

# Analysis by Surface Plasmon Resonance of the Influence of Valence on the Ligand Binding Affinity and Kinetics of an Anti-carbohydrate Antibody\*

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The kinetics of ligand binding by Se155–4, an antibody specific for the *Salmonella* serogroup B *O*-polysaccharide, were studied by surface plasmon resonance. Because trace amounts of oligomers in Fab and single-chain antibody variable domain (scFv) preparations resulted in biphasic binding profiles that were difficult to analyze, all kinetic measurements were performed on purified monomeric fragments and, for certain mutant scFv, dimeric forms. Results obtained with monomeric forms indicated that the relatively low affinity of the antibody was due to rapid dissociation ( $k_{\text{off}} \approx 0.25 \text{ s}^{-1}$ ). Dimeric forms generally showed off-rates that were approximately 20-fold slower and a 5-fold increase in association rate constants to approximately  $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ . Although the association phases for scFv dimers showed good curve fitting to a one component interaction model, the dissociation phases were biphasic, presumably because the availability and accessibility of sites on the antigen always leads to some monovalent attachment. The fast off-rate for dimers was the same as the monomer off-rate. Se155–4 IgG off-rates were very similar to those observed for scFv dimer, whereas the on-rate was the same as that obtained with Fab and scFv monomer.

The relatively weak affinities that characterize protein-carbohydrate interactions make understanding how biological processes are mediated by oligosaccharides difficult. However, it is becoming evident, particularly for certain plant and animal lectins, that specificity is achieved by a combination of multivalence and the geometry of the subunit arrangement. There is now a considerable amount of data available on structural and energetic aspects of protein-carbohydrate interactions (1, 2). General structural features are the stacking of aromatic side-chains against the sugar rings, the presence of hydrogen bond networks in which the sugar OH groups act as both acceptors and donors, and the coordination of multiple hydrogen bonds by water molecules (1). As for their energetics, titration microcalorimetry has indicated that these interactions are usually enthalpy-driven and that water reorganization, especially desolvation, is a key feature of complexation (2). Kinetics of the interactions are less well known but are obvi-

ously important in understanding the subtleties of carbohydrate-mediated biological events. Without consideration of the time factor, analysis of these interactions from a biological standpoint is difficult. The development of surface plasmon resonance techniques has provided an opportunity to explore biomolecular interaction in real time.

We have applied SPR<sup>1</sup> technology to the analysis of antigen binding by the antibody Se155–4. The specificity of Se155–4 is dominated by a 3,6-dideoxyhexose, abequose, presented by the lipopolysaccharide *O*-antigen of *Salmonella* serogroup B bacteria (3). This polysaccharide repeating unit is built from four hexopyranose units:  $\{\rightarrow 2\}[\alpha\text{D-Abe}(1\rightarrow 3)]\alpha\text{D-Man}(1\rightarrow 4)\alpha\text{L-Rha}(1\rightarrow 3)\alpha\text{D-Gal}(1\rightarrow)$ . The structural (4) and energetic (5, 6) aspects of antigen binding to Se155–4 have been well characterized, and an efficient *Escherichia coli* expression system is available for the production of antibody fragments (7, 8).

Initial SPR investigations of antigen binding by wild-type Se155–4 scFv revealed a distinctly biphasic association profile (8). A subsequent study on the isolation of scFv mutants with enhanced binding properties from complementarity-determining region-randomized phage display libraries showed that mutants were selected primarily on their propensity to form dimers (9). It was observed that mutants that existed largely in the dimeric state displayed classical association kinetics, which in turn led to the realization that the wild-type biphasic association kinetics were the result of trace amounts of scFv dimer in the preparations. In this report we describe the detailed analysis by SPR of Se155–4 IgG, Fab, and scFv binding to immobilized antigen.

## EXPERIMENTAL PROCEDURES

**Recombinant DNA Techniques**—Clones encoding Se155–4 Fab and scFv, with the exception of SLA-1, were isolated as described previously (7–9). SLA-1, a mutant in which the original linker was shortened from V<sup>106L</sup>LQPKSSPSVTLFPPSSNGE<sup>1H</sup> to V<sup>106L</sup>LQPKSSPSVTE<sup>1H</sup> was constructed by oligonucleotide replacement. All DNA manipulations were carried out by standard procedures (10).

**Expression and Purification**—Transformed cultures were grown as described previously, and fully active antibody fragments were isolated from periplasmic extracts by affinity chromatography (3). Fab and scFv preparations were analyzed for monomer, dimer, and higher oligomer content by size-exclusion HPLC using a Superdex 75 (Pharmacia) column (9).

**SPR Analysis**—Binding kinetics were determined by SPR using a BIAcore™ biosensor system (Pharmacia Biosensor) (11). BSA-*O*-polysaccharide conjugates (3) were immobilized on research grade CM5 sensor chips in 10 mM sodium acetate, pH 4.5, using the amine coupling kit supplied by the manufacturer. Unreacted moieties on the surface

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<sup>1</sup> The abbreviations used are: SPR, surface plasmon resonance; BSA, bovine serum albumin; Fv, antibody variable domain fragment(s); scFv, single-chain Fv; RU, resonance unit; HPLC, high pressure liquid chromatography.

were blocked with ethanolamine. The serogroup B *O*-polysaccharide is comprised of up to 25 tetrasaccharide repeating units, and the carbohydrate content of the BSA-conjugates indicated that on average each BSA molecule was coupled to four polysaccharide chains.<sup>2</sup> In view of the high degree of polymerization of the polysaccharide, the neoglycoprotein should be quite homogeneous with respect to carbohydrate presentation. Conjugate concentrations of 20  $\mu\text{g/ml}$  and contact times of 5 min at a flow rate of 3  $\mu\text{l/min}$  gave approximately 200 RU of immobilized material, where one RU corresponds to an immobilized protein concentration of  $\sim 1 \text{ pg/mm}^2$  (12). Control BSA surfaces were prepared in an analogous manner. All measurements were carried out in HEPES-buffered saline that contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.3 mM EDTA, and 0.005% Surfactant P-20 (Pharmacia Biosensor). Analyses were performed at 25 °C and at a flow rate of 3  $\mu\text{l/min}$ . Surfaces were regenerated with 13  $\mu\text{l}$  of 10 mM HCl at a flow rate of 3  $\mu\text{l/min}$ . When purified monomers and dimers were assayed, they were prepared using a Superdex 75 column equilibrated with HEPES-buffered saline and were analyzed by SPR immediately after isolation.

For the calculation of association rate constants, samples were appropriately diluted in HEPES-buffered saline and analyzed at several antibody concentrations. Samples which gave a significant buffer-related RU change on injection were also analyzed on a blank BSA surface. For the calculation of dissociation rate constants, relatively high concentrations of antibody were used, and the kinject command was used to inject 10  $\mu\text{l}$  of 100  $\mu\text{M}$  free trisaccharide,  $\alpha\text{-D-Gal}(1\rightarrow2)\text{-}[\alpha\text{-D-Abe}(1\rightarrow3)]\alpha\text{-D-Man}$ , over the sensor chip surface immediately following the sample injection.

**Data Analysis**—Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data (13) using the BIAevaluation 2.0 software (Pharmacia Biosensor). The dissociation rate constant is derived using the equation

$$R_t = R_{t_0} e^{-k_{\text{off}}(t-t_0)} \quad (\text{Eq. 1})$$

where  $R_t$  is the response at time  $t$ ,  $R_{t_0}$  is the amplitude of the response, and  $k_{\text{off}}$  is the dissociation rate constant. Dissociation of two components can be treated as the sum two independent events where each is described by an equation of the above form. The association rate constant can then be derived using the equation

$$R_t = \frac{k_{\text{on}} C R_{\text{max}} (1 - e^{-(k_{\text{on}} C + k_{\text{off}}) t})}{k_{\text{on}} C + k_{\text{off}}} \quad (\text{Eq. 2})$$

where  $R_t$  is the response at time  $t$ ,  $R_{\text{max}}$  is the maximum response,  $C$  is the concentration of ligate in solution,  $k_{\text{on}}$  is the association rate constant, and  $k_{\text{off}}$  is the dissociation rate constant. A two component association is treated as the sum of two independent events, each described by an equation of the above form.

Rate constants were also calculated by using linear transformations of the sensorgram data and the equation

$$K_s = k_{\text{on}} C + k_{\text{off}} \quad (\text{Eq. 3})$$

where  $K_s$  is the slope of a  $d\text{RU}/dt$  versus RU plot. A range of concentrations are analyzed, and a plot of  $K_s$  versus  $C$  has a slope of  $k_{\text{on}}$  and a Y-intercept of  $k_{\text{off}}$ .

Affinities were calculated from rate constants and from analysis of equilibrium binding. By measuring equilibrium resonance units as a function of ligand concentration, binding data can be analyzed by Scatchard plots using the equation

$$\frac{R_{\text{eq}}}{C} = K_d R_{\text{max}} - K_d R_{\text{eq}} \quad (\text{Eq. 4})$$

where  $R_{\text{eq}}$  is the equilibrium resonance units,  $R_{\text{max}}$  is the resonance signal at saturation,  $C$  is the concentration of free protein, and  $K_d$  is the association constant. A plot of  $R_{\text{eq}}/C$  versus  $C$  has a slope of  $-K_d$ .

## RESULTS

**Antibody Fragment Oligomerization**—Size exclusion HPLC showed that although the wild-type scFv product was almost exclusively monomeric, many of the better binding mutants isolated by phage display from partially randomized libraries (9) had a tendency to dimerize. Two amino acid substitutions in the heavy chain (M34I and G109S in mutant B5-1) gave a

product that was almost entirely dimer. Dimers were of the type in which the light chain variable domain of one scFv interacts with the heavy chain variable domain of a second scFv (9, 14).

**Effect of Oligomerization and Aggregation on Binding Profiles**—Trace amounts of dimers or higher oligomers in antibody fragment preparations were observed to dramatically affect the sensorgram profiles obtained with BSA-*O*-polysaccharide surfaces. Prior to size-exclusion HPLC, the wild-type scFv gave distinctly biphasic association and dissociation kinetics (Fig. 1A), whereas analysis of monomeric fractions obtained by HPLC showed rapid association and dissociation kinetics with little evidence of biphasic behavior (Fig. 1B). With purified monomer, much higher concentrations were required to achieve the levels of binding obtained with unfractionated scFv containing dimer. Fab preparations also contained traces of aggregates that caused biphasic association patterns if not removed by HPLC. Mutant scFv that existed primarily as dimers, such as B5-1 (Fig. 1C), showed monophasic association phases. Contamination of dimer preparations with trace amounts of monomer did not compromise the results because at identical concentrations, dimer response was approximately 100-fold higher than of its monomeric equivalent. In selecting representative scFv mutants, isolated from phage libraries (8, 9), for SPR analysis, a primary criterion was availability of purified monomer and dimer forms. Obtaining suitable monomeric preparations was more problematic because of the need to eliminate all dimer.

**Dissociation Rate Constant Determinations**—The dissociation kinetics of wild-type Fab and scFv as well as several mutant scFv isolated from phage display libraries (8, 9) were determined. In all instances, the dissociation data were collected in the presence of free trisaccharide because this significantly increased the rate of release of both monomeric and dimeric scFv from immobilized BSA-*O*-polysaccharide, even at higher antibody concentrations, which should have minimized rebinding (Fig. 2, A and B). The dissociation phases of monomeric Fab and several monomeric scFv preparations showed good curve fitting to the monoexponential decay model (Fig. 3). The  $k_{\text{off}}$  values obtained for all wild-type and mutant forms fell in a narrow range around  $0.25 \text{ s}^{-1}$  (Table I).

The dissociation data, collected in the presence of free trisaccharide, for single-chain Fv containing trace amounts of dimer fit better to the BIAevaluation biexponential decay function describing the dissociation of two components from the immobilized antigen than to the single exponential expression (Fig. 4, A and B). This was also true for dimer (mutant B5-1) dissociation (Fig. 4, C and D) and is thought to result from both monovalent and bivalent attachment of the dimers to the surface. The availability and accessibility of sites on the repeating epitope antigen would always lead to some monovalent attachment, particularly at antibody concentrations that give binding approaching  $R_{\text{max}}$ . In each instance, the faster of the two off-rates was quite similar to the rates obtained with pure monomers, although the short linker scFv (SLA-1), IgG, and B4-3 had somewhat slower rates (Table I).

**Association Rate Constant Determinations**—The extremely fast off-rates associated with Fab and scFv monomer binding resulted in association phases that had very narrow analysis windows at the antibody concentrations required to obtain adequate signals. Nevertheless, in most instances, nonlinear analysis of appropriate parts of sensorgrams gave good curve fitting to the monoexponential association model (Fig. 5A). The narrow analysis windows made linear analysis of the association data more difficult, but rates were obtained for the Fab and two of the scFv mutants (Table I). The values obtained, by

<sup>2</sup> E. Altman, unpublished results.

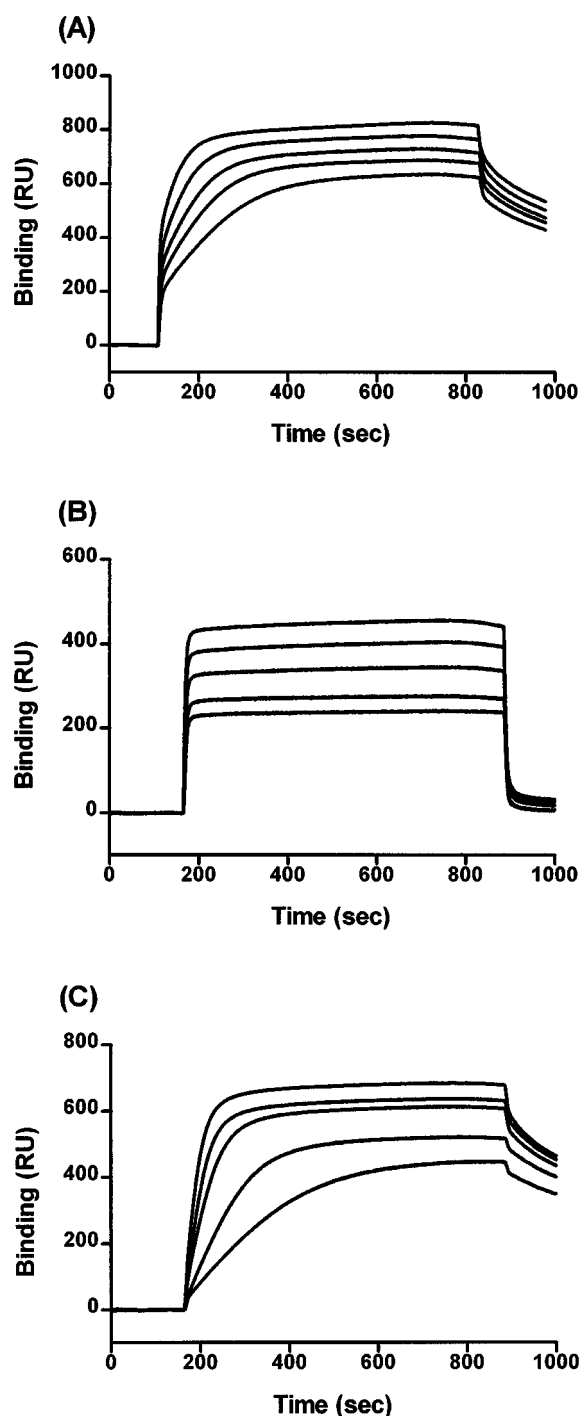


FIG. 1. Relative response versus time for the binding to immobilized BSA-*O*-polysaccharide of wild-type Se155-4 scFv containing trace amounts of dimer (A), monomeric wild-type scFv obtained by size-exclusion HPLC (B), and mutant B5-1, which exists almost entirely in the dimeric form (C). The concentrations are (from bottom to top) 1, 1.5, 2, 3, and 5  $\mu\text{M}$  in A; 0.2, 0.4, 0.8, 1.2, and 1.6  $\mu\text{M}$  in B; and 20, 40, 80, 120, and 200 nM in C.

both methods, for the Fab and scFv fell in a relatively narrow range around  $4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  (Table I). The association profiles of monomeric scFv containing trace amounts of dimer fit well to the biexponential interaction model (Fig. 5B), but analyses of this type did not yield useful information. The initial rapid binding part of the sensorgram, attributed to monomer, was difficult to analyze because of the time factor, and analysis of the second phase, attributed to dimer, was not feasible due to the uncertainties in estimating the dimer concentration.

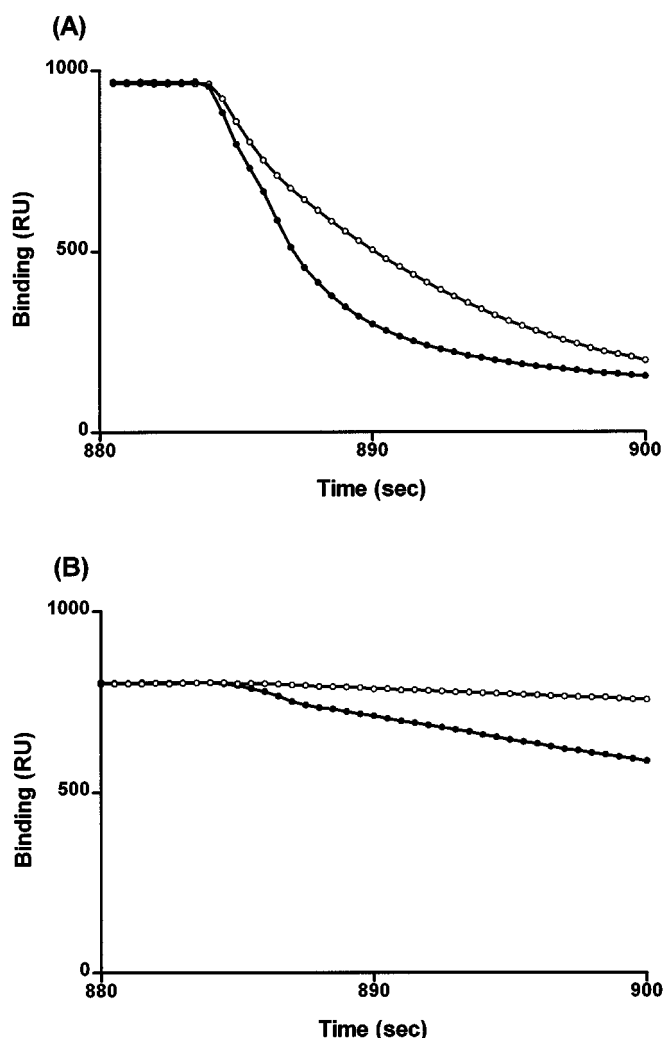


FIG. 2. Effect on off-rate measurement of the immediate injection of free trisaccharide following antibody injection. Primary dissociation data for monomeric SK4 (A) and dimeric B5-1 (B) in the absence (○) and presence (●) of free trisaccharide are shown.

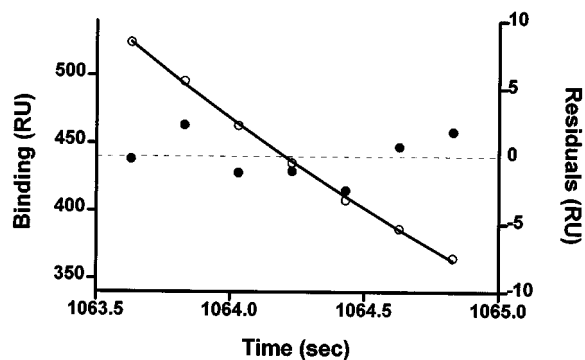


FIG. 3. Curve fitting of monomeric wild-type scFv dissociation phase to the BIA evaluation single-component model. Dissociation data were collected in the presence of free trisaccharide and yielded the monomer off-rates given in Table I.

The binding profiles obtained with IgG and scFv preparations that were predominantly dimer were more amenable to on-rate analysis. Using the nonlinear analysis method, the plotted residuals indicated that the association data fit well to the single exponential function (Fig. 5C). Association rate constants were approximately  $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  for the three scFv dimers that were examined (Table I). Interestingly, Se155-4 IgG was observed to have an association rate constant that was

TABLE I  
Kinetics and affinities of BSA-O-chain binding by monomeric Fab and scFv and IgG as calculated by linear and nonlinear analysis of sensorgrams

		$k_{\text{on}}^a$		$k_{\text{off}}^{\text{fast}}$	$k_{\text{off}}^{\text{slow}}$	$K_D$ monomer <sup>b</sup>	$K_D$ dimer <sup>c</sup>
		Nonlinear analysis <sup>d</sup>	Linear analysis <sup>e</sup>				
		$M^{-1} s^{-1}$		$s^{-1}$	$M^{-1}$	$M^{-1}$	
Fab	B-50	$3.6 \times 10^4 (\pm 5.7)^f$	$3.8 \times 10^4 (\pm 10)$	$3.1 \times 10^{-1} (\pm 1.9)$		$8.4 \times 10^{-6}$	
scFv monomer	SK4	$4.9 \times 10^4 (\pm 12.9)$		$2.0 \times 10^{-1} (\pm 2.3)$		$4.1 \times 10^{-6}$	
	3B1	$3.2 \times 10^4 (\pm 20.3)$		$2.4 \times 10^{-1} (\pm 9.0)$		$7.5 \times 10^{-6}$	
	B5-6	$6.3 \times 10^4 (\pm 8.0)$	$2.3 \times 10^4 (\pm 8.7)$	$2.9 \times 10^{-1} (\pm 2.1)$		$6.7 \times 10^{-6}$	
	B3-20	$3.4 \times 10^4 (\pm 14)$	$4.7 \times 10^4 (\pm 2.6)$	$2.5 \times 10^{-1} (\pm 1.0)$		$5.3 \times 10^{-6}$	
scFv dimer	SLA-1	$2.5 \times 10^5 (\pm 2.3)$	$2.4 \times 10^5 (\pm 0.4)$	$1.2 \times 10^{-1} (\pm 10)$	$1.1 \times 10^{-2} (\pm 3.1)$		$4.5 \times 10^{-8}$
	B5-1	$1.6 \times 10^5 (\pm 4.0)$	$1.5 \times 10^5 (\pm 3.2)$	$2.0 \times 10^{-1} (\pm 0.5)$	$1.3 \times 10^{-2} (\pm 1.5)$		$8.1 \times 10^{-8}$
	B4-3	$1.4 \times 10^5 (\pm 3.8)$	$1.5 \times 10^5 (\pm 4.1)$	$6.7 \times 10^{-2} (\pm 4.9)$	$7.8 \times 10^{-3} (\pm 1.6)$		$5.6 \times 10^{-8}$
IgG	Se155-4	$5.1 \times 10^4 (\pm 2.5)$	$5.5 \times 10^4 (\pm 2.4)$	$1.2 \times 10^{-1} (\pm 0.6)$	$1.2 \times 10^{-2} (\pm 5.9)$		$2.3 \times 10^{-7}$

<sup>a</sup> Calculated on a per site basis.

<sup>b</sup> Calculated using  $k_{\text{off}}^{\text{fast}}$  and the average, where applicable, of on-rates calculated by linear and nonlinear methods.

<sup>c</sup> Calculated using  $k_{\text{off}}^{\text{slow}}$  and the average of on-rates calculated by linear and nonlinear methods.

<sup>d</sup> Calculated by nonlinear analysis of individual sensorgrams.

<sup>e</sup> Calculated from  $k_s$  versus  $C$  plots.

<sup>f</sup> Uncertainties are given as % St.E.

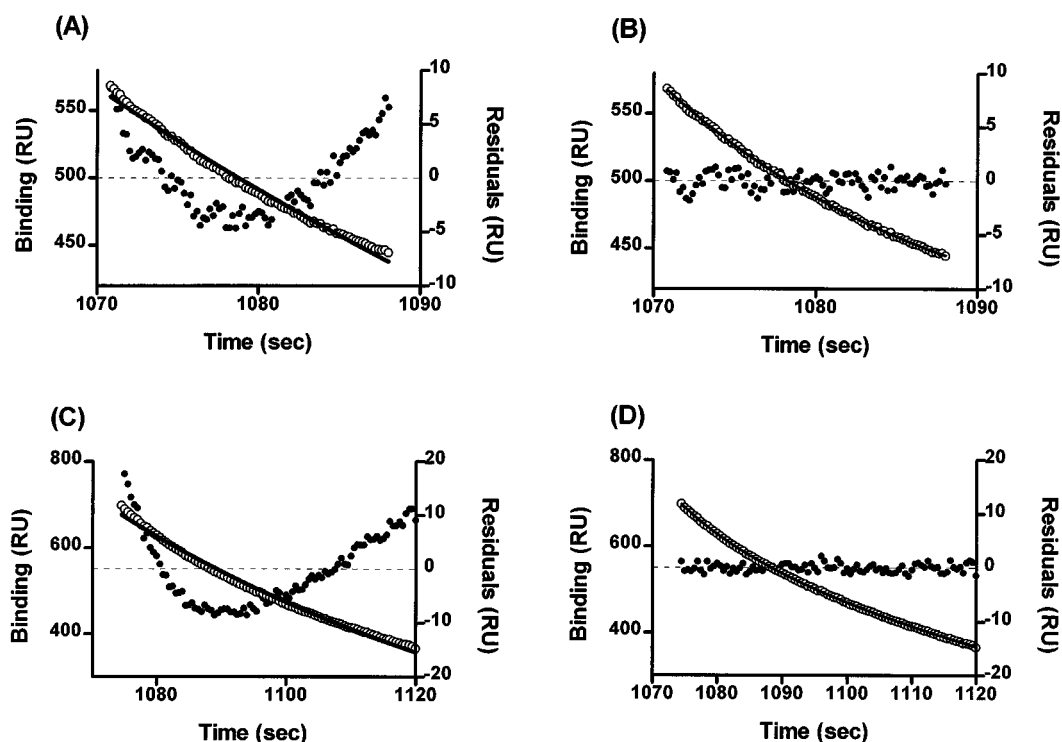


FIG. 4. Curve fitting of wild-type scFv containing trace amounts of dimer (A and B) and B5-1 scFv (C and D) dissociation phases to the BIAevaluation single-component (A and C) and two-component (B and D) models. Analyses were performed on dissociation data collected in the presence of free trisaccharide ligand and generated the two off-rates, given in Table I, for samples containing dimer.

3–5 times slower than the dimeric scFv (Table I). On-rates determined by linear transformation of the data and the concentration dependence of binding were in excellent agreement with those obtained by nonlinear fitting of the primary data (Table I, Fig. 6).

**Equilibrium Binding**—Binding affinities of Se155-4 Fab, monomeric and dimeric scFv, and IgG were also determined by equilibrium binding and Scatchard plot analysis (Fig. 7). The  $K_D$  values thus obtained for Fab and monomeric scFv binding were all very similar (approximately  $6 \times 10^{-6}$  M). Curved Scatchard plots, typical of bivalent interactions (15), were obtained for IgG and dimeric scFv. In such instances, the slope at lower site occupancy was used to derive an affinity for bivalent binding. Unfortunately, the slope at higher occupancy did not give an accurate affinity for monovalent binding because there is still a considerable amount of bivalent binding in this part of

the plot. The much sharper break in the dimeric scFv plot relative to the IgG plot is in agreement with the on-rate differences given in Table I. Dimeric scFv exhibited affinities that were about 100-fold higher, whereas the affinity of IgG was only 25-fold higher than that of the monovalent fragments (Table II). Thus, equilibrium binding analysis can provide an alternative means of determining on-rates from the  $K_D$  values derived from Scatchard plots and off-rates measured in the presence of free trisaccharide (Table II). This may be the most accurate method of determining  $k_{\text{on}}$  for monovalent fragments that rapidly reach  $R_{\text{eq}}$  at the analyte concentrations required for adequate binding.

**Comparison of SPR and Microcalorimetry Results**—A comparison of affinities determined by SPR methods and titration microcalorimetry is given in Table III. Derivation of affinities from kinetic constants and equilibrium binding gave compara-

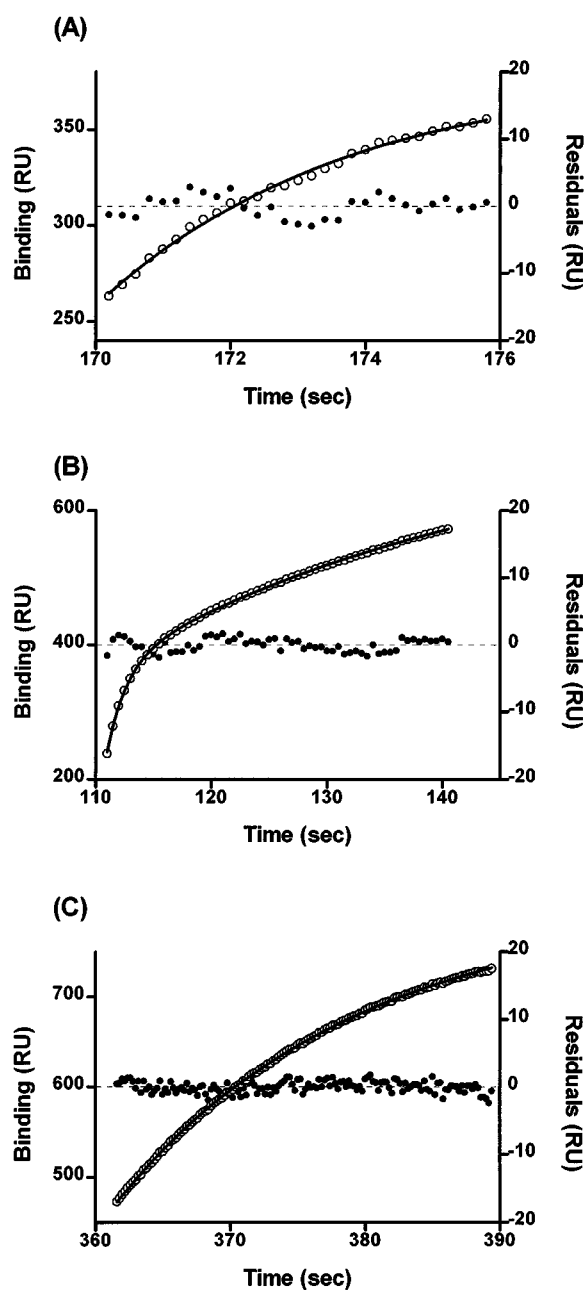


FIG. 5. Nonlinear analysis of wild-type scFv and B5-1 scFv association phases. Monomeric wild-type scFv (A) and wild-type scFv containing trace amounts of dimer (B) were fitted to the BIAevaluation one-component and two-component models, respectively. B5-1 dimer (C) was fitted to the one-component model. The monoexponential association model was used to generate the  $k_{on}$  values presented in Table I.

ble results. For monovalent fragments, the BSA-*O*-polysaccharide binding affinities were also in reasonably good agreement with those obtained for solution binding of the trisaccharide epitope by titration microcalorimetry. If the IgG  $K_D$  was calculated using the monovalent off-rate ( $k_{off}$  fast), SPR and titration microcalorimetry gave similar affinities,  $2.6 \times 10^{-6}$  M and  $4.8 \times 10^{-6}$  M, respectively. However, the B5-1 dimer affinity calculated using the fast off-rate was over 10-fold higher than that measured by calorimetry.

#### DISCUSSION

The kinetics of complex association and dissociation is an aspect of carbohydrate recognition by proteins that has been virtually inaccessible, but it is only when this component is also considered that a complete picture will emerge as to how car-

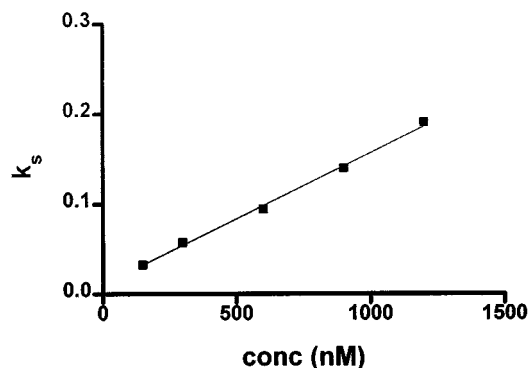


FIG. 6. Linear regression analysis of  $k_s$  versus  $C$  plots for dimeric B5-1 on-rate determination. The slopes of these plots yielded the the  $k_{on}$  values presented in Table I. *conc*, concentration.

bohydrates exert their biological activity. It is possible to obtain rate constants for protein-carbohydrate interactions by fluorescence (16) and NMR (17) methods, but these approaches are not generally applicable. Shinohara *et al.* (18) demonstrated that SPR could be used to study the specificities of different lectins for various glycopeptides but did not attempt to analyze the contribution of lectin valence to observed rate constants. The present investigation represents the first comprehensive effort to derive kinetic constants for a protein-carbohydrate interaction by means of SPR and is another demonstration that SPR is a very useful tool in the study of low affinity interactions. Other examples are peptide/major histocompatibility complex binding to T-cell receptors (19, 20) and transient cellular interactions involving cell adhesion molecules (21).

The data presented here show that extreme care must be exercised in the interpretation of SPR data for protein-sugar interactions, largely because of the repetitive nature of most carbohydrate ligands. Failure to recognize bivalent and multivalent interactions can lead to misinterpretation of results and overestimations of the intrinsic affinities of these systems. Others have also cautioned that multimeric interactions may be a common source of incorrect results when measuring low affinity interactions by SPR (21). Another important precaution is that samples be run on control surfaces, because at the protein concentrations required for analysis of low affinity binding, the amounts of free protein entering the dextran matrix of the sensor chip are sufficient to give a substantial increase in RUs. When properly collected, the SPR data yielded affinity measurements that were in good agreement with those obtained by titration microcalorimetry. Using equilibrium binding data obtained by SPR, the  $K_D$  values of Se155-4 Fab and wild-type scFv were estimated to be  $6.5 \mu\text{M}$  and  $6.8 \mu\text{M}$ , respectively, for interaction with *O*-polysaccharide, compared with microcalorimetry values of  $4.8 \mu\text{M}$  for Fab and  $7.7 \mu\text{M}$  for scFv, with trisaccharide ligand.

The observation that the biphasic association profiles obtained with some scFv preparations are characteristic of monovalent fragments containing trace amounts of dimer or higher oligomer necessitates some reinterpretation of earlier results for Se155-4 binding (8, 9). It is now clear that previous analyses of biphasic association profiles were conducted on the part of the sensorgram resulting from binding of the minor dimeric component. Although the faster on-rates exhibited by dimers partially compensated for the overestimation of binding analyte concentrations, the reported on-rates for the wild-type Se155-4 scFv were, nonetheless, somewhat lower than the actual monomer values.

The results presented here address the question of how much of an affinity gain is conferred by the presence of a second combining site. Crothers and Metzger (22) proposed that the

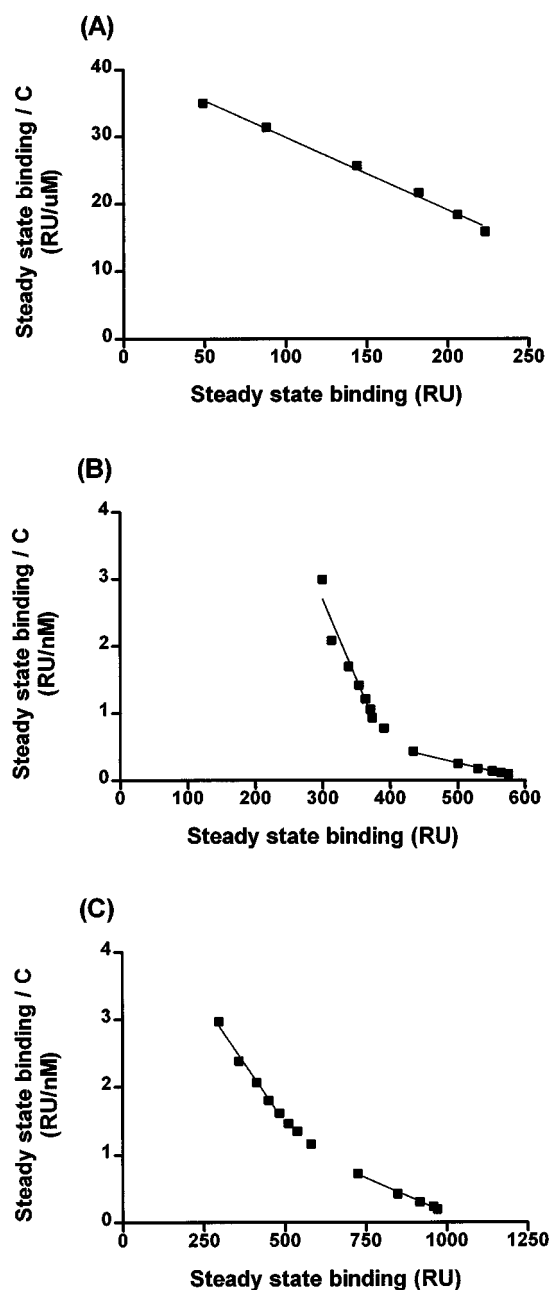


FIG. 7. Scatchard plots for the determination of affinities from equilibrium binding. A, monomeric wild-type scFv. B, dimeric B5-1. C, Se155-4 IgG.

observed affinity of a molecule such as IgG is the product of two binding constants, the first being that of a monovalent fragment of the antibody and the second being the dimensionless constant for the second site, once the first site is occupied. A gain is only obtained if the antigen is multivalent or attached to a surface. However, Jencks (23) has pointed out that the binding energy of a dimeric molecule contains an interaction energy term and is not simply the sum of the two component binding energies. A comparison of monomeric and dimeric Se155-4 scFv showed that divalency led to a 5-fold increase in association rate. A similar finding was reported for IgG3 binding to a multivalent carbohydrate antigen, and the effect was attributed to the tendency of IgG3 to aggregate (24). However, Se155-4 IgG did not exhibit a significantly faster on-rate than the monovalent forms. This may reflect a more unfavorable interaction energy because of less flexibility or more unfavorable steric interactions associated with the IgG form. The dis-

TABLE II  
Affinities and rate constants determined by analysis of equilibrium binding

		$K_D^a$	$k_{off}^b$	$k_{on}^c$
		M	$s^{-1}$	$M^{-1} s^{-1}$
Fab	B-50	$6.5 \times 10^{-6}$	$3.1 \times 10^{-1}$	$1.8 \times 10^4$
scFv monomer	SK4	$6.8 \times 10^{-6}$	$2.0 \times 10^{-1}$	$3.0 \times 10^4$
	3B1	$7.6 \times 10^{-6}$	$2.4 \times 10^{-1}$	$3.8 \times 10^4$
	B5-6	$6.1 \times 10^{-6}$	$2.9 \times 10^{-1}$	$4.8 \times 10^4$
	B3-20	$5.8 \times 10^{-6}$	$2.5 \times 10^{-1}$	$4.3 \times 10^4$
scFv dimer	SLA-1	$7.2 \times 10^{-8}$	$1.1 \times 10^{-2}$	$1.5 \times 10^5$
	B5-1	$4.1 \times 10^{-8}$	$1.3 \times 10^{-2}$	$3.2 \times 10^5$
	B4-3	$5.0 \times 10^{-8}$	$7.8 \times 10^{-3}$	$1.6 \times 10^5$
IgG	Se155-4	$1.4 \times 10^{-7}$	$1.2 \times 10^{-2}$	$8.7 \times 10^4$

<sup>a</sup> Derived from Scatchard plots.

<sup>b</sup> Data from Table I.  $k_{off}$  fast rates for Fab and scFv monomer;  $k_{off}$  slow rates for scFv dimer and IgG.

<sup>c</sup> Calculated by division of column 1  $K_D$  values by column 2 off-rates.

sociation data, collected in the presence of free trisaccharide, indicate that divalency decreases off-rates approximately 20-fold, which in combination with the faster on-rates translates into a 100-fold increase in functional affinity due to scFv bivalency. However, the dimer dissociation rates in the absence of free ligand, which are approximately  $2 \times 10^{-3} s^{-1}$  (9), may give a more accurate indication of the effect of divalency *in vivo*. If these off-rate values are used in affinity calculations, a 1000-fold gain is conferred by scFv bivalency.

In some instances at least, the considerable amount of monovalent attachment of bivalent molecules as binding approaches  $R_{max}$  enables the calculation of monovalent or intrinsic affinities. Although it is difficult to derive on-rates from the association phases of sensorgrams for analyte concentrations giving near  $R_{max}$  binding, analysis of the dissociation phases yields both monomeric and dimeric off-rates. Analysis of binding at low analyte concentration gives accurate measurement of dimer on-rates. For Se155-4 IgG, the fact that the IgG on-rate was the same as that of the monovalent Fab fragment made it possible to calculate an intrinsic affinity from rate constants that was in good agreement with that obtained by microcalorimetry. This should be possible for other multivalent interactions with low intrinsic affinities, such as lectin-carbohydrate binding, provided the off-rates are not too rapid to be measured by SPR and that the monomer and dimer on-rates are similar.

The extremely fast dissociation rates were the most striking feature of the binding kinetics described here. Presumably, this is typical of protein-carbohydrate interactions in general and is the reason for their low intrinsic affinities. Fluorescence (16) and NMR (17) methods have given on-rates for other protein-carbohydrate interactions that are similar to those reported here. These methods have also indicated that fast off-rates are responsible for the low affinities of protein-carbohydrate interactions and that differences in binding constants are primarily the result of differences in off-rates. It is not known if there is a biological reason for these fast rates or if they result from the types of atomic interactions that drive protein-carbohydrate interactions. It may reflect the fact that higher intrinsic affinities are not necessary because repeating epitopes and clustering of glycoproteins and glycolipids on membrane surfaces give large effective ligand concentrations and permit adequate binding through multivalent attachment. Monomeric affinities that are not higher than those needed for exertion of biological function may safeguard against self-recognition.

In summary, SPR has been shown to provide new insights into the subtleties of protein-carbohydrate interactions. The affinity gain conferred by bivalency has been quantitated, and it was shown that scFv dimers offer an advantage over whole

TABLE III  
Comparison of affinities calculated from rate constants determined by SPR from analysis of equilibrium binding by SPR and by titration microcalorimetry

		$k_{\text{off}}/k_{\text{on}}^a$	$K_D$ , M Scatchard <sup>b</sup>	Calorimetry <sup>c</sup>
Fab	B-50	$8.4 \times 10^{-6}$	$6.5 \times 10^{-6}$	$4.8 \times 10^{-6}$
scFv monomer	SK4	$4.1 \times 10^{-6}$	$6.8 \times 10^{-6}$	$7.7 \times 10^{-6}$
	3B1	$7.5 \times 10^{-6}$	$7.6 \times 10^{-6}$	$9.1 \times 10^{-6}$
	B5-6	$6.5 \times 10^{-6}$	$6.1 \times 10^{-6}$	
	B3-20	$5.3 \times 10^{-6}$	$5.8 \times 10^{-6}$	
scFv dimer	SLA-1	$4.5 \times 10^{-8}$ ( $8.0 \times 10^{-7}$ )	$7.2 \times 10^{-8}$	
	B5-1	$8.1 \times 10^{-8}$ ( $9.1 \times 10^{-7}$ )	$4.1 \times 10^{-8}$	$1.3 \times 10^{-5}$
	B4-3	$5.6 \times 10^{-8}$ ( $9.1 \times 10^{-7}$ )	$5.0 \times 10^{-8}$	
IgG	Se155-4	$2.3 \times 10^{-7}$ ( $2.6 \times 10^{-6}$ )	$1.4 \times 10^{-7}$	$4.8 \times 10^{-6}$

<sup>a</sup> Data from Table I. Affinities in brackets calculated using  $k_{\text{off}}$  fast rates.

<sup>b</sup> Data from Table II.

<sup>c</sup> Data from Sigurskjold *et al.* (6) and Deng *et al.* (8, 9).

antibodies in this regard. The observation that rapid off-rates are responsible for the low intrinsic affinity of Se155-4 and probably carbohydrate-binding proteins in general raises the possibility of engineering higher affinity by manipulating the dissociation rate constants.

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**Analysis by Surface Plasmon Resonance of the Influence of Valence on the Ligand Binding Affinity and Kinetics of an Anti-carbohydrate Antibody**  
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