stated experiments were performed at 25°C using HBS buffer [10mM HEPES (pH 7.4), 150 mM NaCl, 1mM CaCl2, and 1mM MgCl2] at a flow rate of 10 μL.min⁻¹. Human CD2 was directly coupled to Research Grade CM5 sensor chips (BIAcore AB) using the Amine Coupling Kit (BIAcore) as previously described (10). Kinetics measurements were performed at a flow rate of 50 μL.min⁻¹ and confirmed at three different immobilization levels of CD2, in order to rule out mass transport artefacts.

Affinity, kinetic and thermodynamic properties were determined as described (20). Equilibrium thermodynamic parameters were obtained by measuring the affinity over a range of temperatures (5 to 37°C), and fitting the non-linear form of the van’t Hoff equation to these data (21)

\[
\Delta G = \Delta H_{T_0} - T \Delta S + \Delta C_p (T - T_0) - T \Delta C_v \ln(T/T_0)
\]

where T is the temperature (in K); T₀ is an arbitrary reference temperature (e.g. 298.15K); \( \Delta G \) is the free energy of binding at the standard state (all components at 1 mol/L⁻¹); \( \Delta H_{T_0} \) is the enthalpy change at T₀ (kcal.mol⁻¹); \( \Delta C_p \) is the heat capacity change (kcal.mol⁻¹K⁻¹) at constant pressure; and \( \Delta S \) is the entropy change at the standard state.

\( \Delta G \) was calculated from the affinity constant (K_D) using the equation

\[
\Delta G = RT \ln (K_D/C)
\]

where R is 1.987 cal.mol⁻¹K⁻¹; K_D is expressed in mol/L; and C is the standard state concentration (1 M).

The activation enthalpy of dissociation (\( \Delta^\ddagger H_{diss} \)) was determined by measuring the k_off over a range of temperature (10-30°C) and plotting ln(k_offT) against 1/T, the slope of which equals -\( \Delta^\ddagger H_{diss}/R \) (20). The \( \Delta^\ddagger H_{act} \) was calculated from the relationship \( \Delta^\ddagger H_{act} = \Delta^\ddagger H_{diss} + \Delta H \).

In experiments varying the ionic strength a series of HBS stocks were prepared with the indicated NaCl or KF concentration. CD58 samples were diluted in the appropriate HBS buffer and the same HBS buffer was used as the running buffer.

**Isothermal Titration Calorimetry (ITC)**—These measurements were performed at 25°C using a MicroCal VP-ITC unit (MicroCal Inc.) as described previously (22). Samples were dialysed extensively into HBS. CD58 at 245 μM was injected in 10 μL aliquots into a cell containing 1345 μL of CD2 (at 35 μM), and the heat release was measured. The heat of dilution was obtained by injection of CD58 into HBS and subtracted prior to data analysis. The titration data were fitted by non-linear curve fitting using the Origin software supplied with the instrument to obtain the K_D, stoichiometry, and \( \Delta H \). \( T \Delta S \) was determined by the relationship \( T \Delta S = \Delta H - RT \ln(K_D/C) \).

**RESULTS AND DISCUSSION**

**Thermodynamics of human CD2 binding to CD58**—In order to investigate whether CD2 binding to CD58 is accompanied by energetically significant conformational changes we undertook a detailed thermodynamic analysis. The binding of CD58 to CD2 was analysed by surface plasmon resonance. The affinity constant (K_D) was measured by equilibrium binding analysis (10). The binding free energy of an interaction (\( \Delta G \)), which can be calculated from the affinity constant (see Experimental Procedures), is comprised of enthalpic (\( \Delta H \)) and entropic (-T\( \Delta S \)) components (\( \Delta G = \Delta H - T \Delta S \)).
The relative contribution of enthalpic and entropic components can be determined by measuring the dependence of \( \Delta G \) on temperature, a procedure termed van’t Hoff analysis (see Experimental Procedures). For protein/protein interactions \( \Delta H \) and \( \Delta T \Delta S \) typically vary with temperature, and this variation is measured as the change in heat capacity (at constant pressure) or \( \Delta C_p \). The binding energy of the CD2/CD58 interaction was measured over the temperature range of 5 to 37°C and \( \Delta H \), \( -\Delta T \Delta S \), and \( \Delta C_p \) were determined by fitting the non-linear form of the van’t Hoff equation to the data (Fig. 1A). From several different determinations the \( \Delta H \) at 25°C was determined to be \(-11.5 \pm 0.2\) kcal.mol\(^{-1}\) (mean ± SEM, \( n = 3 \)), which is highly favourable. The corresponding entropic component (-\( \Delta T \Delta S \)) was \(+4.4 \pm 0.2\) kcal.mol\(^{-1}\), which is unfavourable (Table I).

The \( \Delta C_p \) determined by van’t Hoff analysis was -118 cal.mol\(^{-1}\)K\(^{-1}\) (Table I). This falls at the high end of values typically reported for protein/protein interactions, which range from -1000 to 0 cal\(^{-1}\)mol.K\(^{-1}\), with an average value of \(-300\) cal\(^{-1}\)mol.K\(^{-1}\) (23). A negative \( \Delta C_p \) which is typical of protein/protein interactions, is thought to be the result of the tendency of water to form an ordered ‘shell’ adjacent to non-polar surfaces which ‘melts’ at higher temperatures (24). Upon binding, the burial of non-polar surfaces disrupts this shell, ejecting the water into free solution, with favourable entropic and unfavourable enthalpic effects. Increasing the temperature ‘melts’ the shell so that these effects are gradually lost. The relatively high \( \Delta C_p \) measured for the CD2/CD58 interaction is consistent with the fact that the binding interfaces are highly polar and that, as a result, a comparatively small amount of non-polar surface is buried upon binding.

It is possible to estimate \( \Delta C_p \) from structural data using the empirically-determined relationship

\[
\Delta C_p = (0.32 \pm 0.04) \times \Delta A_{np} - (0.14 \pm 0.04) \times \Delta A_p
\]

where \( \Delta A_{np} \) is the change in the buried non-polar surface area and \( \Delta A_p \) is the change in the buried polar surface area. Using an implementation of the Lee and Richards algorithm developed by Hubbard (25-27), the \( \Delta A_{np} \) and \( \Delta A_p \) were estimated from the crystal structure of the CD2/CD58 complex to be 660 and 690 Å\(^2\), respectively. Using these values the calculated \( \Delta C_p \) is \(-116\) cal.mol\(^{-1}\), which is in good agreement with the experimental value obtained from van’t Hoff analysis (Table II).

Several studies have reported discrepancies between \( \Delta H \) measured indirectly by van’t Hoff analysis (\( \Delta H_{diss} \)) and \( \Delta H \) measured directly by calorimetry (\( \Delta H_{cal} \)) (28-30). We therefore measured \( \Delta H \) directly using ITC (Fig. 2). The \( \Delta H \) and -\( \Delta T \Delta S \) thus measured were similar to those determined by van’t Hoff analysis (Table I). It has been suggested that differences between \( \Delta H_{cal} \) and \( \Delta H_{diss} \) indicate the presence of linked equilibria that contribute to \( \Delta H_{cal} \) but not \( \Delta H_{diss} \). Recent studies dispute this, however, arguing that \( \Delta H_{cal} \) should equal \( \Delta H_{diss} \) and that differences are more likely to be the result of experimental artefact (31-33).

When conformational changes are required for binding this may result in a high activation enthalpy of association (\( \Delta H_{ass} \)), which is a measure of the net number of bonds that need to be broken in order to form the transition state complex (34). Because the binding kinetics were too fast to measure the \( \Delta H_{ass} \) directly, we determined the \( \Delta^1H_{diss} \) and calculated the \( \Delta^1H_{ass} \) from the relationship \( \Delta^1H_{ass} = \Delta^1H_{diss} - \Delta^1H_{diss}/R \) (20). Using this approach the \( \Delta^1H_{ass} \) thus estimated was \(5.3 \pm 0.8\) kcal.mol\(^{-1}\), which is relatively small (35). While this does not support conformational change it does not rule it out either, since it is possible that new bonds, such as long-range electrostatic interactions, are formed in the transition state which compensate for the bonds that are broken (36). The preponderance of charged residues in the binding interface supports this possibility, leading us to test below whether long-range electrostatic interactions accelerate binding.

Our results show that, at physiological temperatures, the CD2/CD58 interaction is enthalpically driven and accompanied by unfavourable entropic changes. This contrasts with most protein/protein interactions (23), which are accompanied by favourable entropic and enthalpic changes (Fig. 3). It is similar to what has been observed with protein/protein interactions that are known to be accompanied by conformational adjustments and a reduction in conformational flexibility, such as T cell...
receptor/ligand (Fig. 3), and gp120/CD4 (37) interactions. However the unfavourable entropic change is relatively modest in comparison with these examples, and it is possible that it arises from sources other than changes in conformational flexibility. For example it may arise purely from solvent effects, such as the trapping of water molecules in the binding interface. We therefore investigated the source of the unfavourable entropic change.

Entropic changes accompanying protein/protein interactions are the sum of favourable changes in solvent (water) entropy and unfavourable changes in protein entropy. Changes in solvent entropy arise mainly from the burial of non-polar surfaces (i.e. the hydrophobic effect). This arises from the movement of water adjacent to hydrophobic surfaces, where it adopts an organized, shell-like structure, into free solution, where it is more disorganised. Changes in protein entropy arise from loss of rotational and translational freedom and conformational flexibility. In order to investigate the extent to which unfavourable entropic changes arise from changes in conformational flexibility we used the approach suggested by Spolar and Record (38) to dissect total entropy change ($\Delta S$) into three main components.

$$\Delta S = \Delta S_{\text{HE}} + \Delta S_{\text{RT}} + \Delta S_{\text{OTHER}} \quad (39)$$

where $\Delta S_{\text{HE}}$ is the entropy change associated with the hydrophobic effect, $\Delta S_{\text{RT}}$ is the change associated with loss of translational and rotational freedom of the interacting proteins, and $\Delta S_{\text{OTHER}}$ is the change arising from other sources, including a reduction in conformational flexibility upon binding. $\Delta S_{\text{HE}}$ arises from the burial of non-polar surface area; it can be calculated from structural data using the empirically-determined relationship:

$$\Delta S_{\text{HE}} = 0.32A_{\text{np}} \ln (T/386) \quad (3)$$

where $A_{\text{np}}$ is the buried non-polar surface area (in Å$^2$). As noted previously, $A_{\text{np}}$ is estimated to be 660 Å$^2$ for the CD2/CD58 interaction.

Based on empirical measurements Spolar and Record estimated $\Delta S_{\text{RT}}$ to be -50 cal mol$^{-1}$K$^{-1}$ for a 1:1 protein/protein interaction.

Since for protein/protein interactions the $\Delta S$ is temperature dependent, there exists a temperature ($T_s$) where $\Delta S = 0$. This can be calculated using the relationship

$$T_s = T*e^{\Delta S_{\text{RT}}/\Delta C_p} \quad (4)$$

where $T$ is an arbitrary temperature (e.g. 298.15 K) and $\Delta C_p$ is the entropy change at that $T$. Using the $\Delta S_{\text{RT}}$ and $\Delta C_p$ values determined above the $T_s$ was calculated to be 261 K for the CD2/CD58 interaction. The $\Delta S_{\text{HE}}$ at $T_s$ was calculated using equation 3 to be 83 cal mol$^{-1}$K$^{-1}$.

$\Delta S_{\text{OTHER}}$ at $T_s$ can thus be calculated from the relationship

$$\Delta S = 0 = \Delta S_{\text{OTHER}} + \Delta S_{\text{HE}} + \Delta S_{\text{RT}} \quad (5)$$

If one assumes that $\Delta S_{\text{RT}}$ is temperature independent over this temperature range, as suggested by Spolar and Record (38), then $\Delta S_{\text{OTHER}}$ is calculated to be -33 cal mol$^{-1}$K$^{-1}$ (Table II). This negative $\Delta S_{\text{OTHER}}$ value indicates that binding is accompanied by a reduction in conformational entropy. Spolar and Record (38) show that the number of residues ($\mathcal{R}$) undergoing the transition from a flexible to a folded or rigid state can be estimated from the empirical relationship

$$\mathcal{R} = \Delta S_{\text{OTHER}}/-5.6 \quad (40)$$

suggesting that ~6 or more residues are converted from a flexible to a stable state upon CD2 binding to CD58.

Recently it has been argued that a better estimate of $\Delta S_{\text{RT}}$ for protein/protein association would be the cratic entropy (41), which is -8 cal mol$^{-1}$ K$^{-1}$ (Rln1/55.5) for a bimolecular interaction in water in the standard state. Substituting this value into equation 5 would give -74.6 cal mol$^{-1}$K$^{-1}$ for $\Delta S_{\text{OTHER}}$ and substituting this in equation 6 gives a value of 13 for $\mathcal{R}$ (Table II). Thus dissection of the binding entropy suggests that CD2 binding to CD58 is accompanied by a reduction in conformational flexibility involving 6-13 residues. While these changes might occur anywhere in the protein complex, structural studies support the notion that this reduction in conformational flexibility involves the BC, C’C” and FG loops of CD2 (17,42) and the C’C” and FG loops of CD58 (14). As noted above, CD2 binding to CD58 is accompanied by and driven by a large favourable enthalpy change. Such favourable enthalpy changes may arise from an increased number of contacts forming at the interface and/or from the trapping of solvent within the binding interface. The binding interface of the CD2/CD58 complex is approximately 1150 Å$^2$ (14). While this is at the
low end of the range for protein/protein interactions (23,43), there appear to be numerous contacts; ten salt bridges and five hydrogen bonds were proposed based on analysis of the crystal structure (14). Site-directed mutagenesis studies of the CD2/CD58 interaction suggest that a centrally positioned CD2 tyrosine and surrounding charged residues make major contributions to the binding energy (44-47). Interestingly, the CD2/CD58 binding interface exhibits poor surface shape complementarity (14), suggesting that water molecules bridge the binding surfaces and raising the possibility that trapped water contributes to the favourable enthalpy changes.

Effect of electrostatic interactions on CD2 binding to CD58

Proteins typically associate in solution with a rate constant of $10^5$-$10^6$ M$^{-1}$s$^{-1}$ (36,48). This rate may decrease if binding requires conformational adjustments (37,49). Given the evidence from structural and thermodynamic studies that CD2 binding to CD58 is accompanied by conformational change, it is notable that the association rate constant of the CD2/CD58 interaction is not particularly slow ($k_{on} \geq 4 \times 10^5$ M$^{-1}$s$^{-1}$) (10). The association may be accelerated, sometimes dramatically (50), by favourable long-range electrostatic interactions. The mechanism is believed to involve accelerated collisions and/or steering proteins into the correct orientation for binding (36,48,51). Given that the CD2/CD58 binding interface is highly charged (14), we investigated the contribution of long-range electrostatic interactions to binding by examining the effect of varying solution ionic strength.

We initially varied the ionic strength by changing the NaCl concentration. Increasing the NaCl concentration from 150 mM to 1500 mM resulted in a $\sim$6 fold decrease in affinity (Fig. 4A), consistent with a previous report (45). This effect was primarily a result of an effect on the $k_{on}$ (Fig. 4B), consistent with screening of long-range electrostatic interactions. Recent studies have shown that electrostatic screening results in a linear relationship between $\ln k_{on}$ and $1/(1 + \kappa_a)$, where the latter is proportional to the ionic strength (36). In the case of the CD2/CD58 interaction this relationship was clearly not linear (Fig. 4C), suggesting that the effect of NaCl was not solely the result of electrostatic screening. One possible explanation was that the Na and/or Cl ions were disrupting the structure of CD2 and/or CD58 either by direct interactions with the proteins or through effects on solvent structure. Since such effects are dependent on the salt we used a different salt (KF) to vary the ionic strength. In contrast to the effect of NaCl, the affinity and kinetics of CD2 binding to CD58 showed no significant change when the concentration of KF was varied from 150 mM to 1500 mM (Fig. 4A-C). It follows that the effect of varying NaCl on binding is not the result of changes in ionic strength and must result from another mechanism. Interestingly, the rat CD2/CD48 interaction was unaffected by varying the NaCl concentration (52), implying that rat CD2 and/or CD48 are resistant to this effect. Given that the surface charge distributions on CD2/ligand binding surfaces are not conserved across species, and that the ligand binding surfaces of the human CD2/ligand pair are more highly charged, it is not surprising that they exhibit differential sensitivity to NaCl (12,14,53).

The absence of an effect when varying KF concentration demonstrates that long-range electrostatic interactions have no net effect on the CD2/CD58 interaction. This is somewhat unexpected given that the binding interfaces in the complex are highly charged with excellent charge complementarity (14). However, it remains possible that there are some favourable electrostatic interactions that are balanced by unfavourable electrostatic interactions. To investigate this further we used site-directed mutagenesis to disrupt a subset of electrostatic interactions. We have recently generated a number of human CD2 mutants in which single charged residues in the binding interface were mutated to alanine (18). Since it was not possible directly to measure $k_{on}$ we calculated it from the $K_D$ and $k_{off}$ (Table III). While mutation of CD2 residues K41, K51 and K91 to alanine had no significant effect on the $k_{on}$, mutation of D31 to alanine led to a five fold decrease in the $k_{on}$ from $4 \times 10^5$ to $0.8 \times 10^5$ M$^{-1}$s$^{-1}$ (Table III). Interestingly, D31 forms a salt bridge with the CD58 residue R44 in the bound complex (14). Taken together, these data suggest that a favourable electrostatic interaction involving D31 and R44 accelerates CD2 binding to CD58 but that other, unfavourable interactions inhibit association. Interestingly, there is also evidence that the rat CD2/CD48 interaction is also characterised by balancing unfavourable and...
favourable electrostatic interactions (52); whereas this interaction is unaffected by changes in salt concentration, mutation to alanine of individual charged residues in the binding interface resulted in both decreases and increases in affinity (52).

Given the highly-charged nature of the CD2/CD58 binding interface, our finding that long-range electrostatic interactions do not accelerate binding is unexpected, and in striking contrast to the barnase/barstar interaction, where electrostatic attractions between highly charged surfaces accelerate binding by several orders of magnitude (50). One difference between the CD2/CD58 and barnase/barstar interfaces is that the latter is less heterogenous; barnase is predominantly positively charged and barstar is uniformly negatively charged. In contrast, the CD2 and CD58 binding surfaces each carry an intricate mixture of negative and positive charges (12,14,53). We suggest that the presence of heterogenous charges on each surface results in a complex electrostatic potential energy landscape with balancing favourable and unfavourable long-range electrostatic interactions. Given that it has no effect on binding kinetics, what is the functional significance of charged nature of the CD2/CD58 binding interface? We have previously shown, in an analysis of the rat CD2/CD48 interaction, that charged residues that contribute little to the binding affinity may nevertheless contribute to binding specificity, by imposing a requirement for charge complementarity on the other surface (52). The reason proposed for their small contribution to affinity is that, in order for them to form favourable interactions (e.g. salt bridges) with the ligand, existing favourable electrostatic interactions (e.g. with water, salt or adjacent residues) need to be broken (54). The molecular interactions that mediate transient cell-cell interactions need to be low affinity to facilitate detachment (55). We suggest that CD2/CD58 binding interface is charged because electrostatic complementarity enables the interaction to be both weak and specific.

Conclusion

In this report we show that the CD2/CD58 interaction is accompanied by an unfavourable entropy change. Dissection of the entropy change using the approach of Spolar and Record (38) suggests that the unfavourable entropy change results in part from a reduction in conformational flexibility. Taken together with previous structural studies, these findings suggest that energetically significant structural rearrangements accompany binding. Despite the highly-charged binding interface, we show that long-range electrostatic interactions have no net favourable effect on CD2 binding to CD58, probably because of the presence of balancing favourable and unfavourable electrostatic interactions.

REFERENCES

38. Spolar, R. S., and Record, M. T., Jr. (1994) Science 263(5148), 777-784


**FOOTNOTES**

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1The abbreviations used are: APC, antigen presenting cell; CD, cluster of differentiation; CHO, Chinese hamster ovary; Cp, heat capacity at constant pressure; HBS, hepes buffered saline; Ig, immunoglobulin; ITC, isothermal titration calorimetry; NK, natural killer; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; pep-MHC, peptide loaded major histocompatibility complex; SPR, surface plasmon resonance; TCR, T cell receptor; WT, wild type.
FIGURE LEGENDS

FIG. 1. Thermodynamic analysis of the CD2/CD58 interaction by SPR. (A) The $K_D$ for the CD2/CD58 interaction was measured at the indicated temperatures converted into the standard state free energy change ($\Delta G$). Values for $\Delta H$, $-T\Delta S$ and $\Delta C$ ($\pm$ SE of fit) at 25°C were derived by fitting the non-linear form of the van’t Hoff equation to the data (see Experimental Procedures). Also shown (dashed line) is a fit of the linear form of the van’t Hoff equation where $\Delta C = 0$. (B) The $k_{off}$ was measured at the indicated temperatures and an Eyring plot constructed (20), the slope of which yields the $\Delta H_{diss}$ (see Experimental Procedures). The error bars indicate SEM (n≥3).

FIG. 2. Thermodynamic analysis or the CD2/CD58 interaction by ITC. The top panel shows the heat release during the titration of aliquots of CD58 into CD2 at 25°C, corrected for baseline drift. The bottom panel shows the integrated heat of binding for the reaction plotted against the amount of injected CD58. This data was fitted to a single binding site model, after subtracting the heat of dilution data, yielding the values shown for $K_D$, stoichiometry, $\Delta H$ and $-T\Delta S$. The fit SE values were <2%.

FIG. 3. Comparison of thermodynamics of protein/protein interactions. Comparison of the thermodynamic parameters measured here for the CD2/CD58 interaction with the values (mean ± SD) reported for 30 protein/protein (23) and 9 TCR/peptide-MHC (20,49,56-59) interactions.

FIG. 4. The dependence of binding on ionic strength. The dependence of (A) $K_D$ and (B) $k_{on}$ on the concentration of NaCl or KF. The $K_D$ was measured directly whereas $k_{on}$ was calculated from the relationship $k_{on} = k_{off}/K_D$ following direct measurement of the $k_{off}$. The error bars indicate SEM (n≥3) and were determined by error propagation for the $k_{on}$. (C) A plot on ln$k_{on}$ versus $1/(1+\kappa a)$ where $\kappa$ is the inverse Debye length and $a$ is the minimal distance of approach of the interacting proteins; $1/(1+\kappa a)$ is proportional to ionic strength (36).
### TABLE I

**Thermodynamic properties of the CD2/CD58 interaction**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value$^a$</th>
</tr>
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<tbody>
<tr>
<td>ΔCp (cal.mol$^{-1}$ K$^{-1}$)$^b$</td>
<td>-118.0 ± 24</td>
</tr>
<tr>
<td>ΔCp (calculated)$^c$</td>
<td>-116.0 ± 1.4</td>
</tr>
<tr>
<td>ΔHVH (kcal.mol$^{-1}$)$^b$</td>
<td>-11.5 ± 0.2</td>
</tr>
<tr>
<td>ΔHITC$^d$</td>
<td>-12.5 ± 0.02</td>
</tr>
<tr>
<td>-TΔSVH (kcal.mol$^{-1}$)$^b$</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>-TΔSITC$^e$</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Δ$^f$H$_{diss}$ (kcal.mol$^{-1}$)$^f$</td>
<td>16.8 ± 0.6</td>
</tr>
<tr>
<td>Δ$^g$H$_{abs}$</td>
<td>5.3 ± 0.8</td>
</tr>
</tbody>
</table>

$^a$Values shown are mean ± SEM (n ≥ 3). ΔCp is assumed to be temperature independent and all other values are for 25°C (298.15 K).

$^b$ΔCp, ΔHVH and -TΔSVH were measured by van’t Hoff analysis (Fig 1A).

$^c$Calculated using the relationship $\Delta C_{P\text{calc}} = (0.32 ± 0.04) \Delta A_{np} - (0.14 ± 0.04) \Delta A_p$

$^d$Measured by isothermal titration calorimetry.

$^e$Calculated from $\Delta H_{ITC}$ and $\Delta G$ measured by ITC (Fig. 2).

$^f$Measured by Eyring analysis (Fig. 1B).

$^g$Calculated from ΔHVH and Δ$^f$H$_{diss}$. 
### Table II

**Dissection of binding entropy ($\Delta S$)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value $^a$</th>
</tr>
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<tbody>
<tr>
<td>$\Delta S_{HE}$ (cal.mol$^{-1}$)$^b$</td>
<td>82.6</td>
</tr>
<tr>
<td>$\Delta S_{RT}$ (cal.mol$^{-1}$)$^c$</td>
<td>-50.0 (-8)</td>
</tr>
<tr>
<td>$\Delta S_{OTHER}$ (cal.mol$^{-1}$)$^d$</td>
<td>-32.6 (-74.6)</td>
</tr>
<tr>
<td>$\Re$ $^e$</td>
<td>6.0 (13)</td>
</tr>
</tbody>
</table>

$^a$ Determined for $T = T_s = 261K$, where $\Delta S = 0$

$^b$ $\Delta S_{HE} = 0.32 A_{np} \ln (T/386)$ with $T = 261K$

$^c$ $\Delta S_{RT}$ was assumed to be -50 (or -8) cal$^{-1}$ mol$^{-1}$ K$^{-1}$

$^d$ $\Delta S_{OTHER}$ was calculated at $T_s = 261K$ where $\Delta S = 0 = \Delta S_{OTHER} + \Delta S_{HE} + \Delta S_{RT}$. Value in bracket assumes $\Delta S_{RT} = -8$ cal$^{-1}$ mol$^{-1}$ K$^{-1}$.

$^e$ $\Re$ was calculated using the equation $\Re = \Delta S_{OTHER} / 5.6$ Value in bracket assumes $\Delta S_{RT} = -8$ cal$^{-1}$ mol$^{-1}$ K$^{-1}$. 
TABLE III

Effect of CD2 mutations on association rate constant

Since the binding kinetics were too fast to allow direct measurement $k_{on}$ was calculated using the relationship $k_{on} = k_{off}/K_D$ and previously determined values for $k_{off}$ and $K_D$ at 25°C (18). Values shown are mean ± SEM ($n \geq 3$) with the SEM calculated by error propagation from $k_{off}$ and $K_D$ SEM values.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$4.0 \pm 0.4 \times 10^5$</td>
</tr>
<tr>
<td>K41A</td>
<td>$2.3 \pm 0.3 \times 10^5$</td>
</tr>
<tr>
<td>K51A</td>
<td>$4.2 \pm 0.4 \times 10^5$</td>
</tr>
<tr>
<td>K91A</td>
<td>$3.1 \pm 0.3 \times 10^5$</td>
</tr>
<tr>
<td>D31A</td>
<td>$0.8 \pm 0.2 \times 10^5$</td>
</tr>
</tbody>
</table>
\[ \Delta C_p = -150 \pm 16 \text{ cal.mol}^{-1}.\text{K}^{-1} \]

\[ -T \Delta S = 4.6 \pm 0.1 \text{ kcal.mol}^{-1} \]

\[ \Delta H = -11.7 \pm 0.1 \text{ kcal.mol}^{-1} \]

Kearney et al. Figure 1A
\[ \ln\left(\frac{k_{off}}{T}\right) \]

\[ 1/T \ (K^{-1}) \]

\[ \Delta H_{diss} \ 18.9 \pm 0.3 \ \text{kcal.mol}^{-1} \]

Figure 1B Kearney et al
Figure 2 Kearney et al

- $\Delta H = -12.5 \text{ kcal.mol}^{-1}$
- $-T\Delta S = 4.3 \text{ kcal.mol}^{-1}$
- Stoichiometry = 0.967
- $K_D = 10^{-6}$ M
Figure 3 Kearney et al
Figure 4 Kearney et al
The contribution of conformational adjustments and long-range electrostatic forces to the CD2/CD58 interaction
Alice Kearney, Adam Avramovic, Monica A. A. Castro, Alexandre M. Carmo, Simon J. Davis and P. Anton van der Merwe

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