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ARTICLE

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Characterisation of the low affinity interaction between rat cell adhesion molecules CD2 and CD48 by analytical ultracentrifugation

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Abstract CD2 is a cell adhesion molecule found on the plasma membrane of T-lymphocytes. Its counter-receptor in rat is the structurally related CD48. This interaction is believed to contribute to the adhesion of T-cells to other cells such as cytotoxic targets and antigen presenting cells. Cell-cell adhesion involves the formation of multiple cell adhesion molecule complexes at the cell surface and if cell-cell de-adhesion is to occur, these complexes need to be disrupted. The affinities of cell adhesion molecule interactions are suggested to be relatively weak to allow this de-adhesion of cell-cell interactions. The CD2/CD48 interaction has been studied using recombinant extracellular proteins and the affinity of the interaction of soluble recombinant rat CD2–CD48 has been determined (at 37 °C) using surface plasmon resonance (and shown to be weak), with the dissociation constant $K_d = 60-90$ μM. The values determined by surface plasmon resonance results could be affected by the immobilisation of the ligand on the chip and any self-association on the chip. We used three different analytical ultracentrifuge procedures which each allowed the interaction to be studied in free solution without the need for an immobilisation medium. Both sedimentation equilibrium (using direct analysis of the concentration distribution and also modelling of molecular weight versus concentration data) and sedimentation velocity at 5 °C yielded dissociation constants in the range of 20–110 μM, supporting the surface plasmon resonance findings showing that binding between these cell adhesion molecules is relatively weak. These studies also ruled out the presence of any significant self-association of the reactants which could lead to systematic error in the surface plasmon resonance results.

Key words T-lymphocytes · Dissociation constant

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Introduction

One of the features of the cells of the immune system is the wide range of interactions they make with other cells or the extracellular matrix as they migrate through tissues and recirculate between blood and lymph. These interactions are mediated through molecules located on their plasma membranes called cell adhesion molecules or CAMs. Given the transient nature of the majority of these cellular interactions, these interactions must be readily reversible and it seemed likely that they would be of low affinity. The interactions are difficult to study as most of the identified CAMs are membrane proteins and are difficult to isolate in large quantities and also in the absence of detergent (necessary to solubilise them). Thus recent work has focused on expressing the extracellular parts of the proteins and analysing their interactions.

The best characterised CAM is the glycoprotein CD2 and its ligands CD48 and CD58. CD2 contains two immunoglobulin-like domains whose structures have been determined in both rat and human by X-ray crystallography of the recombinant proteins after removal of most of the carbohydrate (Jones et al. 1992; Bodian et al. 1994). The structures of human and rat NH_2 terminal domains have also been independently determined by NMR (Driscoll et al. 1991; Wyss et al. 1993). CD2 interacts with CD48 in rodents and CD58 in humans (reviewed in Davis and van der Merwe 1996). CD58 has not been identified in rodents and if human CD48 interacts with CD2 the interaction is of a much lower affinity than that with CD58 (San-

drin et al. 1993; van der Merwe et al. 1994). The three proteins CD2, CD48 and CD58 all have similar topology in that they contain two IgSF domains with similar features such as the NH_2 terminal domain lacking the disulphide bond normally conserved in IgSF domains and domain two has at least one additional disulphide bond. However the overall sequence similarity is quite low (Davis and van der Merwe 1996).

The binding kinetics and affinity of the interaction of CD2 with CD48 (rat) and CD58 (human) have been previously studied using recombinant proteins corresponding to the (soluble) extracellular parts of the antigens (sCD2 and sCD48 where the prefix "s" means "soluble") and analysis by surface plasmon resonance (SPR) using the BIAcore (Pharmacia, Uppsala, Sweden) apparatus (van der Merwe et al. 1993; van der Merwe et al. 1994). This has shown that the dissociation constant of rat CD2 with CD48 is 60–90 μM at 37 °C with an exceptionally fast dissociation rate ($>4\ s^{-1}$). A slightly higher affinity has been determined for the human CD2/CD58 interaction with a K_d in the range 10–20 μM. Again the dissociation rate was in the same order of $>4\ s^{-1}$. This makes the interaction one of the weakest protein-protein interactions characterised by this technique and the fast dissociation rate limits the number of different methods of analysis possible. Sedimentation equilibrium and velocity centrifugation methods are applicable to such interactions and dissociation constants of the order of 10 μM have previously been determined by ultracentrifugation methods for the dimerisation of many systems including interleukin 8 (Burrows et al. 1994), yeast hexokinase P1 (Hoggett and Kellett 1992), HIV p24 (Rose et al. 1992) and the DNA binding domain of the yeast transcriptional activator GAL4 (Gadhavi et al. 1996).

In this study we have investigated the solution behaviour of sCD2 and sCD48 using sedimentation equilibrium and velocity centrifugation, including the interactions between these molecules *in free solution* i.e. without the need for immobilising either molecule or the need for a third phase (i.e. dextran in the case for surface plasmon resonance or a gel column in the case of affinity chromatography).

Materials and methods

Glycoprotein expression and purification

srCD2 and srCD48 (where the pre-fix "r" means "rat") were expressed in Chinese hamster ovary cell lines with a pEE6.hcmv-GS vector and purified by monoclonal antibody affinity chromatography (Davis et al. 1993; Gray et al. 1993; McAlister et al. 1996). Sample integrity was monitored using 15% SDS-PAGE gels.

Ultracentrifugation experiments

Experiments on srCD2, srCD48 and equimolar mixtures of each were performed at 5 °C in a Beckman Optima XL-A analytical ultracentrifuge equipped with scanning absorption optics. Samples were dialysed against 10 mM Hepes, 140 mM NaCl and 0.02% NaN_3 pH 7.4 buffer and loaded into 12 mm path length standard charcoal-filled Epon double sector cells with quartz windows. For concentration determinations, extinction coefficients at 280 nm, ε_{280} of 1300 $ml \cdot g^{-1} \cdot cm^{-1}$ and 1440 $ml \cdot g^{-1} \cdot cm^{-1}$ (i.e. within about ±5% of each other), were determined experimentally from amino acid analysis at 280 nm for srCD2 (van der Merwe et al. 1993) and srCD48 (McAllister et al. 1996) respectively and a mean value of 1370 $ml \cdot g^{-1} \cdot cm^{-1}$ was taken for a 1:1 complex of srCD2–srCD48. A solvent density ρ of 1.00910 g/ml was determined for the hepes buffered saline using an Anton Paar (Graz, Austria) DMA O2 C precision densimeter.

Partial specific volume $\bar{v}$ of the reactants

This is necessary for interpretation of the ultracentrifuge data records, both sedimentation velocity (correction of the sedimentation coefficient to standard solvent conditions), and, more importantly for sedimentation equilibrium (as part of a buoyancy correction term for the evaluation of molar masses and dissociation constants). Selection of the value to be used for the partial specific volumes ($\bar{v}$) of both srCD2 and srCD48 needs some care because of the expected high carbohydrate content of these glycoproteins (Davis et al. 1993). It can be evaluated from the weight average of the partial specific volume of the protein component ($\bar{v}_p$) and that of the carbohydrate component ($\bar{v}_c$):

$$\bar{v}_p = (1 - f_c) \cdot \bar{v}_p + f_c \cdot \bar{v}_c \quad (1a)$$

where f_c is the weight fraction of carbohydrate. $\bar{v}_p$= 0.738 ml/g and 0.732 for srCD2 and srCD48 respectively (calculated from the amino acid sequence using the "consensus" formula of Perkins (1986) and $\bar{v}_c$ for the carbohydrate component can be reasonably taken as ~0.63 ml/g (see e.g. Shire 1992)). f_c in Eq. (1a) is obtained from direct measurement of the buoyant molar mass of the glycoprotein, $M' = \{M(1-\bar{v}\rho)\}$ and calculation (from the amino acid sequence values for M_p and $\bar{v}_p$) of the corresponding value of M'_p $\{=M_p(1-\bar{v}_p\rho)\}$ (Shire 1992):

$$f_c = (M' - M'_p)/(M' + (\bar{v}_p - \bar{v}_c)\rho M'_p) \quad (1b)$$

where ρ (g/ml) is the density of the buffer used.

Sedimentation equilibrium analysis

srCD2, srCD48 and the complex were run at rotor speeds of 17,000, 16,000 and 13,000 rpm respectively. Samples reached equilibrium after ~11 h and this was verified by the overlay of two radial scan traces acquired four hours apart. Baselines were obtained by overspeeding at the end of each sedimentation equilibrium run and recording the residual absorbance. Data were initially analysed using Beckman (Palo Alto, USA) data analysis software. The IDEAL1 model was initially used to estimate apparent

weight-average molar masses ($M_{w,app}$) and to indicate any non-ideality or associative/dissociative behaviour of the srCD2, srCD48 as well as the mixtures thereof. This routine represents the equilibrium solute distribution in terms of a single, monodisperse, thermodynamically ideal species according to the relation:

$$A(r) = A(r_F)\exp[H \cdot M \cdot (r^2 - r_F^2)] + E \quad (2)$$

Where $A(r)$ is the absorbance at radius r, $A(r_F)$ is the absorbance at reference radius r_F, M is the molar mass of the particle and E is the baseline correction. H is the constant $(1-\bar{v}\rho)\omega^2/2RT$ where ω is the angular velocity of the rotor (rad/s), R is the universal gas constant and T is the absolute temperature (K). In the case of a thermodynamically non-ideal or heterogeneous system, Eq. (2) yields the apparent weight average molar mass, $M_{w,app}$, *between the radial positions chosen for the analysis*, which for monomeric or weakly associating systems gives a reasonable approximation for the whole solute distribution between cell meniscus and base.

Data records for the srCD2–srCD48 mixture were then analysed to determine the dissociation constant, K_d(μM) of the interaction. Advantage was taken of the fact that the molar masses (and uv absorption coefficients – see above) of the reactants are similar, even in the glycosylated state as shown below. The interaction can be treated therefore as an effective "dimerisation" with an effective monomer molar mass M_1 the mean of the two reactants. K_ds were obtained in two ways.

Extraction of K_d from direct analysis of the concentration distribution

Firstly using the Beckman model ASSOC4, which for a reversible dimerisation fits the equilibrium solute distribution to a form of Eq. (III27) of Kim et al. (1977):

$$A(r) = A_1(r_F)\exp[H \cdot M_1 \cdot (r^2 - r_F^2)] + A_1^2(r_F)\, k_{2,app} \exp[H \cdot (2M_1)(r^2 - r_F^2)] + E \quad (3)$$

where $k_{2,app}$ (M^{-1}) is the apparent association constant and A_1 is the absorbance due to "monomer" at the reference radial position r_F. The corresponding apparent molar association constant, $K_{2,app} = (M_1/2) \cdot k_{2,app}$ (Kim et al. 1977) and the apparent molar dissociation constant, $K_{d,app} = 1/K_{2,app}$. The reason for the subscript "app" is that because of the relatively high concentrations needed to investigate the suspected weak affinity between srCD2 and srCD48, non-ideality will not be negligible and the association/dissociation constants must therefore be regarded as "apparent ones". The corresponding value of K_d free of these effects can be obtained by extrapolation to "infinite dilution" ($c = 0$).

Extraction of K_d from a plot of M_w versus loading concentration c

K_d was also estimated from a global fit of the weight average molar masses M_w plotted versus ultracentrifuge cell loading concentration, expressed in (monomer) molar concentration terms C ($=c/M_1$) according to:

$$M_w = \frac{M_1}{C}\left[2C + \left(K_d - \sqrt{\{K_d^2 + 8CK_d\}}\ \ 4\right)\right] \quad (4)$$

To obtain M_w used in Eq. (4) from the apparent molar masses, $M_{w,app}$ – the parameter directly measured by sedimentation equilibrium – it is necessary to know the extent of thermodynamic non-ideality of the system, as represented by the thermodynamic or "osmotic pressure" second virial coefficient B ($=B'/2$, where B' is the "sedimentation" or "light scattering" virial coefficient used in early treatments):

$$M_{w,app} = M_w/(1 + 2B_i M_i c) \quad (5)$$

The factor $2B_iM_i$ can be evaluated for the individual reactants ($i=1$) and the complex ($i=2$) from their known molecular mass and molecular dimensions (and an estimated swollen specific volume, v_s, of 1 ml/g) using the routine COVOL (Harding & Horton, in preparation) based on the tri-axial ellipsoid theory of Rallison and Harding (1985). A full treatment of the non-ideality of the complex in the presence of the free reactants is currently unwieldly, so the assumption is made that the non-ideality is due entirely to the complex and there are no contributions from the reactants or cross-terms involving the reactants and complex.

Sedimentation velocity analysis

All samples were centrifuged at 40,000 rpm with radial scans being taken at 45 minute intervals throughout the 12 hour experiment. Sedimentation coefficients were evaluated using second moment boundary analysis. Low concentration sedimentation coefficients were determined using the *Svedberg* program (Philo 1994) which fits the Faxén type equation 2.94 from Fujita (1975) to the raw data. This is advantageous for low molar mass proteins – such as the srCD2–srCD48 system, which may not have fully sedimented away from the meniscus or which have broad sedimenting boundaries. All sedimentation coefficients were standardised in terms of water at 20 °C using the relation (van Holde 1985):

$$s_{20,w} = s_{obs}[(1-\bar{v}\rho_{20,w})/(1-\bar{v}\rho_{T,b})] \cdot (\eta_{T,w}/\eta_{20,w})(\eta_b/\eta_w) \quad (6)$$

where s_{obs} is the measured sedimentation coefficient in the experimental temperature, T, $\rho_{T,b}$ is the solvent density at the temperature of the experiment and $\rho_{20,w}$ the density of water at 20 °C, and $\eta_{T,w}/\eta_{20,w}$ is the viscosity of water at temperature T relative to its viscosity at 20 °C and η_b and η_w are the relative viscosities of the solvent and water at a given temperature.

Sedimentation coefficients determined at several different concentrations can be used to estimate a dissociation constant using locally written software (*SA-Plot*, P. Fellows, Univ. of Leicester, 1995) which uses the extension of the theory of Gilbert and Gilbert (1973) described by

Emes and Rowe (1978). *SA-Plot* incorporates a further extension of this approach which uses the full general equation for concentration dependence (Rowe 1992). The concentration can be calculated from the degree of dissociation (α)

$$\alpha = -K_d c + (K_d c M_1)^2 + 8 c^2 K_d c M_1 / 4 c^2 \quad (7)$$

where K_d is the dissociation constant (M), c is the total concentration (g/ml) and M_1 is the monomer molar mass. The concentration of the monomer (c_m) and complex (c_d) can then be found from Eqs. (8) and (9) as follows.

$$c_m = \alpha c \quad (8)$$

$$c_d = (1-\alpha) c \quad (9)$$

Using these values, sedimentation coefficients for each species can be calculated using Eqs. (10) and (11)

$$s^c = s^0 (1 - g c) \quad (10)$$

where s^c is the sedimentation coefficient at finite solute concentration c, s^0 is the sedimentation coefficient at infinite dilution and g(c) is a general function of concentration (Rowe 1977)

$$g(c) = \frac{k_s - [(c \bar{v}_s)^2 (2\phi_p - 1)]/\phi_p^2}{k_s c - 2 c \bar{v}_s + 1} \quad (11)$$

k_s is the limiting linear sedimentation velocity regression coefficient (in the absence of associative phenomena), $\bar{v}_s$ is the swollen partial volume and ϕ_p is the maximum packing fraction (by volume). *SA-Plot* utilises Eqs. (7)–(11) to compute the weight averaged sedimentation coefficient for the species as a function of total protein concentration and then to iteratively calculate the sum of the square of residuals between experimental data and calculated curves for assumed K_d values until an optimal fit is found by the user (see Emes and Rowe 1978 for further details of this procedure).

Results

From measured sedimentation equilibrium buoyant masses of 8391 g/mol and 8267 g/mol respectively, weight fractions of carbohydrate (from Eq. (2)) and corresponding partial specific volumes for srCD2 and srCD48 could be evaluated (Table 1). The molecular weights, M_w, of srCD2 and srCD48 were then estimated from extrapolation of $M_{w,app}$ to zero concentration which yielded values of (28.3±0.4) kg/mol (or kDa) for srCD2 and (28.7±0.2) kg/mol for srCD48 (Table 1) indicating both molecules are very similar in mass and justifying the approximation used in the analysis of complex formation between these species as a self-association with an effective monomer molar mass of 28.5 kg/mol.

The variation of $M_{w,app}$ with concentration (Fig. 1) in both cases follows well the predicted form of Eq. (5) with $B_1 = 1.82 \times 10^{-4}$ ml · mol · g^{-2} and $2B_1M_1 = 10.35$ ml/g. This $2B_1M_1$ value was an average as determined using the

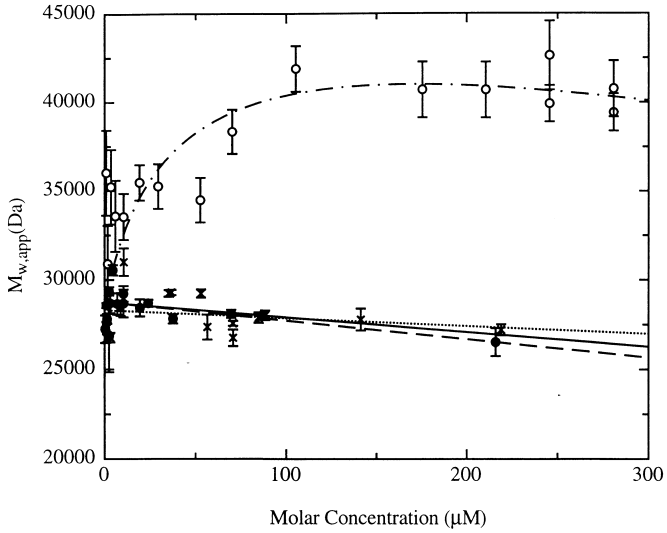

Fig. 1 Apparent weight-average molar mass of srCD2, srCD48 and srCD2–srCD48 as determined using sedimentation equilibrium. (×) srCD2; (●) srCD48; (○) srCD2–srCD48; non-linear least square fits to data for srCD2 (· · · ·) and srCD48 (– – – –); predicted regression for srCD2 and srCD48 with 2BM of 10.4 ml/g (——); fit to srCD2–srCD48 data with combined Eqs. (4) and (5) (– · – · –) (1 μM = ~0.03 mg/ml). Where complex is referred to, the concentration is of *total* protein

Table 1 Molar masses, carbohydrate weight fractions and partial specific volumes

Reactant	Molar mass (g/mol)	f_c	$\bar{v}$ (ml/g)
srCD2	28.3±0.4	0.307	0.705
srCD48	28.7±0.2	0.233	0.708

crystallographic axial molecular dimensions of 85×23×25 Å (Jones et al. 1992) for deglycosylated srCD2 and 94×49×67 Å for glycosylated srCD2 and likewise for srCD48. The agreement with the experimental trend is encouraging and indicates that the individual srCD2 and srCD48 by themselves show no detectable self-association.

Sedimentation equilibrium evaluation of the K_d for the srCD2–srCD48 interaction

An interaction between the equimolar mixtures of srCD2 and srCD48 is clearly indicated by the increase in $M_{w,app}$ as a function of the total loading concentration (Fig. 1). The expected complex molar mass of ~57 kg/mol is not reached and instead reaches a maximum value of <45 kg/mol consistent with a low affinity interaction and the relatively large effects of non-ideality. Visual inspection of Fig. 1, which for convenience is expressed on a molar concentration basis (with respect to monomer molar mass) suggests a K_d in the order of ~50 μM.

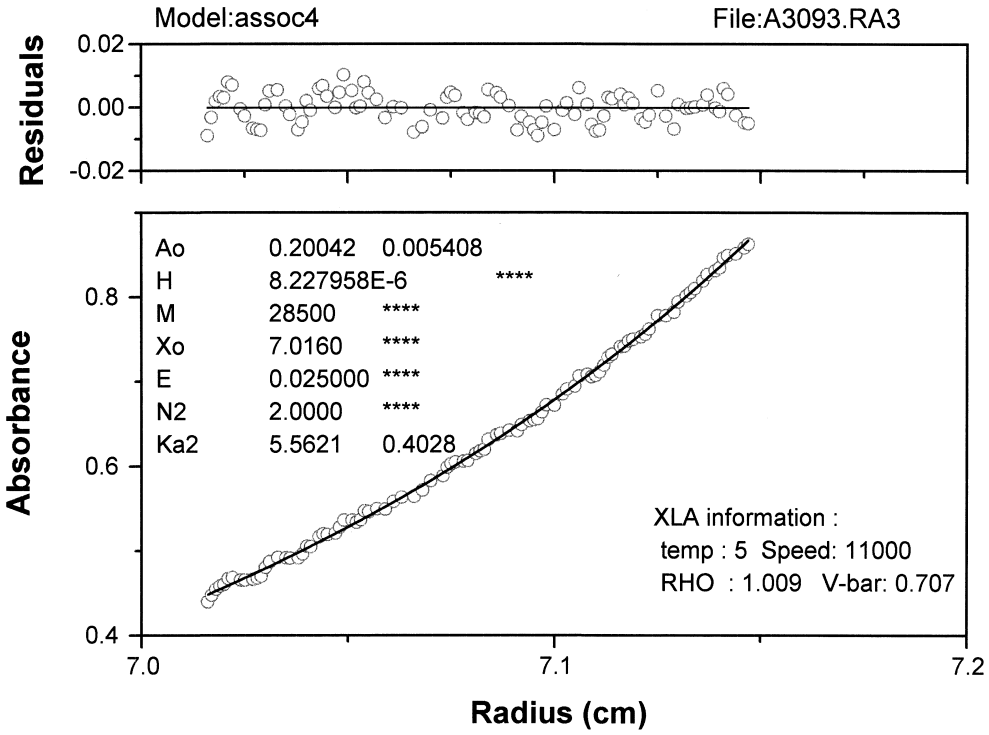

Fig. 2 ASSOC4 monomer-dimer model fitted to raw sedimentation equilibrium data of srCD2–srCD48

The K_d was first estimated from direct representation of the concentration distributions in the ultracentrifuge cell (Fig. 2) using the model ASSOC4 (cf. Eq. (2)) to yield the apparent K_d followed by extrapolation of $K_{d,app}$ to zero concentration (Fig. 3). A value for K_d of 22 ($\pm$11) μM was obtained in this manner.

The $M_{w,app}$ versus c data were then fitted according to Eqs. (4) and (5). Assuming the non-ideality is due entirely to the complex with the reactants linked end-to-end (with deglycosylated dimensions 175×25×23 Å and glycosylated dimensions of 175×66×54 Å) we obtain a mean $\sim 2B_2M_2 = 13.5$ ml/g in Eq. (5). Equation (4) then yields a value for K_d of (103±32) μM, somewhat higher than the value obtained by the direct concentration distribution fitting procedure. In performing a fit to the data of Fig. 1 only those data points at 50 μM or less were employed, since those at higher concentrations were recorded at off-peak wavelengths (>280 nm) to avoid Lambert-Beer law restrictions and possible turbidity. Another possible problem is that we have assumed that the charge contribution to the $2B_2M_2$ term is negligible: if this were not so, this would lead to an overestimate of K_d. However, this possibility too can be ruled out since any charge effects are likely to be suppressed by the presence of low molecular weight electrolyse (salt) in the solvent (ionic strength ~0.14). The difference may reflect problems concerning the application of IDEAL1 – which does not consider the complete distribution of solute in the ultracentrifuge cell – to heterogeneous systems or, more likely, just reflects the precision with which a dissociation constant can be truely measured at these interaction strengths. A further estimate for K_d using sedimentation velocity data therefore proved useful.

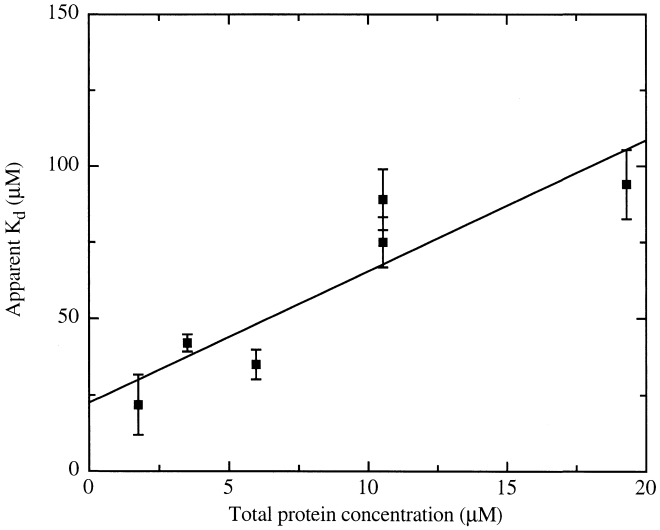

Fig. 3 Concentration dependence of the apparent dissociation constants as estimated using the ideal self-association model ASSOC4

Sedimentation velocity evaluation of K_d for the srCD2–srCD48 interaction

Sedimentation velocity experiments on the individual reactants *and* the complex all yielded, single sedimenting boundaries. Using the SVEDBERG data analysis program (Philo 1994) very good fits to the symmetrical, sedimenting boundaries were obtained (Fig. 4). Extrapolating to infinite dilution yielded very similar weight-average sedi-

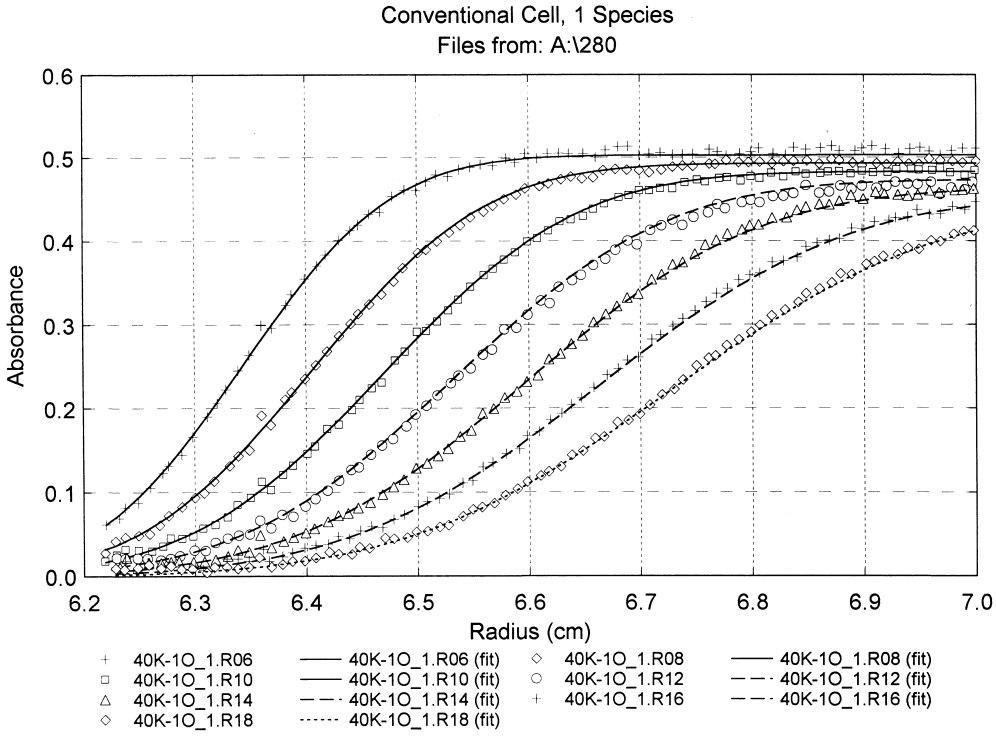

Fig. 4 Fit of raw sedimentation velocity data for srCD2–srCD48 using the programme SVEDBERG (Philo, 1994). Rotor speed = 40 000 rpm. Scan interval = 60 min

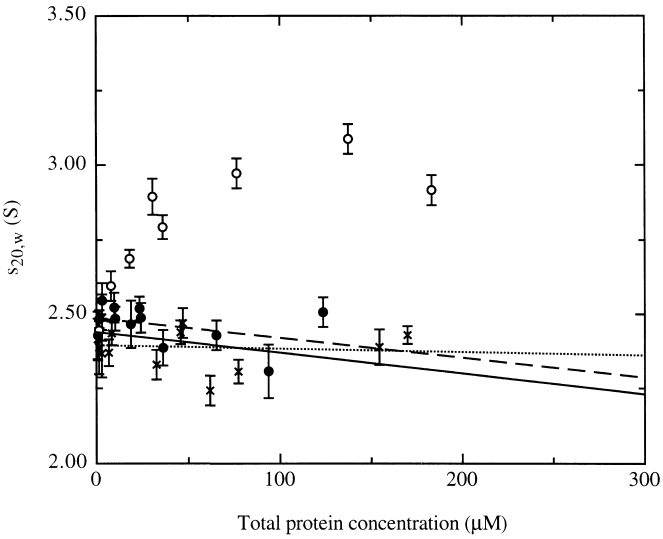

Fig. 5 Weight-average sedimentation coefficients (corrected for buffer density and viscosity at 20 °C) as a function of concentration for srCD2, srCD48 and srCD2–srCD48. (×) srCD2; (●) srCD48; (○) srCD2–srCD48; non-linear least squares fits to the equation $s^c = s^o (1 - k_s c)$ to data for srCD2 (· · · ·) and srCD48 (– – – –). Average regression line for the two: (——); this corresponds to a k_s (monomer) = 5 ml/g

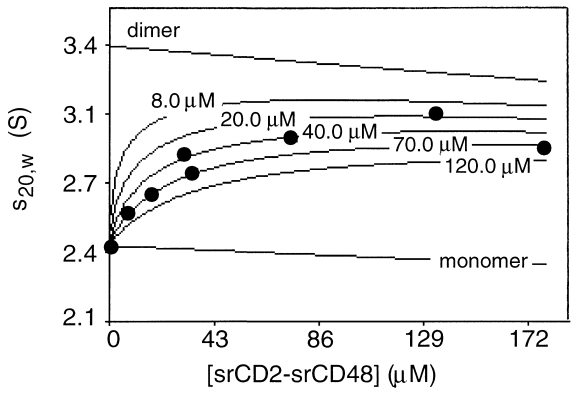

Fig. 6 Weight-average sedimentation coefficients (●) modelled iteratively to Eqs. (7)–(11) [with k_s (monomer) set as 5 ml/g; k_s (dimer as 8.5 ml/g)], for values of the dissociation constant K_d in the range 8–120 μM using the software *SA-Plot*

mentation coefficients of (2.39 ± 0.02) S for srCD2 and (2.48 ± 0.02) S for srCD48, further confirming our treatment of the srCD2–srCD48 interaction as an effective monomer-dimer equilibrium. Further, the presence of a single symmetric boundary for the complex is consistent with a rapid monomer-dimer equilibrium (Gilbert and Gilbert 1973).

The similarity in sedimentation coefficients for the reactants indicates the similarity in size and shape of both molecules. It is also evident from Fig. 5 that srCD2 and srCD48 do not self-associate as they have a negative dependence on concentration (k_s). As shown in Fig. 5 this is strongly comparable to an estimated regression coefficient of 5 ml/g for these species calculated using the relation of Rowe (1977)

$$k_s = 2\bar{v}\left\{\frac{\bar{v}_s}{\bar{v}} + P^3\right\} \qquad (12)$$

where P is the "frictional ratio due to shape" or the "Perrin shape function": this latter parameter can be estimated

Table 2 K_d Evaluations for the srCD2–srCD48 complex

Method	K_d (μM)	Temp. (°C)	Ref.
Sedimentation equilibrium, Eq. (3)	22±11	5	This study
Sedimentation equilibrium, Eq. (4)	103±32	5	This study
Sedimentation velocity	45±25	5	This study
Surface plasmon resonance	25±10	20	van der Merwe (personal communication)
Surface plasmon resonance	60–90	37	van der Merwe et al. (1993)
Microcalorimetry	20–30	25	see van der Merwe and Barclay (1996)

from hydrodynamic modelling. An increase in the sedimentation coefficient of the srCD2–srCD48 complex (Fig. 6) shows hetero-association and at infinite dilution extrapolates back to the sedimentation coefficients of the monomers. These data were used to determine a K_d using SA-PLOT prior to which monomer and dimer extrapolated sedimentation coefficients, their k_s and partial swollen volumes needed to be defined. A dimer sedimentation coefficient was estimated using frictional coefficients determined from preliminary bead modelling studies using hydro (García de la Torre et al. 1994) and ATOB (Byron, 1997) and the k_s was determined for the dimer using Eq. (13) as mentioned earlier. The dimer $s^0_{20,w}$ used was 3.96 S which is very likely to correspond to the 'head-to-head' interaction topology for srCD2–srCD48 (van der Merwe et al. 1995). We are presently examining this behaviour more closely using hydrodynamic bead modelling but this will be considered in more detail elsewhere (Silkowski et al., in preparation). Iterative modelling with SA-PLOT yielded a K_d of (45±25) μM which is in good agreement with those values estimated from sedimentation equilibrium (Table 2).

Discussion

The interaction affinity of srCD2–srCD48 at 5 °C, as determined using ultracentrifugation techniques, is relatively low from the three ultracentrifuge methods used (Table 2) with a "consensus" value of between 20–110 μM. This weak interaction confirms the observations of van der Merwe et al. (1993, 1994). Using the method of surface plasmon resonance (SPR), based on principles completely different from the ultracentrifuge procedures described here, they obtained values of (25±10) μM at 25 °C (P.A. van der Merwe, personal communication) and (60–90 μM) at 37 °C. Unfortunately there are no ultracentrifuge K_d determinations available as yet at these higher temperatures (these first ultracentrifuge determinations were performed at low temperature because of the uncertain stability of the complex and its reactants during the long time course (>24 h) of a sedimentation equilibrium experiment) nor are there surface plasmon resonance determinations at 5 °C. Nonetheless both techniques do appear reasonably consistent. One feature which may have affected the accuracy of the ultracentrifuge K_d determinations, and is particularly associated with glycoproteins, is the difficulty in accurately calculating partial specific volumes due to the heterogeneity of the carbohydrate regions of the molecules. A 1% error in $\bar{v}$ could lead to as much as a 20% difference in K_d as determined from ASSOC4. The $\bar{v}$ values used in this study have low error as the measured $\bar{v}$ corresponds very well to the calculated $\bar{v}$ assuming 30% glycosylation (Perkins 1986; Davis et al. 1993).

Some difference in the K_d determined by the two methods would not be surprising due to the different environments imposed in the "biosensor" SPR method and in the ultracentrifuge. The main differences between SPR and ultracentrifugation are that SPR can give kinetic data as well as affinity constants but also that one ligand has to be immobilised onto the chip. The immobilisation step in the SPR should ideally not however affect the observed affinity constants although mass action effects may alter the kinetics. However it is possible that the immobilisation process disrupts the binding site affecting the observed affinity. In addition, any self-association of the immobilised ligand at the high concentrations present on the chip could also affect the affinity through steric effects. It is possible to minimise these problems by coupling the ligand indirectly e.g., through an antibody, and doing the measurements in the reverse orientation and at various concentrations (discussed in van der Merwe et al. 1993). In this regard ultracentrifugation has the advantage in that it does not involve immobilisation and self-association can be quantified. In this case no self-association of the individual reactants was observed and, further, the affinity observed for the srCD2/srCD48 interaction by the three ultracentrifuge procedures was similar to that determined by SPR confirming that this interaction has indeed a particularly low affinity.

The relatively low affinity interaction – as confirmed by this study – may be typical of other cell surface leukocytes (van der Merwe and Barclay 1994). Another area of interest, which the ultracentrifuge can help to elucidate, is the study of how the extracellular domains of cell adhesion molecules interact. The srCD2–srCD48 interaction is ideally suited to hydrodynamic study and will possibly provide models for interactions between other CAMs. The dimensions of srCD2 have been determined by X-ray crystallography (Jones et al. 1992) and sedimentation velocity analysis and neutron scattering studies have been interpreted using hydrodynamic bead modelling (Silkowski et al., in preparation). Site-directed mutagenesis and NMR analysis indicated that CD2 and CD48 interact in a head-to-head fashion as originally indicated from crystal contacts observed in X-ray crystallography of CD2 (Jones

et al. 1992). This is currently being analysed to test whether this interaction orientation can be detected from the analysis of hydrodynamic properties as a paradigm for the study of other cell adhesion molecules.

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