Impact of Salt Bridges on the Equilibrium Binding and Adhesion of Human CD2 and CD58*

Marco V. Bayas†1, Alice Kearney§1, Adam Avramovic®, P. Anton van der Merwe‡1,2, and Deborah E. Leckband¶1‡3

From the †Department of Chemistry and §Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 and ¶Sir William Dunn School of Pathology, University of Oxford, OX1 3RE Oxford, United Kingdom

This study describes quantitative investigations of the impact of single charge mutations on equilibrium binding, kinetics, and the adhesion strength of the CD2–CD58 interaction. Previously steered molecular dynamics simulations guided the selection of the charge mutants investigated, which include the CD2 mutants D31A, K41A, K51A, and K91A. This set includes mutations in which the previous cell aggregation and binding data either agreed or disagreed with the steered molecular dynamics predictions. Surface plasmon resonance measurements quantified the solution binding properties. Adhesion was quantified with the surface force apparatus, which was used previously to study the closely related CD2–CD48 interaction. The results reveal roles that these salt bridges play in equilibrium binding and adhesion. We discuss both the molecular basis of this behavior and its implications for cell adhesion.

The recognition of antigen-presenting cells by T-cells, in the adaptive immune response, involves the simultaneous interaction of large arrays of low affinity surface proteins (1, 2). As a consequence, the intercellular junction is highly dynamic with ligand-receptor bonds breaking and re-forming continuously. This facilitates the efficient aggregation of the different molecular species into a specialized pattern known as the immunological synapse (3). Among the adhesion molecules present in the synapse, CD2 is one of the most extensively studied, and it has become a paradigm for protein–protein interactions in cell adhesion (2).

CD2 is a transmembrane protein expressed on the surface of T lymphocytes. Interactions with its ligands, CD58 in humans and CD48 in rodents, increase the adhesion strength between the T-cell and antigen-presenting cells and thereby enhance antigen recognition (4, 5). CD2 binds its ligands with low affinity, because of its fast off-rates ($k_{\text{off}} > 1 \text{s}^{-1}$). The association constant for the human CD2–CD58 interaction is $K_A \sim 10^5 \text{M}^{-1}$, which is 3 orders of magnitude lower than typical values of antibodies, hormone receptors, and proteases (1, 6). Additionally, charge complementarity is postulated to make an important contribution to the binding specificity of CD2 (7–11).

The extracellular segment of CD2 and its ligands comprises the following two immunoglobulin superfamily domains: a membrane-distal V-set domain (D1) and a membrane-proximal C2-set domain (D2) (9, 12). The interacting surfaces are located in the V-set N-terminal domains. The contact area is a relatively flat surface made up of the G, F, C, C’, and C” β-strands and the intervening FG, CC’, and C’C” loops (Fig. 1) (6–8). The crystal structure of the CD2-CD58 complex also showed that most of the residues in these regions are charged and form several inter-protein salt bridges (11). The only relatively hydrophobic residues at the interface are Phe-46 and Pro-80 of CD58 and Tyr-86 of CD2 (Fig. 1).

To determine the role of these salt bridges in binding equilibrium and cell aggregation, different groups have investigated the impact of point mutations on the CD2/CD58 binding affinity and on cell aggregation as measured by erythrocyte rosetting formation. Based on these assays, the salt bridges can be classified as critical or noncritical (13) (see Table 1). On the basis of rosetting assays (7, 8), critical salt bridges are those that significantly decrease or even abrogate cell aggregation when eliminated, whereas noncritical salt bridges are those whose elimination does not significantly affect rosetting. It should be noted, however, that the rosetting assays involved sheep red blood cells and that sheep and human CD58 are not identical. Alternatively, in steered molecular dynamics simulations of forced CD2-CD58 bond rupture (13), noncritical salt bridges are the ones that broke first in the simulation, whereas the critical ones rupture latest in the simulation period and bear the greatest loads (Table 1). One residue (Lys-91) appeared to form a novel salt bridge during dissociation. Table 1 summarizes the conclusions of different assessments of the impact of salt bridges on CD2-mediated cell adhesion and shows that there is some, but not complete, agreement between these different approaches.

Central to this study is the question of which molecular parameters determine cell adhesion strength. It is often assumed that the binding affinity scales the adhesion. However, the strengths of single, noncovalent bonds are determined by the height and width of the unbinding barrier in the intermolecular potential (14). Despite numerous investigations of the strengths of single molecular linkages, biological adhesion rarely involves single bonds. Yet few quantitative studies address the adhesion between surfaces bridged by multiple, parallel protein bonds. The latter is more relevant to intercel-
multimer junctions, where there is dynamic exchange between bound and unbound states. This is distinctly different from single molecule behavior. However, whether adhesion between surfaces bridged by multiple bonds is dictated by the affinity, Gibbs free energy, or binding kinetics has not been addressed. Here we report a quantitative investigation of the impact of single charge mutations on equilibrium binding, kinetics, and the adhesion strength of the CD2-CD58 interaction. Previously steered molecular dynamics simulations (13) guided the selection of the charge mutants investigated, which include the CD2 mutants D31A, K41A, K51A, and K91A (Fig. 1). Surface plasmon resonance measurements quantified the solution binding properties. Adhesion was quantified with the surface force apparatus, which had been used previously to study the closely related murine CD2-CD48 interaction (15). The results reveal roles that these salt bridges play in equilibrium binding and adhesion (resisting force). We discuss both the molecular basis of this behavior and its implications for cell adhesion.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The nickel-chelating lipid, NTA di-lauryl glycerol ester, was custom synthesized by Neuftech Chemicals (Vancouver, Canada). DPPE and DTPC were from Avanti Polar Lipids (Alabaster, AL). Aqueous solutions were prepared with water purified with a Milli-Q UV filtration system (18 mgeq/LS; Millipore). High purity salts were purchased from Aldrich. Mica was from S&J Trading Co., Ltd.

**Protein Production**—cDNA constructs encoding extracellular regions of wild type human CD2 and CD58 with C-terminal oligohistidine tags were generated by PCR. The C termini of the encoded CD2 and CD58 were SCPEKHHHHHHH and TCISSHHHHHH, respectively. The 5’ primers of CD2 and CD58 were TAGTAGTCTAGACCTAGCAGCAACCAGGAC and TAGTAGTCTAGACTCAAGCAGCGGACCAGTACG, respectively. These included an XbaI restriction site. The 3’ primers were CTAGAATCCGAATTCAATGATGGTGATGATGGCTGCTTGGGATACAGGTTGTCAAAA-TGAT, respectively. The latter added an oligohistidine tag followed by a stop codon and an EcoRI restriction site. These constructs were digested with XbaI and EcoRI enzymes and cloned into the glutamine synthetase expression vector pEE14 (16). The plasmids were transfected into Chinese hamster ovary K-1 cells using the FuGENE transfection kit (Roche Applied Science) according to the manufacturer’s instructions. The clones expressing high levels of soluble protein were obtained as described previously (17). Expressed proteins were purified from tissue culture supernatant using Ni-NTA-agarose (Qiagen) and, immediately prior to binding studies, further purified by gel filtration on a Superdex S75 column (Amersham Biosciences), as described previously (18).

CD2 mutants were generated by overlapping PCRs. The 5’ and 3’ primers for CD2 described above were used with the following pairs of complementary primers: for D31A, AGTGATGATATTGCCGATATAAAATGGGAAA and TCCCCATTTATGCCTGGGATACAGGTTGTGCTGAT; for K41A, AAAACTTCAGACGCAAGGCCCAGC and TTGTGATGATGGCTGCTTGGGATACAGGTTGTCAAAA-TGAT, respectively. The latter added an oligohistidine tag followed by a stop codon and an EcoRI restriction site. These constructs were digested with XbaI and EcoRI enzymes and cloned into the glutamine synthetase expression vector pEE14 (16). The plasmids were transfected into Chinese hamster ovary K-1 cells using the FuGENE transfection kit (Roche Applied Science) according to the manufacturer’s instructions. The clones expressing high levels of soluble protein were obtained as described previously (17). Expressed proteins were purified from tissue culture supernatant using Ni-NTA-agarose (Qiagen) and, immediately prior to binding studies, further purified by gel filtration on a Superdex S75 column (Amersham Biosciences), as described previously (18).

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![FIGURE 1. The ligand binding surface of CD2.](image)

The membrane-distal V-set domain of CD2 is depicted with the β-strands shown as arrows. The side chains of the charged residues that are mutated in this study are depicted in red. Also depicted in yellow is Tyr-86, which has been shown to be critical for CD58 binding (11, 31). This figure was made using DS Viewer Pro version 6 (Accelrys Software Inc.).

| Residue | Location | Rosetting assay (% wild type)* | Dissociation constant $K_d/K_d(WT)^b$ | SMD*c |
|---------|----------|-------------------------------|---------------------------------------|--------|
| Asp-31  | C        | ND                            | >47                                   | C      |
| Asp-32  | C        | 0                             | >127                                  | C      |
| Lys-34  | C        | 11                            | >47                                   | NC     |
| Lys-41  | C        | 56                            | 14                                    | NC     |
| Lys-43  | C        | 4                             | >63                                   | NC     |
| Arg-48  | C        | 0                             | >57                                   | C      |
| Lys-51  | C        | 8                             | 10.3                                  | C      |
| Glu-95  | G        | 87                            | 3.33                                  | NC     |
| Lys-91  | G        | 0                             | ND                                    | Transient |

*Data are from Ref. 8.

*Data are from Ref. 31.

*Data are from Ref. 13: C, critical; NC, non-critical; ND, not done. Lys-91 formed a transient salt bridge during the simulated detachment.

4 The abbreviations used are: NTA, nitritoltriacetic acid; SFA, surface force apparatus; DPPE, dipalmitoyl phosphatidylethanolamine; DTPC, ditritanoyl phosphatidylcholine; h, human; SMD, steered molecular dynamics.
were confirmed dideoxy sequencing. Correct folding of the CD2 mutants was confirmed by binding to a series of CD2 monoclonal antibodies, as described previously (19).

Surface Plasmon Resonance Measurements of Equilibrium Binding Parameters—These studies were performed on a BIAcore 2000 (BIAcore AB) (20). Unless otherwise stated, experiments were performed at 25 °C using HBS buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂) at a flow rate of 50 μl·min⁻¹. Human CD2 was directly coupled to research grade sensor chips (BIAcore AB) using the amine coupling kit (BIAcore AB) as described previously (19). Kinetic measurements were performed at a flow rate of 50 μl·min⁻¹ and confirmed at three different immobilization levels of CD2, to rule out mass transport artifacts.

Dissociation constants, kinetic rates, and thermodynamic properties were determined as described (21). Equilibrium thermodynamic parameters were obtained by measuring the dissociation constant over a range of temperatures (5–37 °C) and fitting the thus determined binding free energy ΔG to the nonlinear form of the van’t Hoff Equation 1 (22).

\[
\Delta G = \Delta H_{0} - T\Delta S_{0} + \Delta C_{p}(T - T_{0}) - T\Delta C \ln\left(\frac{T}{T_{0}}\right) \quad (\text{Eq. 1})
\]

where \( T \) is the absolute temperature; \( T_{0} \) is an arbitrary reference temperature (e.g. 298.15 K); \( \Delta G \) is the standard state binding free energy (all components at 1 mol·liter⁻¹); \( \Delta H_{0} \) is the enthalpy change at \( T_{0} \) (kcal·mol⁻¹); \( \Delta C_{p} \) is the enthalpy change (kcal·mol⁻¹) at constant pressure; and \( \Delta S \) is the entropy change at the standard state.

\( \Delta G \) was calculated from the association constant (\( K_{A} \)) by using Equation 2,

\[
\Delta G = -RT \ln(K_{A} \times \mathcal{C}) \quad (\text{Eq. 2})
\]

where \( R \) is 1.987 cal·mol⁻¹·K⁻¹; \( K_{A} \) is expressed in M⁻¹; and \( \mathcal{C} \) is the standard state concentration (1 M).

The activation enthalpy of dissociation (\( \Delta H_{\text{diss}} \)) was determined by measuring \( k_{\text{off}} \) over a range of temperatures (10–30 °C) and plotting \( \ln(k_{\text{off}}(T)/T) \) against \( 1/T \), the slope of which equals \( -\Delta H_{\text{diss}}/R \) (21, 23).

Immobile Protein Monolayer Assembly and Characterization—The surface density (proteins/μm²) of protein adsorbed onto planar, supported NTA-DLGE-containing membranes was determined using a homemade surface plasmon resonance instrument based on the Kretschmann configuration (24, 25). These experiments used a 390-Å gold film evaporated on a clean glass slide. The slide was initially coated with a 15-Å chromium adhesion layer by resistive evaporation at a base pressure <10⁻⁶ torr. The gold-coated slide was then incubated overnight in a 1 mM solution of octadecanethiol in alcohol to form a self-assembled octadecanethiol monolayer on gold. An outer lipid layer containing 75 mol % NTA-DLGE and 25 mol % DTPC was then prepared by Langmuir-Blodgett deposition onto the octadecanethiol monolayer from the water-air interface of a Langmuir trough (NIMA, Coventry, UK) at 25 °C and a constant surface pressure of 34 mN/m.

After establishing the base-line resonance angle, the protein solution was injected into the cell at ~15 μl·min⁻¹ and incubated under static conditions for 2 h. The concentration of the protein solution was the same as used to prepare protein layers for SFA measurements. The surface plasmon resonance cell was then flushed with buffer to remove unbound and nonspecifically bound protein. From the final change in the resonance angle, we determined the protein coverage (25). After a 2-h incubation, the surface densities of CD58 and CD2 were ~1 and ~1.5 x 10⁴ molecules/μm², respectively.

Protein Monolayers for Force Measurements—The proteins were immobilized on planar-supported lipid bilayers deposited onto mica sheets (15). The first lipid layer was gel phase DPPE at 43 Å²/lipid. The outer lipid layer contained 75 mol % NTA-DLGE and 25 mol % DTPC. The latter was deposited onto the DPPE film from an aqueous subphase containing 100 mM NaNO₃, 50 mM Tris base, 2 mM Ca(NO₃)₂, 50 μM NiSO₄, at pH 7.5. The average lipid density was ~65 Å²/lipid. The histidine-tagged proteins were immobilized to the NTA-functionalized membranes from ~70 μg·ml⁻¹ solutions (15). The disks were then rinsed gently with buffer to remove nonspecifically bound protein. Finally the disks were mounted in the surface force apparatus, while keeping the samples under solution at all times.

Force Measurements of CD2-CD58 Adhesion—The force measurements were performed with a Mark II SFA (26). The SFA quantifies the force between two macroscopic, crossed hemi-cylindrical silica disks as a function of their separation distance (26). The absolute intersurface distance is determined within ±0.1 nm by interferometry (27). The force normalized by the geometric average radius of the disks \( R = (R_{1}R_{2})^{1/2} \) is determined within \( F/R = ±0.1 \text{ mN/m} \) from the deflection of a sensitive spring supporting one of the disks. Measurements were conducted at 25 °C in a temperature-controlled room. The DTPC monolayer is fluid at this temperature.

With the resulting molecular assembly (Fig. 2), the absolute distance (\( D \)) between the lipid bilayers was determined as follows. We measured the total change in the absolute thickness (\( T \)) of the molecular layers between the DPPE monolayers after the deposition of the NTA-DLGE-containing layer and protein immobilization. Thus \( D = T - 2 \times t_{\text{NTA-DLGE}} \), where \( t_{\text{NTA-DLGE}} \) is the thickness of the NTA-containing layer (Fig. 2). The latter was determined by x-ray reflectivity to be 31 Å (28). Thus, \( D = 0 \) corresponds to contact between the dehydrated membrane surfaces.

In the experiments, the normalized force \( F_{c}/R \) versus the separation \( D \) was determined as the disks were brought into contact and then separated. The normalized force between the hemicylinders corresponds to the energy per area between flat plates \( E_{s} \) according to the Derjaguin approximation (29) as shown in Equation 3,

\[
F_{c}(D) = 2\pi R E_{s}(D) \quad (\text{Eq. 3})
\]

The normalized force \( F_{c}/R \) is therefore independent of the force geometry.

The adhesion energy density is determined from the force required to separate the surfaces, the pull-off force (\( F_{pu} \)), and the Derjaguin-Mueller-Toporov theory (29). This relates the adhesion energy per area (\( E_{s} \)) to \( F_{pu} \) by \( E_{s} = F_{pu}/2\pi R \) (29). We
measured $F_{po}/R$ after different intersurface contact times. In this study, the contact time is defined by the time from the initial detection of the steric repulsion until the disks jump apart. In adhesion measurements of ligand-receptor interactions, we also normalize the adhesion energy per area by the protein surface density to correct for differences in the protein surface densities. The latter is an estimate of the bond energy.

RESULTS

Analysis of the Solution Binding Properties of CD2 Mutants—
Mutant forms of the human CD2 ectodomain were expressed in CHO-K1 cells at yields (3 mg liter$^{-1}$) comparable with wild type CD2. All mutants were shown to bind to at least one of a panel of monoclonal antibodies specific for human CD2 (data not shown). The binding of CD58 to the CD2 mutants was analyzed by surface plasmon resonance. The association constants ($K_A$) were determined from equilibrium binding analysis (Fig. 3), which yielded the values shown in Table 2.

CD58 bound D31A CD2 with a 20-fold lower affinity than wild type CD2 and to the other three mutants with a 2–3-fold lower affinity than wild type CD2. From the $K_A$ values, we obtain the binding free energy change ($\Delta G$) (Table 2), which is the sum of enthalpic ($\Delta H$) and entropic contributions ($-T \Delta S$). The effect of the CD2 mutations on the enthalpy and entropy changes accompanying CD58 binding were determined by van’t Hoff analysis (Supplemental Material). Interestingly, although the D31A mutation results in an unfavorable change in $\Delta H$, all three of the other mutations resulted in more favorable enthalpy changes (Table 2). Thus the lower affinity of CD58 for these mutants results from unfavorable entropy changes (Table 2).

We next examined the effect of the mutations on the rate of CD58 dissociation from CD2 (Fig. 4). The D31A and K51A mutations increased $k_{off}$ by ~4–5-fold, whereas the K41A and K91A mutations increased $k_{off}$ by ~2-fold (Table 3). According to transition state theory (23), $k_{off}$ can be used to calculate the energy barrier for dissociation. This is the activation energy for dissociation, which has entropic and enthalpic contributions. The latter is the activation enthalpy of dissociation ($\Delta H_{diss}^\alpha$) and can be determined from plots of $\ln(k_{off}/T)$ versus $1/T$ (23) (Supplemental Material). The D31A mutation decreased $\Delta H_{diss}^\alpha$ significantly, whereas the other mutations resulted in smaller decreases in $\Delta H_{diss}^\alpha$ (Table 3).

Force Measurements of CD58 Adhesion to CD2 Variants—
Fig. 5 shows the normalized force-distance profile between the membrane-bound monolayers of wild type CD2 and CD58. This illustrates the general features of the measured interactions between CD58 and all five CD2 variants. In the advancing profile (decreasing $D$), the repulsive force increases monotonically at $D < 150$ Å because of steric repulsion between the end-on oriented protein layers, which are ~75 Å in length. Upon separation, the proteins adhere, and the force decreases.
TABLE 2
Equilibrium analysis of CD58 binding to CD2 mutants
The values are means ± S.E. derived from three independent experiments. All values are for 25 °C.

|         | WTCD2       | D31A CD2    | K41A CD2    | K51A CD2    | K91A CD2    |
|---------|-------------|-------------|-------------|-------------|-------------|
| $K_a$ ($\times 10^{-3}$ M$^{-1}$) | 140 ± 12    | 7 ± 2       | 48 ± 5      | 48 ± 4      | 63 ± 1      |
| $\Delta G$ (kcal mol$^{-1}$)  | -7.1 ± 0.03 | -6.1 ± 0.01 | -6.4 ± 0.05 | -6.4 ± 0.05 | -6.5 ± 0.06 |
| $\Delta F$ (kcal mol$^{-1}$)  | -11.5 ± 0.2 | -9.0 ± 0.3  | -12.3 ± 0.3 | -12.1 ± 0.3 | -12.2 ± 0.3 |
| $-7\Delta S$ (kcal mol$^{-1}$) | 4.4 ± 0.2   | 3.9 ± 0.4   | 5.7 ± 0.4   | 5.7 ± 0.4   | 5.7 ± 0.2   |

At the minimum ($F < 0$), which is also the pull-off force, the bonds yield, and the surfaces jump out of contact.

Because the proteins diffuse laterally, the measured pull-off forces increase with the surface contact time, as more proteins form intermembrane linkages (30). The adhesion was measured as a function of contact time between 0 and ~30 min. Fig. 6 shows the time dependence of the normalized adhesion energies. All CD2 variants exhibited similar asymptoting adhesion with time. The data were fit, using nonlinear regression, by the empirical function: $E_a = \alpha(1 - e^{-t/t_{\text{lag}}})$. Extrapolation to $t \to \infty$ gives the limiting adhesion energy $\alpha$, which depends on the receptor identity, surface density, and lateral mobility (15, 30). The fitted parameters for $\alpha$ and the normalized adhesion energies are shown in Table 4.

The fitted values for $\alpha$ were compared using a $t$ test, to determine the statistical significance of apparent differences in adhesion energies relative to both wild type CD2 and each other. For a given pair ($\alpha_1$ and $\alpha_2$), the null hypothesis is $\alpha_1 = \alpha_2$. Table 5 shows the $t$ statistics values calculated for each pair. The numbers above the diagonal are the $t$ values, whereas those below are the corresponding $p$ values for the two-tailed case. The null hypothesis is rejected when $p < 0.05$.

Based on the force data, WTCD2 generated the strongest adhesion, whereas the K51A gave the weakest. The K51A mutation decreased the adhesion energy to ~58% of the wild type interaction. On the other hand, the mutations D31A and K91A only decreased the adhesion energy to ~69 and ~79% of the wild type value, respectively. The mutation K41A had the weakest effect, with an adhesion energy ~85% that of WTCD2.

The normalized adhesion energies were calculated by dividing the adhesion energy per area by the surface density of CD58. The surface density of CD2 (wild type and mutants) was $14.3 \pm 1.8 \times 10^3$ molecules/µm$^2$. We assumed that the maximum number of adhesive bonds is determined by the receptor with the lower surface density, which is CD58 in our experiments. The homophilic CD2 interaction was relatively weak (data not shown). The estimated bond energy was $2.4 \pm 0.2$ kcal mol$^{-1}$, which is ~47% that of the WTCD2-CD58. There was no similar
homophilic CD58 binding. Table 4 summarizes the normalized adhesion energies for the different protein pairs.

The distances corresponding to the minima in the force curves reflect the dimensions of the protein complexes under maximum tension. During separation, we observed that the surfaces first slowly moved apart over ~20 Å prior to the final, abrupt pull off at an average distance of 130 ± 10 Å. Therefore, at 110 Å < D < 130 Å, the two membranes began separating without additional force, until they finally and abruptly pulled apart at ~130 Å. Because it was difficult to precisely determine the initial point of final bond rupture, the standard deviation in the measurement is relatively large. It is worth noting that 20 Å is roughly the distance that the protein complex reorients in the SMD simulations prior to bond failure (13).

To determine how the protein mediated adhesion scales with thermodynamic and kinetic parameters, we plotted the adhesion versus the different kinetic rates and bond energies determined from the surface plasmon resonance measurements. These are summarized in Fig. 7. The normalized adhesion energy scales best with ln $k_{\text{off}}$ (Fig. 7C). It also scales reasonably well with interaction free energy or $\Delta G$ (Fig. 7B) and the activation enthalpy of dissociation or $\Delta H_{\text{diss}}^\ddagger$ (Fig. 7F). It does not scale with ln $k_{\text{on}}$ (Fig. 7D), with $K_A$ (Fig. 7A), or with $\Delta H$ (Fig. 7E).

**DISCUSSION**

**Effect of CD2 Mutations on Solution Binding Properties**—Mutating the different charged residues on the binding face of CD2 had differential effects on the affinity of CD2 for CD58, namely D31A had the most profound effect, decreasing the affinity 20-fold, whereas K41A, K51A, and K91A decreased $K_A$ values by 2–3-fold. Asp-31, Lys-41, and Lys-51 form salt bridges with CD58 in the CD2/CD58 crystal structure (11). Interestingly, Lys-91 did not form a salt bridge in the structure of the complex, but steered molecular dynamic simulations predicted that a transient salt bridge forms during unbinding (13). One possible explanation for the substantial effect of the D31A mutation is the proximity of Asp-31 to Tyr-86 (Fig. 1), which other mutagenesis studies indicate is critical for CD58 binding (31).

The mutation D31A had the greatest effect on $k_{\text{off}}$, increasing it ~5-fold. It is noteworthy that K51A had a more substantial effect on $k_{\text{off}}$ than either K41A or K91A. A possible structural basis for this is that Lys-51 is right next to Arg-48, which also

| TABLE 4 |
| --- |
| **Comparison of adhesion energy with free energy and simulations** |
| | Adhesion energy per area | Normalized adhesion energy* | $\Delta G$ | SMD prediction |
| | mJ/m$^2$ | kcal/mol | kcal/mol$^{-1}$ |
| WTCD2 | 0.338 ± 0.013 | 5.1 ± 0.2 | −7.1 ± 0.03 |
| K41A | 0.29 ± 0.013 | 4.4 ± 0.2 | −6.4 ± 0.06 | NC |
| K91A | 0.266 ± 0.011 | 4.0 ± 0.2 | −6.5 ± 0.06 | Transient |
| D31A | 0.216 ± 0.016 | 3.3 ± 0.2 | −5.1 ± 0.01 | C |
| K51A | 0.195 ± 0.020 | 2.96 ± 0.30 | −6.4 ± 0.05 | C |
| WTCD2-K41A | 0.29 ± 0.013 | 4.4 ± 0.2 | −6.4 ± 0.06 | Transient |
| WTCD2-K51A | 0.195 ± 0.020 | 2.96 ± 0.30 | −6.4 ± 0.05 | C |
| WTCD2-K91A | 0.266 ± 0.011 | 4.0 ± 0.2 | −6.5 ± 0.06 | Transient |
| WTCD2-D31A | 0.216 ± 0.016 | 3.3 ± 0.2 | −5.1 ± 0.01 | C |
| WTCD2-K51A | 0.195 ± 0.020 | 2.96 ± 0.30 | −6.4 ± 0.05 | C |

*The normalized adhesion energy is the adhesion energy per area divided by the surface density of the receptor with the lowest surface coverage (CD58). The surface density of CD58 was $9.5 \times 10^3$ molecules/μm$^2$.
forms a salt bridge (11). The K51A mutation may therefore disturb an additional salt bridge.

The equilibrium binding measurements show that K41A, K51A, and K91A mutations increase the favorability of interaction. This could reflect the proximity of Asp-31 to the critical residue Tyr-86 (31) or its contribution to the energy profile along the unbinding trajectory (13).

Role of Charged Amino Acids in Adhesion—Because the SFA quantifies the range of the interaction as well as the magnitude of adhesion, we compared the membrane separation at bond failure with the protein complex dimensions predicted on the basis of crystallographic data and structural models. The measured distance of 130 ± 10 Å agrees quantitatively with the predicted complex dimensions (33). This confirms directly that CD2 and CD58 adhere in the predicted head-to-head orientation. These results also agree with prior SFA measurements of the mouse CD2-CD48 interaction (15). However, the normalized adhesion energy for the hCD2-CD58 interaction is ~8 times that measured for murine CD2-CD48 (15).

A fundamental question in cell adhesion is whether the physical bonds that stabilize protein complexes in solution are the same load-bearing bonds that stabilize the complexes under force. The trends in the adhesion and Gibbs free energies are similar, with the exception of the K51A mutant (Table 4). The K41A and K91A mutations had modest effects on both the adhesion and free energy (Table 4). Although K91A appears to have a greater impact on adhesion, the normalized adhesion energies of both mutants are statistically indistinguishable (Table 5), in agreement with the equilibrium binding data. The D31A mutation also significantly altered both the adhesion and ΔG. By contrast, the effect of mutating Lys-51 was mixed, i.e. the K51A mutation substantially decreased adhesion and resetting (7, 8), but it only altered ΔG by 0.7 kcal·mol⁻¹.

The trends predicted by SMD simulations (Table 4) and the linear correlation between adhesion and ln koff (Fig. 7) give some insight into the possible basis of the apparently different effects of K51A on adhesion and the Gibbs free energy. A recent study shows that the adhesion strength increases with the height of the unbinding barrier when the pulling rate exceeds a critical value determined by the intrinsic bond relaxation time (34). At slower loading rates, the adhesion correlates increasingly with ΔG. As the pulling rate transitions from the thermodynamic to the kinetic regime, the activation barrier increasingly influences adhesion, as observed in this study. The slightly better correlation with ln koff than ΔG (Fig. 7) is as predicted in this crossover region (34).

An alternative explanation for differing effects of mutations on the affinity versus adhesion is that some load-bearing interactions in forced bond dissociation might not play the same role in adhesion as in equilibrium binding. For example, binding in solution could follow multiple pathways in which different salt bridges contribute to the complex association, alignment, and stabilization. The application of a directional external force could bias the unbinding trajectory (32) and constrain dissociation to a smaller subset of trajectories in which a particular salt bridge(s) dominates the energy landscape. The correlation with ln koff suggests that the former mechanism (see above) operates in this case.

Force measurements also demonstrated the possibility that CD2 forms homophilic bonds. Mouse CD2 exhibited some hysteresis between the advancing and receding curves between identical proteins, suggesting a weak attraction (15). However,
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the proteins did not adhere (15). On the other hand, human CD2 monolayers adhered. The homophilic hCD2 adhesion is 2-fold weaker than the hCD2-hCD58 interaction, and four times stronger than the murine CD2-CD48 adhesion (15). Interestingly, both human and rat CD2 packed head-to-head in crystals, and this was used as an early model for the heterophilic CD2 interactions (1). The crystal structure of human CD2 does not show any well defined salt bridges at the protein interface. Instead, residues Gly-90, Tyr-86, and Phe-54 appear in inter-protein contacts (12). The measured homophilic CD2 binding suggests that these contacts are physically possible. Despite our finding that this interaction is comparable with that of the murine CD2–CD48 bond (15), a physiological role for homophilic CD2 adhesion has not been identified.

In summary, we used force measurements solution binding studies and genetically engineered CD2 variants to investigate the role of salt bridges in the human CD2-CD58 interaction. We confirmed the impact of salt bridge elimination predicted by steered molecular dynamics simulations. Furthermore, with the measured kinetic and thermodynamic bond parameters, we determined how adhesion between membranes bridged by multiple protein pairs scales with the thermodynamic and kinetic parameters of the bonds.

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